

Variation in antibacterial activity, thymol and carvacrol contents of wild populations of *Thymus daenensis* subsp. *daenensis* Celak.

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Abstract

Thymus daenensis subsp. *daenensis* is an endemic aromatic and medicinal plant of Iran. The study was conducted to determine variations of antibacterial activity, thymol and carvacrol content in different populations of *Thymus daenensis*. The flowering aerial parts of *Thymus daenensis* were collected from ten locations in two provinces, Iran. The antibacterial activity of the extract was tested assays against four pathogens (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Escherichia coli*). The extract was characterized using HPLC. The amounts of thymol and carvacrol, the main components, varied 13.89 to 35.89 and 7.45 to 23.53 mg/g extract, respectively. The highest content of thymol in all investigated samples was obtained from Sheykhshaban population. The extract of Sheykhshaban population showed the strongest antibacterial activity. This population has the highest elevation in comparison with others. So, the higher altitudes might improve the contents with more effective antimicrobial activity.

Keywords: *Thymus daenensis* subsp. *daenensis*, wild population, antibacterial activity, thymol.

Abbreviations: HPLC: High performance liquid chromatography. GPS: Global Positioning System. DEM: Digital Elevation Model. GIS: Geographic Information System. UV: Ultra Violet. DMSO: Dimethyl sulfoxid. MIC: Minimal Inhibitory Concentration.

Introduction

The genus *Thymus* L. belongs to the mint family (Lamiaceae), and consists of about 215 species of herbaceous perennials and small shrubs in the world. The Mediterranean region can be described as the center of the genus (Cronquist, 1988; Heywood, 1993; Morales, 2002; Stahl-Biskup and Saez, 2002). Fourteen *Thymus* species has been reported in Flora Iranica (Jalas, 1982; Stahl-Biskup and Saez, 2002), four of which, *T. carmanicus* Jalas, *T. daenensis* Celak subsp. *daenensis* Celak, *T. daenensis* Celak subsp. *lancifolius* (Celak.) Jalas, *T. persicus* (Roniger ex Reach. F.) and *T. trautvetteri* Klokov & Desj.-Shost have been known to be endemic (Rechinger, 1982). *T. daenensis* subsp. *daenensis* is an endemic subspecies of Iran. This subspecies generally grows in high altitudes in Zagros mountains range (Rahimmalek et al., 2009). The areal parts and volatile constituents of thyme, a perennial dwarf shrub, are used as a medicinal herb. *Thymus* species are commonly used for herbal tea, flavoring agents (condiment and spice) and medicinal purposes (Stahl-Biskup and Saez, 2002). Infusion and decoction of aerial parts of *Thymus* species are used to produce a tonic, carminative, digestive, antispasmodic, anti-inflammatory, and expectorant and for the treatment of colds in Iranian traditional medicine (Zargari, 1990; Nickavar et al., 2005; Ghasemi Pirbalouti, 2009). Recent studies have shown that *Thymus* species have strong antibacterial, antifungal, antiviral, anti-parasites, spasmolytic and antioxidant activities (Rahimmalek et al., 2009b; Jordan et al., 2009). The pervious study showed that essential oil and extract of *T. daenensis* exhibited antimicrobial activities against *Candida albicans* (Ghasemi Pirbalouti et al., 2009a), *Listeria monocytogenes*

(Ghasemi Pirbalouti et al., 2009b), *Campylobacter jejuni* and *Campylobacter coli* (Ghasemi Pirbalouti et al., 2010a), *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Ghasemi Pirbalouti et al., 2010b), *Escherichia coli* O157:H7 (Ghasemi Pirbalouti et al., 2010c) and *Saprolegnia parasitica* (Ghasemi Pirbalouti et al., 2009c). The aromatic and medicinal properties of the genus *Thymus* have made it one of the most popular medicinal plants (Nickavar et al., 2005). It is believed that these activities are to some extent caused by the constituents. Therefore, there is a considerable research interest towards the compositional analysis of *Thymus* essential oil and extract (Stahl-Biskup and Saez, 2002). It has been reported that essential oil yield and their components in plants is related to genetic (Mohammad Shafie et al., 2009), climate, edaphic, elevation and topography (Pourhohit and Vyas, 2004; Rahimmalek et al., 2009a) and genotyp (G), growing conditions (E) and their interaction (G x E) (Basu et al., 2009). Chemical polymorphisms or chemotypes have been reported for many medicinal plants (Mockute et al., 2001; Russell and Southwell 2003; Curado et al., 2006; Mohammad Shafie et al., 2009). Recent findings showed that some of the medicinal plant characteristics can be affected by genetic and ecological factors such as precipitation, temperature, plant competition and nitrogen content in the soil (Letchamo et al., 1995). For example, higher altitude, variation in soil type had profound effect in the spread and amount of volatile constituents of *T. serpylloides* in Southeastern Spain (Letchamo et al., 1995). However, there are no reports in assessment of major compounds (thymol and carvacrol) of *T.*

