Comparative study of the volatile constituents of *Thymus serpyllum* L. grown at different altitudes of Western Himalayas

Shweta Goyal^{1,2} · Rakshit Pathak^{1,2} · H. K. Pandey¹ · Anjali Kumari¹ · Geeta Tewari² · N. S. Bhandari³ · Madhu Bala¹

Received: 14 January 2020 / Accepted: 21 May 2020 / Published online: 13 June 2020
© Springer Nature Switzerland AG 2020

Abstract

Thymus serpyllum L., a well-known aromatic plant of the Himalayan region is often used in pharmaceutical, cosmetic, flavoring and fragrance industries because of its phenolic and non-phenolic constituents. The amount of these compounds varies with the origin, the climatic conditions and the developmental stage of harvesting time of the plant. Under different environmental conditions, plant specimen of the same species respond differently through production and accumulation of the primary and secondary metabolites. In the present work, *T. serpyllum* L. was collected from its natural habitat and grown at different agro-climatic zones, i.e., Auli (higher Himalayas; 2744 m asl), Pithoragarh (lower Himalayas; 1524 m asl) and Haldwani (foothill areas; 412 m asl). The essential oil from fresh aerial parts of cultivated plants at full blooming stage was obtained using Clevenger apparatus and analyzed by GC–FID and GC–MS. The amount of thymol was also quantified by HPTLC. The data were statistically analyzed using MS Excel and SPSS 16.0. The percentage yield of the essential oils varied from 0.20 to 0.84%. Thymol was the major compound found in all the three cultivations, being the highest for Haldwani (84.63%) followed by Auli (50.80%) and Pithoragarh (41.15%) cultivations. Camphor was the second major compound, only identified in Pithoragarh (36.34%) region. Alpha-thujene, *p*-cymene, alpha-terpineol, (*E*)-caryophyllene, beta-bisabolene, alpha-pinene and carvacrol were also identified in significant amounts. HPTLC quantification suggested the similar pattern of thymol percentage as obtained from GC–FID. The study revealed that there was a significant difference in the terpenoid constituents of the plant grown at different agro-climatic zones. *Thymus serpyllum* grown at lower altitude area (warm climatic zone) can be a potential source of thymol along with high essential oil yield.

Keywords *Thymus serpyllum* L. · Essential oil · Climatic conditions · Thymol · GC · GC/MS · HPTLC

1 Introduction

Thymus serpyllum (wild thyme or Breckland thyme) belonging to the family Lamiaceae is distributed throughout Kashmir and Kumaun Region of Uttarakhand [1]. It is a perennial short shrub having purplish flowers present at the tip of the stem, highly branched stems and forms a mat-like structure. Flowering occurs from May to September. It grows well on dry and stony ground grasslands. Traditionally, the shrub is used to prepare herbal formulations such as cough syrups, infusions, oil, tinctures and

infusions [2]. The aerial parts of the plant are also used as condiments, spices and herbal tea [3]. *Thymus* species also show antiviral, antifungal, anti-parasitic, antioxidant and antimicrobial activities [3–5].

Previous investigations on the essential oils (EOs) of the shrub confirmed the presence of secondary metabolites such as aromatic terpenoids, flavonoids and phenolic acid [3, 6–15]. The essential oils derived from various *Thymus* species differ in their chemical composition. The most common constituents of the essential oil are thymol and carvacrol. Besides this, the non-phenolic constituents such

✉ Geeta Tewari, geeta_k@rediffmail.com | ¹Defence Institute of Bio-Energy Research (DRDO), Haldwani, Uttarakhand 263139, India. ²D.S.B Campus, Kumaun University, Nainital, Uttarakhand 263002, India. ³S. S. J. Campus, Kumaun University, Almora, Uttarakhand, India.

as *p*-cymene and linalool have also been reported to be found in the EOs of *Thymus* [14, 15]. However, the percentage of these chemical constituents might vary by origin, climatic conditions, soil conditions, the developmental stage/season and harvesting time of the plant [16]. The essential oil content varied between 0.1 and 0.6% [17]. A number of reports are available on the essential oil composition of wild *T. serpyllum* collected from India, Spain, Altai region, Lithuania, Estonia and Iran [18–25]. Different collections showed percentage variation in contents of essential oil, which largely depends on the plant origin and the growing conditions as well as ecological factors [18]. The literature survey revealed that the essential oil composition of *T. serpyllum* has been investigated from India [9, 10]. According to Farsam et al. [21], the quality of essential oils can be affected by agronomical practices and the high quality of essential oil is one of the main requirements of pharmaceutical and food industry. To the best of our knowledge, no work has been reported on the comparative essential oil composition of *T. serpyllum* grown at different altitudes of Uttarakhand. Therefore, the aim of the present study was to compare the chemical composition of these essential oils.

2 Materials and methods

2.1 Collection and cultivation

The samplings of *Thymus serpyllum* L. were collected from Jhalla village toward Malari, Chamoli district, Uttarakhand

(Altitude 3100 m, Latitude 30° 41' N, Longitude 79° 54' E) in the year 2016, and initially maintained at the Defence Institute of Bio-Energy Research (DIBER) Field station, Pithoragarh experimental field. The plant specimens were identified at Botanical Survey of India (BSI) (Accession No. 118592). After maintaining the specimen for 2 years, the species was vegetatively populated and transferred in the experimental plots of the DIBER Field station of Auli, Pithoragarh and Haldwani with the spacing 20 × 20 cm² in triplicate (Table 1). Before transferring the plants in the plots, the seedlings were rooted in horticulture substrate and then planted in open field experimental plots. Table 1 shows the geographic data of cultivation sites. Various intercultural operations like weeding, irrigation and Farm Yard Manure (FYM) application were performed in experimental plots. Plants were harvested at their respective full blooming stages from the selected location followed by washing, and then, the fresh material was used for further analyses.