daenensis subsp. *daenensis* essential oil growing in different climatic regions of the country. The aims of this study were (1) to evaluate the antibacterial properties of the extract of wild populations of *T. daenensis*, grown in various geographical regions of Iran, (2) to determine the variation of thymol and carvacrol contents of different populations, (3) to assess the relationships between variations of thymol and carvacrol contents and the environmental factors involved in different geo-ecological regions.

Results and discussion

Characterization of soil and climatic conditions of natural habitats

The results showed that most of natural habitats were in high altitudes (2000-2800 m above sea level). The soil and climatic information of selected regions was summarized in Table 1.

Thymol and carvacrol contents

The major compounds (thymol and carvacrol) of the ten accessions were determined. The amounts of thymol and carvacrol of flowering areal parts (mean of four replicates) ranged from 13.89 mg/g to 35.89 mg/g and 7.45 mg/g to 23.53 mg/g in all populations, respectively (Table 2). Previous studies on another Iranian *Thymus* species showed that the main components of the oils and extracts were carvacrol and thymol (Rustaiyan et al., 2000; Sefidkon et al., 2002; Rasoli and Mirmostafsa, 2003; Sajjadi and Khatamsaz, 2003; Nickavar et al. 2005; Mojab and Nickavar, 2006; Nejad Ebrahimi et al., 2008). In this study, higher amount of thymol was observed in comparison with carvacrol content (Table 2). Nickavar et al. (2005) reported that the essential oil of the aerial parts of *Thymus daenensis* subsp. *daenensis* have twenty six components, which represented about 99.7% of the total detected constituents. In their study the major compounds were: thymol (74.7%), p-cymene (6.5%), b-caryophyllene (3.8%) and methyl carvacrol (3.6%).

Antibacterial test

Preliminary screening of the *in vitro* antimicrobial activity of ten extracts from different localities against four pathogens microorganisms was studied using the paper disc agar diffusion technique. The results showed significant variation in the antimicrobial properties of extracts (Table 2). The extracts showed strong activity (inhibition zone ≥ 20 mm) and moderate activity (inhibition zone $< 20-12$ mm). Attending to this, the major effectiveness was achieved by the extracts from Shaykhshaban population from Chaharmahal va Bakhtiari province. However, more precise data on the antimicrobial properties were obtained through determination of bacteriostatic concentrations. The minimum inhibitory concentration (MIC; μg extract/mL medium) (against four microorganisms) of ten extracts are shown in Table 2. The extract with the most bacteriostatic properties were: Shaykhshaban, Dezak and Daran populations with $\text{MIC} \leq 19$ $\mu\text{g/mL}$ against three strains tested. In present study, comparison of the amounts of thymol of *Thymus daenensis* subsp. *daenensis* extract in different geographic conditions showed that there are some qualitative and quantitative