2.2 Morphological variations of the plants

Cultivation of the plants under different geographic conditions brings a change in their chemical constituents as well as physicochemical properties. The length of stem, shape and size of the leaves is affected by geographic conditions where they have been grown. The morphological data of the plant samples evaluated in this study are given in Tables 2 and 3.

2.3 Isolation of essential oil

Five hundred gram of the fresh aerial parts of *T. serpyllum* (TS) from each location was hydrodistilled in a Clevenger apparatus. The hydrodistillation of the plants from each location was repeated three times with the same amount of fresh plant material. The obtained essential oils (EOs) were separated from water, then dried over anhydrous sodium sulfate and stored in dark sealed vials at low temperature (4 °C) prior to gas chromatography–flame ionization detector (GC–FID), gas chromatography–mass

Table 1 Geographic data of cultivation sites of *T. serpyllum*

Parameters	Auli (12 °C; 188% humidity)	Pithoragarh (26 °C; 121% humidity)	Haldwani (29 °C; 115% humidity)
Altitude (m)	2744	1524	412
Latitude	30.52° N	29.58° N	29.22° N
Longitude	79.57° E	80.22° E	79.52° E

Table 2 Morphological characteristics of *T. serpyllum* L. plants

<i>Thymus serpyllum</i> L.	Locations	Length of stem (cm) ± SD	Length of leaf (cm) ± SD	Width of leaf (cm) ± SD	Length of sub-branch (cm) ± SD	Color of flower ± SD
	Auli	20.73 ^a ± 0.21	0.27 ^a ± 0.15	2.17 ^a ± 0.49	0.57 ^a ± 0.21	Purple
	Pithoragarh	31.93 ^b ± 0.61	0.63 ^b ± 0.21	0.50 ^b ± 0.10	4.97 ^b ± 3.07	Purple
	Haldwani	16.73 ^c ± 1.76	0.60 ^b ± 0.10	0.30 ^b ± 0.10	7.23 ^b ± 0.96	Purple
SE		0.88	0.13	0.24	1.52	–

The mean values followed by different alphabets (a–c) in superscript within the column are significantly different at a significance level of 0.05; SD = standard deviation

Table 3 Variation in the fresh weight, percent moisture content and percent ash of *T. serpyllum*

Locations	Plot size (m × m)	Fresh plant weight (kg) ± SD	Moisture of fresh plant (%) ± SD	Moisture of dried plant (%) ± SD	Ash (%) ± SD
Auli	2 × 2	1.33 ^a ± 0.15	51.36 ^a ± 0.03	5.15 ^a ± 0.03	9.12 ^a ± 0.04
Pithoragarh		3.43 ^b ± 0.21	54.27 ^b ± 0.11	7.76 ^b ± 0.02	7.49 ^b ± 0.12
Haldwani		3.87 ^c ± 0.15	62.07 ^c ± 0.02	9.19 ^c ± 0.02	11.14 ^c ± 0.03
SE		0.14	0.05	0.02	0.06

The mean values followed by different alphabets (a–c) in superscript within the column are significantly different at a significance level of 0.05; SD = standard deviation

spectrometry (GC–MS) and high-performance thin-layer chromatography (HPTLC) analyses.

2.4 Physicochemical properties of essential oil

2.4.1 Physical properties of the essential oil

Authenticity of the essential oils was done by evaluating its various physical properties such as % oil yield, color, density and refractive index (RI) (density measurement by densitometer, DA-135N and RI by refractometer, RA-600). These parameters give an idea about the purity of essential oils and differentiate the pure essential oil from its adulterant [26, 27].

2.4.2 GC–FID and GC–MS analysis of essential oils

The qualitative and quantitative analyses of the EOs were done using GC–FID and GC–MS, respectively. The gas chromatography analyses were done with GC–FID Model 2010 (Shimadzu, Japan) equipped with a CombiPAL AOC-20i/s Autosampler (CTC Analytics, Zwingen, Switzerland). The compounds were separated on a Rxi®-5Sil-MS capillary column (30 m length, 0.25 mm internal diameter and 0.25 µm film thickness). The carrier gas was nitrogen. Injector and detector temperature were set at 260 °C and 290 °C, respectively. GC analysis of the essential oil of one location (Pithoragarh) was done using different model (Nucon 5765) set as identical conditions as above. For identification of the constituents, GC–MS analysis was done using model GC–MS QP-2010 (Shimadzu Scientific Instruments, Kyoto, Japan) equipped with a CombiPAL AOC-20i/s Autosampler (CTC Analytics, Zwingen, Switzerland). The compounds were separated on a Rxi®-5Sil-MS capillary column (30 m length, 0.25 mm internal diameter and 0.25 µm film thickness). The carrier gas was helium at a flow rate of 16.3 mL/min with a split ratio of 100:1. The injector temperature was 260 °C. The column oven temperature program used was: 50 °C (hold for 2 min) to 280.0 °C at 10 °C/min hold for 23 min. The ion source temperature and interface temperature were set at 230 °C and 270 °C, respectively, and the MS mode

was electron impact (EI). The compounds were separated by GC and further fragmented by mass spectrometer and identified by comparing the mass spectra obtained with NIST14 and WILEY8.LIB from the US National Institute of Technology, Standards (NIST) mass spectra libraries, calculating retention indices (Ri) relative to the homologous alkane series (C₉–C₃₃) and literature data [28]. Quantification of EO components was done by GC–FID peak area %.

2.5 High-performance thin-layer chromatography (HPTLC) fingerprints of essential oils from different locations

Each plant was identified by its unique fingerprint pattern. HPTLC has been proved to be an efficient tool for developing it. The chemical picture obtained from fingerprint represents the type of ecosphere where the plants have grown. The different secondary metabolites present in the plants may vary in quantity and quality depending on its growing conditions [29]. Therefore, in the present work, the HPTLC fingerprint profile of the essential oils of *T. serpyllum* grown at different agro-climatic zones has been developed. The 1.5 µL and 2 µL essential oil samples after dilution with toluene were applied in the form of 8 mm bands in three replicates through CAMAG linomat V sample applicator on Si60F254 (20 × 10) (Merck, Germany) of 0.2 mm thickness. After injection, the TLC plates were developed in Twin trough chamber saturated for 20 min with 20 mL of toluene/ethyl acetate (9.3:0.7 v/v) as mobile phase. After development, the plates were dried at room temperature, kept in a photo-documentation chamber (Camag Reprostar TLC Scanner 3) and images were captured under white light, UV light at 254 and 366 nm, respectively. Further, densitometric scanning was done through Vision Cat Software. After that, the plate was derivatized with freshly prepared vanillin–sulfuric acid reagent and heated at 105 °C on a TLC heater plate to observe the development of color of bands which was further photo-documented under white light to capture the image. The color and Rf of the bands were recorded.

2.6 Quantification of major compound

Thymol was the major compound identified in the GC–FID and GC–MS analyses. Further, it was quantified using HPTLC. For this, standard thymol was purchased from Sigma-Adrich. Standard solution (1 mg/mL) of thymol was prepared in toluene and was applied on TLC plate as 2 μ L, 4 μ L, 6 μ L, 8 μ L, 10 μ L in the form of 8 mm bands through CAMAG linomat V sample applicator on Si60F254 (20 $\times$ 10) (Merck, Germany) of 0.2 mm thickness. Along with this, 1.5 μ L essential oil samples after dilution with toluene were applied on the same plate in replicates. After injection, the TLC plates were developed in a Twin trough chamber with 20 mL of toluene: ethylacetate (9.3:0.7 v/v) as mobile phase and saturated for 20 min. After development, the plates were dried at room temperature, kept in kept in a photo-documentation chamber (Camag Reprostar TLC Scanner 3) and the images were captured under white light, UV light at 254 and 366 nm, respectively. Further, densitometric scanning was done through Vision Cat Software. After that, the plate was derivatized with freshly prepared vanillin–sulfuric acid reagent and heated at 105 °C on a TLC heater plate to observe the development of color of bands which was further photo-documented under white light to capture the images. The calibration curve was plotted using peak areas against corresponding concentrations. Linearity was tabulated by analyzing standard solutions at different concentrations. The peak area and the concentration of thymol were further subjected to regression analysis to evaluate the regression equation and correlation coefficients.

2.7 Statistical analyses

Experimental data were processed using Microsoft Excel (mean $\pm$ standard deviation; SD). The significance level of correlation coefficients was checked on probability levels of $p < 0.05$ and $p < 0.01$. The mean values were compared by Duncan test. All statistical analyses were performed using SPSS 16.0.

3 Result and discussion

3.1 Morphological characteristics of *T. serpyllum*

Due to the increasing demand of medicinal plants in herbal medicines, their conservation and cultivation has become the urgent need for health care nowadays. Cultivation of the plants under different geographic conditions brings a change in their chemical constituents as well as physicochemical properties. The length of stem, shape and size of the leaves is affected by geographic conditions

where the medicinal plants have been grown (Tables 2 and 3).

Primary data revealed that increasing altitude resulted in decreased leaf length (0.60–0.27 cm), sub-branch length (7.23–0.57 cm), fresh weight (3.87–1.33 kg) and moisture content (9.19–5.15%) (Tables 2 and 3). At mid-altitude, the stem length (31.93 cm) was observed to be the highest. *T. serpyllum* grown at higher altitudes (2744 and 1524 m) were observed to be longer (20.73 and 31.93 cm) than grown at foothills (16.73 cm) (412 m). The growth in high altitude might be due to nutrient rich soil. Nitrate nitrogen was observed to be significantly and positively correlated with stem length (Table 4; $r^2 = 0.871$; $p < 0.01$). The herb-age yield of *T. serpyllum* was the highest when grown at Haldwani (3.87 kg) followed by Pithoragarh (3.43 kg) and Auli (1.33 kg) in the same plot size (2 $\times$ 2 m) (Table 3). The length of sub-branch (7.23 cm), fresh weight (3.87 kg), moisture content (62.07 and 9.19%; fresh and dried plant, respectively) and ash (11.14%) were observed to be the highest when *T. serpyllum* was grown at Haldwani (foothills). The other parameters such as stem length and leaf length were observed to be the highest for *T. serpyllum* grown at Pithoragarh (lower Himalayas) (Tables 2 and 3). The width of the leaves was the highest for *T. serpyllum* grown at Auli (higher Himalayas). Color of the flowers was similar, i.e., purple for all the cultivated plants (Table 2).

3.2 Physicochemical parameters of oil

The extracted oils were light yellow to pale yellow in color. Refractive index varied from 1.4819 to 1.4873, while the density of the oils ranged between 0.943 and 0.952 g/mL.