differences between ten localities in Chaharmahal va Bakhtiari and Isfahan provinces of Iran that may have been caused by the genetic differences and different environmental factors. Previous findings on other Lamiaceae plants have shown that the variation of their quantitative extract composition is attributed to the geographic direction (Kokkini et al., 1997; Karousou et al., 1998; Yavari et al., 2010). Karousou et al. (2005) showed that high carvacrol content in two species (*Coridothymus capitatus* Reichenb. fil. and *Satureja thymbra* L.) is associated to the dry dwarf-shrub formations of the lowland, whereas a high thymol content is related to the more mesic timber or highland formations. Also, they reported that the relation between oil composition and the natural habitats of the collected plants suggests the use of natural habitat unit as a tool for the assessment and prediction of variation in essential oil in a single species (Karousou et al., 2005). The highest amount of thymol in all investigated samples was recorded in Shaykhshaban population from Chaharmahal va Bakhtiari province (35.89 mg/g extract), while the lowest was observed in Sabz-e-koh population from the same province (13.89 mg/g extract) (Table 2). Altitude seems to be the most important environmental factor influencing the thymol content, where high thymol contents were obtained at high altitudes (Table 2). Yavari et al. (2010) reported that there were positive relationship between some essential oil characters of *Thymus migricus* and some environmental factors. The influence of environmental factors over p-cymene, gamma-terpinene, linalool and thymol concentration was evidently showed. Essential oil yield was fairly strongly related to the concentrations of Ca^{2+} and K^+ , percentage of organic matter, altitude, temperature, and soil texture (Yavari et al. 2010). A number of studies in the phenol-rich Lamiaceae species *Thymus vulgaris* L. (Gouyon et al., 1986), *T. piperella* L. (Boira and Blanquer, 1998) and *Origanum vulgare* L. (Vokou et al., 1993) have shown that the preponderance of carvacrol or thymol in their essential oils is associated to climatic conditions. The highest amount of carvacrol in all investigated samples was recorded in Larak population from Chaharmahal va Bakhtiari province (23.53 mg/g extract), while the lowest was observed in Koohrang population from the same province (7.45 mg/g extract) (Table 2). The results showed that some of the geographic, climatology and edaphic factors had no significant effects on the thymol and carvacrol content. Shan et al. (2007) reported that a total of 46 spice and herb extracts from different regions contained high levels of phenolics and exhibited antibacterial activity against foodborne pathogens. They suggested that there were highly positive relationships ($R^2=0.73-0.93$) between antibacterial activities and phenolic content of the tested extracts against each bacterium. Some studies claim that the phenolic compounds present in spices and herbs might also play a major role in their antimicrobial effects (Hara-Kudo et al., 2004). However no large scale systematic investigation of the relationship between bacterial inhibition and total phenolic content of spices and herbs has been reported. In this study, most of the antimicrobial activity in extracts from different populations appears to be explainable by phenolic compounds (thymol and carvacrol). These results agree with those reported by other researchers (Consentino et al., 1999; Davidson and Naidu, 2000; Skocibusicet al., 2006; Rota et al., 2008). The Shaykhshaban population with high amounts of thymol proved to be more active with diameters of

Table 1. Geographical and climatic of natural habitats of 10 populations belonging to *Thymus daenensis* subsp. *daenensis*

| Origin | Province | Elevation | Latitude | Longitude | P ^a | T ^b | pH | E.C. ^d | O.C. ^e | P ^f | N ^g | K ^h |
|--------------|----------|-----------|-----------|-----------|----------------|----------------|------|-------------------|-------------------|----------------|----------------|----------------|
| Sabz-e-koh | Ch va Bk | 2292 | 31° 49' N | 50° 51' E | 593 | 15 | 7.60 | 0.62 | 2.79 | 59.1 | 0.288 | 711 |
| Larak | Ch va Bk | 2370 | 32° 34' N | 50° 39' E | 345 | 11.3 | 7.82 | 0.52 | 1.54 | 12.7 | 0.146 | 998 |
| Shahrekord | Ch va Bk | 2045 | 32° 21' N | 50° 53' E | 323 | 11.3 | 8.05 | 0.76 | 1.13 | 15.9 | 0.158 | 741 |
| Dezak | Ch va Bk | 2298 | 32° 06' N | 51° 03' E | 443 | 11.5 | 8.03 | 0.50 | 0.58 | 18.3 | 0.061 | 456 |
| Sheykhshaban | Ch va Bk | 2747 | 32° 35' N | 50° 38' E | 382 | 11 | 7.76 | 0.49 | 0.461 | 11.4 | 0.052 | 402 |
| Koohrang | Ch va Bk | 2479 | 32° 27' N | 50° 17' E | 1415 | 9.3 | 7.58 | 0.60 | 0.71 | 16.3 | 0.073 | 567 |
| Daran | Isfahan | 2303 | 32° 56' N | 50° 26' E | 348 | 12.9 | 7.56 | 0.66 | 1.10 | 56.7 | 0.110 | 528 |
| Semirom | Isfahan | 2302 | 31° 40' N | 51° 33' E | 407 | 11 | 7.78 | 0.74 | 0.94 | 18.7 | 0.106 | 653 |
| Hamgin | Isfahan | 1806 | 31° 55' N | 51° 23' E | 250 | 12.3 | 7.65 | 1.13 | 2.27 | 23.2 | 0.141 | 567 |
| Khansar | Isfahan | 2502 | 33° 09' N | 50° 26' E | 407 | 12.2 | 7.47 | 0.71 | 1.09 | 17.1 | 0.263 | 914 |