3.3 Essential oil yield and composition

Previous investigations on EOs of *T. serpyllum* confirmed the presence of secondary metabolites such as aromatic terpenes, terpenoids, flavonoids and phenolic acid [7, 8, 11, 15]. Furthermore, the non-phenolic constituents such as *p*-cymene and linalool were also found in the EOs of *Thymus* spp. [14, 15]. GC–FID and GC–MS data revealed that the terpenoid constituents in the essential oil could be affected by the harvest season, climatic conditions, developmental stage and geographic region [18]. A total of 29, 21 and 16 components were identified in Auli, Pithoragarh and Haldwani cultivation representing 94.78%, 96.08% and 96.84% of the total oil, respectively (Figs. 1, 2, 3 and Table 6). The oil yield of *Thymus* grown at the lowest altitude and warm climatic zone (Haldwani) was the highest (0.84%) followed by cold climatic zones (Pithoragarh and Auli; 0.26% and 0.20%, respectively) (Table 5). Table 7 suggested that oil yield was positively correlated with the location temperature ($r^2 = 0.697$; $p < 0.05$) and negatively

Table 4 Correlation between soil properties and growth parameters of *T. serpyllum*

	Altitude	Stem length	Leaf length	Leaf width	Sub-branch	Fresh weight	Potassium	Ammonical nitrogen	Nitrate nitrogen	Conductivity	TDS
Altitude	1	0.226	-.657	0.885**	-.866**	-.936**	-.074	0.320	-.124	0.016	0.027
Stem length		1	0.226	-.164	-.038	0.104	-.189	-.495	0.871**	-.951**	-.958**
Leaf length			1	-.750*	0.416	0.761*	-.030	0.040	0.522	-.439	-.459
Leaf width				1	-.799**	-.938**	-.174	0.497	-.491	0.387	0.374
Sub-branch					1	0.891**	0.178	-.612	0.163	-.133	-.154
Fresh weight						1	0.045	-.456	0.414	-.337	-.352
Potassium							1	0.000	-.139	0.109	0.194
Ammonical nitrogen								1	-.459	0.519	0.512
Nitrate nitrogen									1	-.926**	-.918**
Conductivity										1	0.987**
TDS											1

**Correlation is significant at the 0.01 level (2-tailed); *Correlation is significant at the 0.05 level (2-tailed)

Fig. 1 Gas chromatogram of essential oil of *T. serpyllum* cultivated at Auli **Fig. 2** Gas chromatogram of essential oil of *T. serpyllum* cultivated at Pithoragarh

correlated with the altitude ($r^2 = -0.891$; $p < 0.01$). This may be due to the excessive low temperature at higher altitudes that causes cold stress to the plant and result in the loss of plant yield and quality of oil, while in a previous study on *Foeniculum vulgare* from Himalayan region showed that the oil yield was the highest in the collections from higher altitudes (Pithoragarh and Didihat) [30]. In the present investigation, EO of thyme grown at different altitudes showed great variation in the percentage of major compounds such as thymol, α -terpineol, p -cymene, camphor and γ -terpinene. Thymol was the major compound found in all the three locations with the highest percentage in Haldwani (84.63%) followed by Auli (50.80%) and Pithoragarh (41.15%) collection (Table 6). According to Saez, phenolic monoterpenes in thyme are accumulated at

Fig. 3 Gas chromatogram of essential oil of *T. serpyllum* cultivated at Haldwani

warm places, whereas, at cooler places, production of non-phenolic components favored [20]. Altitude ($r^2 = -0.723$; $p < 0.05$) was significantly and negatively correlated with the content of thymol in *T. serpyllum* (Table 7). Camphor was the second major compound in Pithoragarh cultivation (36.34%). Alpha-terpineol (9.93%) was identified as major constituent only in the Auli collection, and the content was found to be positive and significantly correlated with altitude ($r^2 = 0.879$; $p < 0.01$) and humidity ($r^2 = 0.997$; $p < 0.01$) while negatively correlated with temperature ($r^2 = -0.986$; $p < 0.01$) (Table 7). *p*-Cymene (10.80%) was present in the highest amount in Auli cultivation and was observed to be positively and significantly correlated with the altitude ($r^2 = 0.679$; $p < 0.01$) and humidity ($r^2 = 0.920$; $p < 0.01$) while negatively correlated with temperature of the location ($r^2 = -0.880$; $p < 0.01$) (Table 7). γ -Terpinene (1.52–4.45%), β -bisabolene (1.77–2.23%) and carvacrol

(1.82–3.30%) were also present in significant amount in all cultivations (Table 6). Eleven components, namely sabinene, ethyl hexanol, α -phellandrene, β -ocimene, α -terpineol, carvone, carvacrol acetate, α -humulene, allo-aromandendrene, δ -cadinene and β -sesquiphellandrene, were present only in Auli cultivated *Thymus*, while 1-Octen-3-ol, *o*-cymene, 1,8-cineole, terpinen-4-ol, thymol methyl ether and germacrene D were found only in the Pithoragarh collection. Furthermore, two compounds, namely δ -2-carene and carvacrol methyl ether, were present in the Haldwani cultivation. This chemical diversity could be attributed to environmental factors [31].

In Iran, the percentage oil yield of *Thymus pubescens* and *T. kotschyanus* varied in between 0.57 and 0.90%, and when the essential oil was analyzed using GC–MS, the major components were found to be *p*-cymene (21.1% and 20.7%), γ -terpinene (21.9% and 22.7%), thymol (18.7% and 18.7%) and germacrene D (6.0% and 5.1%) before flowering and full flowering stages, respectively [32]. In Lithuania *T. serpyllum*, the percentage oil yield varied from 0.12 to 0.17%. 1,8-Cineole, germacrene B, (*E*)- β -ocimene, α -cadinol and *cis*-*p*-menth-2-en-1-ol were the major components [24]. In Estonia, the oil yield of *T. serpyllum* was found to be 0.6–4.4%. (*E*)-Nerolidol, geranyl acetate, caryophyllene oxide, linalyl acetate, linalool and myrcene were observed as predominant compounds, while thymol and carvacrol were present in low quantity [25]. In a previous study on *T. serpyllum* from Poland, the percentage oil yield varied from 0.21 to 0.60% and carvacrol (37.49%), γ -terpinene (10.79%), (*E*)-caryophyllene (6.51%), *p*-cymene (6.06%), (*E*)- β -ocimene (4.63%) and β -bisabolene (4.51%) were the predominant compounds [17]. When the same species of *Thymus*, i.e., *T. serpyllum* was grown in India at an altitude of 1250 m in the Western Himalayas of Kattyur valley at the experimental farm of the Central Institute of Medicinal and Aromatic Plants, Research Centre, Purara, Uttarakhand, the percentage oil yield came out to be 0.8–1.3% [10]. The essential oil content was found to be the highest in summer season (0.28%) followed by rain (0.25%) and spring seasons (0.18%), whereas it was 0.17% and 0.07% during autumn and winter seasons, respectively [10]. The major components were carvacrol (37.49%), γ -terpinene (10.79%), β -caryophyllene (6.51%), *p*-cymene

Table 5 Physicochemical parameters of oil and percent oil yield

S. No.	Plants	Appearance	Oil (%) $\pm$ SD	RI (25 °C) $\pm$ SD	Density (g/mL) $\pm$ SD
1	T.S. Auli	Light yellow	0.20 ^a $\pm$ 0.01	1.4819 ^a $\pm$ 0.00	0.952 ^a $\pm$ 0.00
2	T.S. Pithoragarh	Light yellow	0.26 ^b $\pm$ 0.01	1.4825 ^b $\pm$ 0.00	0.943 ^b $\pm$ 0.00
3	T.S. Haldwani	Pale yellow	0.84 ^c $\pm$ 0.04	1.4873 ^c $\pm$ 0.00	0.948 ^c $\pm$ 0.00
SE		–	0.02	0.00	0.00

The mean values followed by different alphabets (a–c) in superscript within the column are significantly different at a significance level of 0.05; SD = standard deviation

Table 6 Chemical constituents identified in the EO of *T. serpyllum* cultivated at different altitudes

S. No.	Compound	Class of com-pound	R ^{cal}	R ^{lit}	RT (min) (TS Auli)	TS Auli per-centage of oil $\pm$ SD	RT (min) (TS Pithoragarh)	TS Pithoragarh per-centage of oil $\pm$ SD	RT (TS Haldwani)	TS Haldwani percentage of oil $\pm$ SD
1	α -Thujene	MH	924	924	8.4	2.04 ^a $\pm$ 0.02	8.6	0.63 ^b	8.5	0.12 ^c
2	α -Pinene	MH	930	932	8.7	0.86 ^a $\pm$ 0.01	8.9	0.37 ^b	8.8	0.10 ^c
3	Camphene	MH	945	946	9.3	0.43 ^a	9.6	1.15 ^b $\pm$ 0.10	–	ND
4	Sabinene	MH	969	969	10.3	0.23	–	ND	–	ND
5	β -Pinene	MH	974	974	10.5	0.3	10.7	0.06	–	ND
6	1-Octen-3-ol	AC	981	974	–	ND	10.8	0.15	–	ND
7	3-Octanone	AC	986	979	10.9	1.97 ^a $\pm$ 0.10	–	ND	10.9	0.69 ^b
8	Myrcene	MH	990	989	11	1.76 ^a $\pm$ 0.10	11	0.18 ^b	–	ND
9	Ethyl hexanol	AC	998	997	11.4	0.1	–	ND	–	ND
10	δ -2-Carene	MH	1004	1001	–	ND	–	ND	11.5	0.06
11	α -Phellandrene	MH	1005	1002	11.7	0.21	–	ND	–	ND
12	<i>p</i> -Cymene	MH	1025	1020	12.7	10.80 ^a $\pm$ 0.02	12.8	0.75 ^b	12.7	4.05 ^c $\pm$ 0.02
13	<i>o</i> -Cymene	MH	1027	1022	–	ND	13.3	4.39 $\pm$ 0.01	–	ND
14	Limonene	MH	1029	1024	12.8	0.66 ^a $\pm$ 0.02	13.5	0.93 ^b	–	ND
15	1,8-Cineole	OM	1030	1026	–	ND	13.7	0.73	–	ND
16	β -Ocimene	MH	1045	1044	13.5	0.05	–	ND	–	ND
17	γ -Terpinene	MH	1059	1054	14.2	4.45 ^a $\pm$ 0.01	15.1	2.82 ^b $\pm$ 0.02	14.2	1.52 ^c $\pm$ 0.10
18	Linalool	OM	1099	1095	16.1	0.06	16.6	0.2	16.2	0.06
19	Camphor	OM	1154	1141	–	ND	20	36.34 $\pm$ 0.03	–	ND
20	Terpinen-4-ol	OM	1181	1174	–	ND	21.7	0.07	19.9	0.09
21	α -Terpineol	OM	1195	1186	21.1	9.93 $\pm$ 0.01	–	ND	–	ND
22	Thymol methyl ether	OM	1230	1232	–	ND	23.5	0.54	–	ND
23	Carvacrol methyl ether	OM	1231	1241	–	ND	–	ND	21.1	0.89
24	Carvone	OM	1232	1239	23.9	0.08	–	ND	–	ND
25	Thymol	OM	1279	1289	25.8	50.80 ^a $\pm$ 0.01	26.5	41.15 ^b $\pm$ 0.02	25.8	84.63 ^c $\pm$ 0.02
26	Carvacrol	OM	1322	1298	25.9	3.30 ^a $\pm$ 0.02	26.9	2.70 ^b $\pm$ 0.02	25.9	1.82 ^c $\pm$ 0.01
27	Thymol acetate	OM	1351	1349	28.3	0.10 ^a $\pm$ 0.01	28.9	0.82 ^b	28.3	0.03 ^a
28	Carvacrol acetate	OM	1366	1370	28.5	1.55 $\pm$ 0.03	–	ND	–	ND
29	Copaene	SH	1373	1374	28.7	0.56	–	ND	28.7	0.02
30	(<i>E</i>)-Caryophyllene	SH	1418	1417	30.6	1.57 ^a $\pm$ 0.02	–	ND	30.5	0.47 ^b
31	α -Humulene	SH	1451	1452	32	0.08	–	ND	–	ND
32	Allo-aromandendrene	SH	1454	1458	31.7	0.07	–	ND	–	ND
33	Gemacrene D	SH	1484	1484	–	ND	34	0.25	–	ND
34	β -Bisabolene	SH	1508	1505	34.3	2.23 ^a $\pm$ 0.02	35.1	1.77 ^b $\pm$ 0.02	34.3	2.11 ^c $\pm$ 0.03