^a P: Annual precipitation (mm), ^b T: Average temperature (°C), ^d E.C.: electrical conductivity (dS/m), ^e O.C.: organic carbon (%), ^f P: Available P (mg/kg), ^g N: total nitrogen (%), ^h K: Available K (mg/kg)

inhibition zone ranging from 20-24 mm, MIC≤19 µg/mL against *E. coli*, *S. aureus* and *P. aeruginosa* and MIC≤156 µg/mL against *B. cereus*. *E. coli* was the most susceptible organism; its growth presented strong inhibition by all extracts tested with MIC≤19 µg/mL. Also, our results indicated that *S. aureus* was more sensitive than *B. cereus* and *P. aeruginosa*. These chemical differences can be most probably explained by the variability of the genetic factors as well as the existence of different chemotypes. Sheikhshaban population might be a potential thymol-rich source for mass-cultivation in order to improve commercial purposes.

So, the altitudes between 2400 to 2800 meters above sea level might be introduced as the best location for the production of quantity effective materials for this plant aimed to attain the best possible results. The extracts of wild populations *T. daenensis* have a stronger antibacterial activity as compared to the positive antibacterial standards. The phenolic compounds, such as thymol and carvacrol, are widely reported to possess high levels of antimicrobial activity (Baydar et al., 2004; Nejad Ebrahimi et al., 2008). Several studies have focused on the antimicrobial activity of the essential oils and extracts of thyme in order to identify the responsible compounds (Burt, 2004; Crespo et al., 1990; Nelson, 1997). Thymol and carvacrol, which are the main components of *T. daenensis* essential oil and extract, have been considered as biocidal, resulting in bacterial membrane perturbations that lead to leakage of intracellular ATP and potassium ions and ultimately cell death (Helander et al., 1998; Juven et al., 1994; Ultee et al., 1999). Some studies claim that the phenolic compounds present in spices and herbs might also play a major role in their antimicrobial effects (Hara-Kudo et al., 2004).

There has been no large scale systematic investigation of the relationship between bacterial inhibition and total phenolic content of spices and herbs. Previous studies (Shan et al., 2005) showed that in some spices and herbs a highly positive linear relationship exists between antioxidant activity and total phenolic content. Many herb and spice extracts such as *T. daenensis* and *S. bachtiarica* contained high levels of phenolics and exhibited antibacterial activity. Previous studies (Rasooli et al., 2006) on the antimicrobial activity of the essential oils of some *Thymus* species showed that most of the species which possess large quantities of phenolic monoterpenes, have shown activity against viruses, bacteria, food-derived microbial strains and fungi.

Material and methods

Plant material

The aerial parts (up to ~ 5 cm, 0.05-0.2 kg) of wild populations of *T. daenensis* subsp. *daenensis* Celak (four individuals from each population) were collected from Isfahan and Chaharmahal va Bakhtiari provinces, Centre and Southwest of Iran. Each sample was labeled and its location was recorded using a GPS (Vista Garmin) receiver. The samples of the plants were identified by regional floras and authors with floristic and taxonomic references (Rechinger, 1982), and voucher specimens were deposited at the Herbarium of IAU, Shahrekord Branch, Shahrekord, Iran. The accessions of thyme were transferred from natural habitats at the early flowering stage on April-June 2009. Soil physical and chemical characteristics such as pH, Ec, texture, OC %, and of N, P and K contents were obtained through soil-sampling and analysis. The slope and elevation information were obtained from the DEM using two well-known GIS software packages ILWIS (3.0 Academic). Climatic conditions of natural habitats were determined using the nearest meteorology station.

Sample preparation

Harvested flowering aerial parts (leaves and flowers) were dried at room temperature for one week. The extracts were obtained by stirring 100 mg of ground samples with 30 ml of pure ethanol (analytical grade; Merck, Germany) for 30 min. Samples were filtered by a Whatman no 4. filter paper.