Table 6 (continued)

S. No.	Compound	Class of com-pound	R ^{cal}	R ^{lit}	RT (min)	(TS Auli)	TS Auli per-centage of oil ± SD	RT (min) (TS Pithoragarh)	TS Pithoragarh per-centage of oil ± SD	RT (TS Haldwani)	TS Haldwani percentage of oil ± SD
35	δ-Cadinene	SH	1515	1513	34.6		0.03	–	ND	–	ND
36	β-Sesquiphellandrene	SH	1520	1521	34.9		0.04	–	ND	–	ND
37	Spathulenol	OS	1573	1577	36.9		0.23	–	ND	36.9	0.18
38	Caryophyllene oxide	OS	1577	1582	37.1		0.29	38	0.08	–	ND
Total compounds					29			21		16	
Total identified (%)					94.78			96.08		96.84	

Mode of identification: retention index (RI), MS (GC–MS); R^{cal}: retention indices on Rxi⁵-5SII-MS capillary column; R^{lit}: retention indices [28]; the % of the individual components were calculated based on peak area (FID) response; TS Auli: essential oil of *Thymus serpyllum* L. Auli; TS Pithoragarh: essential oil of *Thymus serpyllum* L. Pithoragarh; TS Haldwani: essential oil of *Thymus serpyllum* L. Haldwani; RT-retention time; ND-not detected; MH-monoterpene hydrocarbons; AC-aliphatic compounds; OM-oxygenated monoterpenes; SH-sesquiterpene hydrocarbons; OS-oxygenated sesquiterpenes; mean values in each row followed by different alphabets (a–c) at superscript are significantly different at $p < 0.05$ according to Duncan's multiple range test

Table 7 Correlation between altitudes of locations, major constituents, fresh weight and physical properties of oil of *T. Serpyllum*

	Thymol	α-Terpineol	p-Cymene	Camphor	Oil yield	Refractive index	Density	Altitude	Fresh weight	Moisture	Ash	Temperature	Humidity
Thymol	1.000	–.306	0.017	–.672*	0.953**	0.950**	0.267	–.723*	0.451	0.888**	0.968**	0.459	–.376
α-Terpineol		1.000	–.947**	–.500	–.570	–.585	0.813**	0.879**	–.979**	–.710*	–.064	–.986**	0.997**
p-Cymene			1.000	–.752*	–.276	–.292	–.943**	0.679*	–.876**	–.445	0.260	–.880**	0.920**
Camphor				1.000	–.424	–.409	–.875**	–.027	–.352	–.255	–.831*	0.350	–.435
Oil yield					1.000	0.998**	–.019	–.891**	0.690*	0.980**	0.851**	0.697*	–.629
Refractive index						1.000	–.041	–.900**	0.699*	0.985**	0.845**	0.710*	–.643
Density							1.000	0.456	–.710*	–.194	0.477	–.712*	0.770*
Altitude								1.000	–.936**	–.960**	–.531	–.946**	0.912**
Fresh weight									1.000	0.807**	0.218	0.992**	–.988**
Moisture										1.000	0.746*	0.817*	–.760*
Ash											1.000	0.228	–.138
Temperature												1.000	–.996**
Humidity													1.000

**Correlation is significant at the 0.01 level (2-tailed); *Correlation is significant at the 0.05 level (2-tailed)

(6.06%), (*E*)- β -ocimene (4.63%) and β -bisabolene (4.51%) [10]. *T. serpyllum* grown in Jammu and Kashmir at an elevation of 12,000 feet above sea level in Pir Chanassi was observed to be rich in thymol (16.5–18.8%), borneol (1.6–3.1%), ledol (0.2–7.3%), safrole (1.1%), 1,8-cineole (14.0–18.0%), spathulenol (1.3–2.1%) and thymoquinone (2.6%) [33]. The percentage of oxygenated monoterpenes (OM) was the highest in the *T. serpyllum* cultivated at medium altitude (Pithoragarh), while the content of monoterpene hydrocarbons (MH) was the lowest in the same cultivation (Fig. 4).

Fig. 4 Variation in the compound classes of the cultivated *T. serpyllum*. Different alphabets showed significant difference at a significant level of $p < 0.05$ according to Duncan's Multiple range test

3.4 Fingerprints of essential oil

As previously discussed, the authenticity of the essential oil is an important aspect. Besides physicochemical properties, obtaining the fingerprint pattern of the essential oil also authenticates and identifies the plant. HPTLC is a versatile tool for obtaining the chemical fingerprint of the plant. The HPTLC chromatogram of essential oil of *T. serpyllum* L. and before derivatisation showed a single intense band (R_f 0.56) under UV-254 nm in all the samples, suggesting it to be the major compound, but when derivatized with vanillin–sulfuric acid, the essential oil of TS showed 4 intense colored bands of the terpenoid compounds (Fig. 5). The fingerprint pattern can act as a biomarker for its chemical identity. Although the pattern of a fingerprint is same for each plant, the chemotypes and their concentration vary according to the growing conditions, developmental stage and climatic conditions. These fingerprint patterns can give information on the purity of the oils which can be used in various herbal formulations [34].