Reagents and chemicals

Methanol (HPLC grade), ethanol (analytical grade), acetonitrile (analytical grade) and water (HPLC grade) were purchased from Merck Co (Darmstadt, Germany). The standard of thymol and carvacrol acid were purchased from ROTH (Karlsruhe, Germany).

Preparation of standard solution

Stock standard solutions were prepared by accurately weighing 22.3 mg thymol reference standard and 16.4 mg carvacrol into separate 50 ml volumetric flasks and dissolving in acetonitrile/water (50:50, v/v). Working standard solutions (1, 2.5 and 5 ml) were prepared by dilution

Table 2. The amounts of thymol and carvacrol (mg/g extract), zones of growth inhibition (mm) and minimum inhibitory concentration (MIC) (mg/mL) of different population of *Thymus daenensis* subsp. *daenensis*

| Population | Thymol (mg/g extract) | Carvacrol (mg/g extract) | Zones of growth inhibition (mm) | | | | Minimum inhibitory concentration (mg/mL) | | | |
|-------------|-----------------------|--------------------------|---------------------------------|-------------------------|-------------------------|-------------------------|--|------------|------------|------------|
| | | | <i>E.c</i> ^a | <i>S.a</i> ^b | <i>P.a</i> ^c | <i>B.C</i> ^d | <i>E.c</i> | <i>S.a</i> | <i>P.a</i> | <i>B.C</i> |
| Sabz-e-koh | 13.89 | 10.55 | 17.6 | 20.3 | 16.6 | 22.6 | < 0.019 | < 0.019 | > 0.312 | 0.039 |
| Larak | 25.94 | 23.54 | 20.0 | 17.0 | 23.6 | 19.0 | < 0.019 | 0.039 | 0.039 | 0.019 |
| Shahrekord | 23.05 | 7.49 | 20.0 | 19.6 | 17.6 | 12.6 | < 0.019 | < 0.019 | > 0.312 | > 0.312 |
| Dezak | 19.39 | 7.68 | 21.6 | 21.0 | 12.6 | 23.6 | < 0.019 | < 0.019 | < 0.019 | > 0.312 |
| Sheykshaban | 35.89 | 12.01 | 20.3 | 22.3 | 23.6 | 24.6 | < 0.019 | < 0.019 | < 0.019 | 0.156 |
| Koohrang | 21.66 | 7.45 | 21.3 | 13.3 | 18.6 | 16.3 | < 0.019 | > 0.312 | 156 | 0.078 |
| Daran | 25.51 | 7.65 | 19.6 | 20.3 | 21.0 | 21.3 | < 0.019 | < 0.019 | > 0.312 | < 0.019 |
| Semirom | 14.47 | 16.27 | 22.3 | 19.6 | 16.3 | 20.6 | < 0.019 | < 0.019 | 0.312 | 0.156 |
| Hamgin | 25.51 | 8.17 | 21.0 | 22.0 | 24.6 | 21.3 | 0.039 | < 0.019 | 0.156 | < 0.019 |
| Khansar | 24.86 | 7.60 | 21.3 | 20.0 | 17.3 | 18.3 | < 0.019 | < 0.019 | 0.078 | > 0.312 |

^a *E.c*: *Escherichia coli*, ^b *P.a*: *Pseudomonas aeruginosa*, ^c *S.a*: *Staphylococcus aureus*, ^d *B.c*: *Bacillus cereus*

from the stock standard solution. The mixture was stirred carefully and refluxed in a water bath at 90 °C for 1 hr.