3.5 Quantification of major compound

HPTLC is a rapid, versatile and economic technique for the identification and quantitative determination of the compounds. Thymol was the major compound found in all the three essential oils (Fig. 6). The presence of thymol and its quantity was also confirmed using its standard in HPTLC. Thymol showed absorption maxima at an absorbance of 274 nm. The results revealed that the R_f

Fig. 5 Derivatized TLC plate showing fingerprint pattern of *T. Serpyllum* EOs under white light

Fig. 6 TLC of standard thymol and the *T. Serpyllum* EO samples under UV-274 nm

Table 8 Rf value and area percent of the peaks of standard and oils

Track	Sample	Max Rf	Area
1	Thymol (reference)	0.483	0.01386
2	Thymol (reference)	0.483	0.02035
3	Thymol (reference)	0.484	0.02517
4	Thymol (reference)	0.484	0.02874
5	Thymol (reference)	0.486	0.03141
6	Thymol (TS Auli sample)	0.484	0.02644
8	Thymol (TS Pithoragarh sample)	0.484	0.02592
10	Thymol (TS Haldwani sample)	0.483	0.02624

of standard thymol coincides with that of the intense pink band in the sample at 0.484 ± 0.002 (Table 8). When samples were scanned at the same wavelength, a sharp band was observed confirming the presence of thymol in each plant sample. For the quantification of thymol in the samples, calibration curve was plotted at different concentrations. The calibration curve was found to be linear in the concentration range of (2000–10000 ng/spot). Densitogram scanned at 274 nm shows peak area of standard thymol and thymol in the samples (Fig. 7).

Linear regression data showed that the value of r^2 was 0.986. The lowest amount that could be detected was $0.34 \mu\text{g/spot}$ and the lowest quantity that could be determined was $1.04 \mu\text{g/spot}$ (Table 9). The amount of thymol was found to be the highest in the essential oil of TS from Haldwani ($752.7 \mu\text{g/mg}$) followed by Auli ($494.1 \mu\text{g/mg}$) and the lowest in the case of Pithoragarh ($397.1 \mu\text{g/mg}$) (Table 10).

4 Conclusions

The study revealed that the plant yield, oil percent and major components of the essential oil were the highest in the case of *T. serpyllum* cultivated Haldwani and the lowest in the *T. serpyllum* from Pithoragarh. Camphor was identified only in the essential oil of *T. serpyllum* cultivated at Pithoragarh. Quantification by HPTLC gave similar results as the peak area % of GC/FID response for thymol. The study also suggested that warm climatic condition is suitable for cultivation of *T. serpyllum* in terms of thymol and oil production. Further, comparative study of the comparative biological activity of the essential oil is in progress.

Fig. 7 Densitogram showing peak area of thymol standard (Track 1–5) and the *T. serpyllum* Pithoragarh, Auli, Haldwani EOs in duplicates

Table 9 Linear regression data for the calibration curves with LOD and LOQ

S. No.	Calibration curve data	Obtained values
1	Absorption maxima	274 nm
2	Linearity ($\mu\text{g}/\text{spot}$)	(2–10)
3	Regression ($Y=mX+C$)	$2.168 \times 10^{-9}X + 1.083 \times 10^{-2}$
4	Correlation coefficient	0.986
5	Limit of detection (LOD)	0.34 μg
6	Limit of Quantification (LOQ)	1.04 μg
7	Slope	2.168×10^{-9}
8	Intercept	1.083×10^{-2}

Table 10 Amount of thymol present in TS essential oils

S. No.	Linearity range (500–2500 ng/spot)	Thymol (μg) in 1 mg of sample	Thymol (%)
1	TS EO Auli	494.1	49.41
2	TS EO Pithoragarh	397.1	39.71
3	TS EO Haldwani	752.7	75.27

Acknowledgements The financial support from Defence Research and Development Organization (DRDO) is gratefully acknowledged. The authors wish to thank Director DIBER, DRDO for providing

logistics and research facility for successful conduction of the research work.

Compliance with ethical standards

Conflict of interest There is no conflict of interest.

References

1. Mukerjee SK (1940) A revision of the Labiatae of the Indian empire. Rec Bot Surv India 14:93
2. Kayani S, Ahmad M, Zafar M (2014) Ethnobotanical uses of medicinal plants for respiratory disorders among the inhabitants of Gallies-Abbottabad, Northern Pakistan. J Ethnopharmacol 156:47–60
3. Stahi-Biskup E, Saez F (2002) Thyme-the genus *Thymus*. Taylor & Francis, London
4. Davidson PM, Naidu AS (2000) Phyto-phenols. In: Naidu AS (ed) Natural food antimicrobial systems. CRC Press, Boca Raton, pp 265–294
5. Ahmad AM, Khokhar I, Ahmad I, Kashmiri MA, Adnan A, Ahmad M (2006) Study of antimicrobial activity and composition by GC/MS spectroscopic analysis of the essential oil of *Thymus serpyllum*. Internet J Food Saf 5:56–60
6. Miri R, Ramezan M, Javidnia K, Ahmadi L (2002) Composition of the volatile oil of *Thymus transcaspicus* Klokov from Iran. Flavour Fragr J 17:245–246
7. Miguel G, Simoes M, Figueiredo AC, Barroso JC, Pedro LG, Carvalho L (2004) Composition and antioxidant activities of the

- essential oils of *Thymus caespititius*, *Thymus camphoratus* and *Thymus mastichina*. Food Chem 86:183–188
8. Ebrahimi SN, Hadian J, Mirjalili MH, Sonboli A, Yousefzadi M (2008) Essential oil composition and antibacterial activity of *Thymus caramanicus* at different phenological stages. Food Chem 110:927–931
 9. Verma RS, Rahman L, Chanotiya CS, Verma RK, Singh A, Yadav A, Chauhan A, Yadav AK, Singh AK (2009) Essential oil composition of *Thymus serpyllum* cultivated in the Kumaon region of western Himalaya, India. Nat Prod Commun 4(7):987–988
 10. Verma RS, Verma RK, Chauhan A, Yadav AK (2011) Seasonal variation in essential oil content and composition of thyme, *Thymus serpyllum* L. cultivated in Uttarakhand hills. Indian J Pharm Sci 73(2):233–235
 11. Teimouri M (2012) Antimicrobial activity and essential oil composition of *Thymus daenensis* Celak from Iran. J Med Plant Res 6:631–635
 12. Nikolić M, Glamoclija JM, Ferreira ICFR, Calhelha RC, Fernandes A, Marković TLJ, Marković DLJ, Giweli A, Soković M (2013) Chemical composition, antimicrobial, antioxidant and antitumor activity of *Thymus serpyllum* L., *Thymus algeriensis* Boiss. and Reut and *Thymus vulgaris* L. essential oils. Ind Crops Prod 52:183–190
 13. Kirillov V, Stikhareva T, Mukanov B, Chebotko N, Ryazantsev O, Atazhanova G, Adekenov S (2016) Composition of the essential oil of *Thymus serpyllum* L. from Northern Kazakhstan. J Essent Oil Bear PI 19(1):212–222
 14. Piccaglia R, Marotti M (1991) Composition of the essential oil of an Italian *Thymus vulgaris* L. ecotype. Flavour Fragr J 6:241–244
 15. Sefidkon F (2002) Essential oil composition of *Thymus pubescens* and *T. kotschyanus* from Iran. J Essent Oil Res 14:116–117
 16. Mancini E, Senatore F, Del Monte D, De Martino L, Grulova D, Scognamiglio M, Snoussi M, De Feo V (2015) Studies on chemical composition, antimicrobial and antioxidant activities of five *Thymus vulgaris* L. essential oils. Molecules 20(7):12016–12028
 17. Wesołowska A, Grzeszczuk M, Jadczyk D, Nawrotek P, Struk M (2015) Comparison of the chemical composition and antimicrobial activity of *Thymus serpyllum* essential oils. Not Bot Horticult Agrobot 43(2):432–438
 18. Banava YA, Pokrovsky LM, Tkachev AV (1998) Chemical composition of the essential oil of *Thymus serpyllum* L.s.l. growing wild in Altai region. In: International conference on natural products and physiologically active substances (ICNPAS-98). November 30–December 6, p 59, Novosibirsk, Russia
 19. Mathela CS, Agarwal J, Taskinen J (1980) Composition of essential oil of *Thymus serpyllum* Linn. J Indian Chem Soc 57:1249–1250
 20. Saez F (1995) Essential oil variability of *Thyme zygis* growing wild in southeastern Spain. Phytochemistry 40:819–825
 21. Farsam H, Amanlou M, Radpour MR, Salehinia AN, Shafiee A (2004) Composition of the essential oils of wild and cultivated *Satureja khuzistanica* Jamzad from Iran. Flavour Fragr J 19:308–310
 22. Mockute D (2004) 1,8-Cineole-caryophyllene oxide chemotype of essential oil of *Thymus serpyllum* L. growing wild in Vilnius (Lithuania). J Essent Oil Res 16:236–238
 23. Raal A, Paaver U, Arak E, Orav A (2004) Content and composition of the essential oil of *Thymus serpyllum* growing wild in Estonia. Medicina Kaunas 40:795–800
 24. Ložionė K, Venskutonis RP (2006) Chemical composition of the essential oil of *Thymus serpyllum* L. ssp. *serpyllum* growing wild in Lithuania. J Essent Oil Res 18:206–221
 25. Paaver U, Orav A, Arak E, Mäeorg U, Raal A (2008) Phytochemical analysis of the essential oil of *Thymus serpyllum* L. growing wild in Estonia. Nat Prod Res 22(2):108–115
 26. Turek C, Stintzing FC (2013) Stability of essential oils: a review. Compr Rev Food Sci F 12(1):40–53
 27. Do TKT, Minaglou FH, Antonietti S, Fernandez X (2015) Authenticity of essential oils. Trends Anal Chem 66:146–157
 28. Adams RP (2007) Identification of essential oil components by gas chromatography/mass spectrometry, 4th edn. Allured Publishing, Carol Stream
 29. Guerrini A, Sacchetti G (2014) Chemical fingerprinting of medicinal and aromatic plant extracts: HPTLC bioautographic assays as preliminary research tool to match chemical and biological properties. Med Aromat Plants 3(3):1000e152
 30. Punetha D, Tewari G, Pande C, Bhatt S (2019) Effect of climatic conditions on the volatile compounds of the aerial parts of *Foeniculum vulgare* Mill. J Essent Oil Bear PI 22(4):1093–1103
 31. Stahl-Biskup E, Laakso I (1990) Essential oil polymorphism in Finnish *Thymus* species. Planta Med 56:464–468
 32. Sefidkon F, Dabiri M, Mirmostafa SA (2004) The composition of *Thymus serpyllum* L. oil. J Essent Oil Res 16:184–185
 33. Aziz S, Irshad HRM, Asghar SF, Hussain H, Ahmed I (2010) Phytotoxic and antifungal activities of essential oils of *Thymus serpyllum* grown in the state of Jammu and Kashmir. J Essent Oil Bear PI 13(2):224–229
 34. Asha D, Lizzy M (2017) Chemical profiling of *Thymus vulgaris* L. using HPTLC. J Pharmacogn Phytochem 6(4):1017–1023

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.