Identification of phenolic compounds using HPLC

The isolation and analysis method for thymol and carvacrol were conducted according to previously published protocols (Krause and Ternes, 1999; Hajimehdipoor et al., 2010; Shekarchi et al., 2010). The obtained mixture was injected to HPLC system (Kanauer, Germany). An HP 1000 series liquid chromatography system comprising vacuum degasser, quaternary pump, autosampler, thermostatted column compartment and diode array detector was used. Column Machery-NAGEL, Nucleosin-100-5 C18, Loop 20 µl was maintained at 30 °C. Solvents used for separation were water (eluent A) and acetonitrile (eluent B). The gradient program was as follows: 70% A/30% B, 0-5 min; 42% A/58% B, 5-18 min; 70% A/ 30% B, 18-30 min. The calibration curves (correlation coefficient) for thymol and carvacrol were $Y=89322x-382440$ ($r^2=0.998$) and $Y=74919x-247838$ ($r^2=0.994$), respectively. Samples were filtered through a 0.45 µm membrane filter before injection. The flow rate was kept 1 ml min⁻¹. The injection volume was 20 µl, and peaks were monitored at 330 nm. The chromatographic peaks of thymol and carvacrol were confirmed by comparing their retention times and UV spectra with that of their reference standard. Working standard solutions were injected into the HPLC and peak area responses were obtained. Standard graphs were prepared by plotting concentration versus area. Quantification was carried out from integrated peak areas of the samples using the corresponding standard graph.

Bacterial strain

The extracts were screened for antimicrobial activity using the agar diffusion technique (Rota et al., 2004) against four microorganisms of significant importance. The bacterial strains were used to assess the antimicrobial properties of the test samples, two Gram-negative strains: *Staphylococcus aureus* and *Bacillus cereus* and two Gram-negative strains: *Escherichia coli* and *Pseudomonas aeruginosa*. All clinical isolates obtained from Food Microbiology Laboratory, Veterinary Medicine Faculty, IAU, Shahrekord Branch, Iran, and identified using conventional morphological as well as biochemical tests. Stock cultures of bacteria were kept in 20% glycerol PBS (phosphate buffered saline) at -70 °C.

Active cultures were generated by inoculating 100 µl of the thawed microbial stock suspensions into 5 ml nutrient broth (Merck, Germany) followed by overnight incubation at 37 °C. An initial bacterial suspension containing 10⁷ CFU/ml was made from the flask broth culture. Subsequent dilutions were made from the above suspension, which were then used in tests.

Antimicrobial test

BHI agar (Merck, Germany) was used to prepare the culture medium and autoclaved at 121 °C for 15 min. Plates were prepared with 10 ml agar inoculated with 1 ml of each bacterial suspension. Filter paper disks (Whatman No. 1, 6 mm diameter) were impregnated with 60 µl of extract (100 µg/disc), and incubated at 35°C for 18 h. The extracts were dissolved in DMSO (15 µl) before the test for antimicrobial activity. Discs (6 mm diameter) of gentamycin, estereptomycin and tetracyclin (10 µg) were used as positive controls. The scale of measurement was the following (disk diameter included): P≥20 mm zone of inhibition is strongly inhibitory; <20–12 mm zone of inhibition is moderately/mildly inhibitory; and <12 mm is no inhibitory. All the data collected for each assay are the averages of three determinations. The minimal inhibitory concentration (MICs) values were determined by serial dilution assay. The appropriate amount of the extract dissolved in DMSO was added to nutrient broth. All extracts were initially tested at 0.019-10 mg/ml. Each tube was inoculated with 5 ml of bacterial suspension at a density of 10⁷ CFU/ml, and incubated at 37° C for 48 h. The growth of microorganisms was observed as turbidity determination by the measurement of optical density at 600 nm, using spectrophotometer (Eppendorf, AG, Germany). Erythromycin was included as positive control in each assay. DMSO solution (extract-free) was used as a negative control. Control tubes were incubated under the same condition. All assays were carried out in triplicate.

Analysis of data

The differences between experimental groups were compared using one-way ANOVA. All data processing was performed with SPSS software Version 11.5.

Conclusion

In the present study we demonstrated, the potent antibacterial activity of *T. daenensis* subsp. *daenensis* extract against foodborn pathogens strains, which justifies the large use of this plant in traditional medicine. We considered that it would be very useful to promote thymol and carvacrol chemotypes crop culture in order to guarantee the quality of products. In addition, the Iranian *T. daenensis* subsp. *daenensis* might be a potential thymol-rich source for commercial cultivation. However, further research is needed to evaluate the effectiveness of *T. daenensis* subsp. *daenensis* essential oils and extracts in food ecosystems to establish their utility as natural antimicrobial agents in food preservation and safety. It may be concluded that the best geographic localities for the large scale production of major constituents, is the altitudes between 2400 to 2800 meters above sea level. For example Sheikhshaban population or ecological conditions similar to Sheikhshaban might be a potential thymol-rich source for commercial cultivation.

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