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Full Length Research Paper

Comparison of essential oil composition in wild and cultivated populations of *Thymus pubescens* Boiss. & Kotschy ex Celak. from Iran

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Thymus pubescens **Boiss. and Kotschy ex Celak. (Lamiaceae) is a grassy and permanent herb which grows wild in some regions of Iran including West Azarbaijan province. Content, composition and antioxidant activity of the essential oils of both wild populations and cultivated ones were compared in this study. The aerial parts of *T. pubescens* at the beginning of the flowering stage were collected from provenance (*T.pub*-$w_{1=}$ Shahindezh mountains & *T. pub*-$w_{2=}$ Salmas mountains), and from a cultivated field of the Agriculture Research Centre (*T. pub*-F) in West Azarbaijan province in June, 2009. The essential oils were extracted by hydro-distillation in a Clevenger apparatus and yield 0.73 and 0.67% in the wild habitats, and 0.43% in the cultivated one respectively, based on v/w. Analyses of the essential oils by GC/MS allowed to identify 20 and 23 constituents in the wild *Thymus* populations (*T.pub*-w_1, *T. pub*-w_2) representing 98.78 and 98.96% of the oils, respectively. Main constituents of the essential oils were respectively carvacrol (33.49%) (30.16%), thymol (14.19) (15.16), linalool (15.76%) (13.14%), and geranyl acetate (9.59%) (9.08%). Cultivated plants (*T. pub*-F) presented 24 constituents, representing 96.35% of the oil, whose major components were carvacrol (34.37%), thymol (13.48%), borneol (10.17%), linalool (6.4%), 1,3,8-p-menthatriene (8.87%), and pinene (8.48%). Two main chemotypes of essential oils were identified, being carvacrol the main component (30.16 to 34.37%). Furthermore, the antioxidant activity of essential oils of the aforesaid plants using DPPH radical scavenging was determined; all of the extracts manifested almost the same pattern of antioxidant activity as the ascorbic acid (Vit C).**

Key words: *Thymus pubescens*, essential oil, GC/MS, carvacrol-thymol.

INTRODUCTION

There are about 350 species of *Thymus* genus throughout the world, sixteen of which exist in Iran and they grow adventively in Alborz and other mountains (Zagros) of Iran, particularly in Azarbaijan, Gilan, Mazandaran, Ghazvin and Tehran provinces. *Thymus* genus plants are mostly woody stem, aromatic, ever green, durable and subshrubs and are usually found in calcic soil and grass fields throughout Europe Africa and Asia. Of this genus, which belongs to the family Lamiaceae, an important and medicinal species is *Thymus pubescens* Boiss. & Kotschy ex Celak or syn. *T. xylorrhizus* Boiss. & Kotschy. (Ghahreman, 1994; Mehrgan et al., 2008; Mozaffarian, 1996; Rechinger, 1982).

In phytology, *T. pubescens* is a perennial plant widely spread out in Iran and Turkey. This plant has low shrubs

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Table 1. Specifications of provenances of *T. pubescens* Boiss. & Kotschy ex Celak. plants.

Habitats	Height (m)	Species density (%)	Cover crown (Cm^2)	Slope %	Slope direction
1- (*T. pub*-W1) = Shahindezh (Kurdkandi Village)	1930	5	4	30	Northeast
2- (*T. pub*-W2) = Salmas (Broshkhoran Village)	2380	15	6	7	Northeast

with woody based stems, sleeping on the ground to upright. The flower branch is 2 to 13 cm. The flowers are red or purple-blue and are 5 to 8 mm and flowering begins from spring until summer (Jamzad, 2009). Different species of *Thymus* are used to flavor foods. Its essential oil is a strengthening and stomach medication, menstrual stimulant and carminative, treats respiratory problems, pertussis, chronic cough and menstrual pain (Zargari, 1989). All species of *Thymus* are rich in essential oils and often contain phenolic compounds which are strong antiseptics (Naghdi Badi et al., 2003; Sefidkon et al., 2001).

Essential oils contain aroma compounds extracted from different parts of woody and grassy plants (flowers, buds, leaves, seeds, fruits, roots, branches, and plant's skin) which are virtually a complex mixture of hydrocarbons, alcohols, esters, aldehydes, carboxylic compounds and in cases phenylpropanoids. Most hydrocarbons are monoterpene compounds, however sesquiterpenes can also be found. Antibacterial properties of essential oils, are mostly because the presence of phenolic compounds (Burt, 2004; Cassiano, 2007; Holley and Patel, 2005).

The components of the areal parts of *Thymus kotschyanus* and *Thymus pubescens* essential oil collected from Behshahr suburbs in Mazandaran province in full flowering stage have been investigated (Morteza et al., 2006). Pulegone (18.7%), isomenthone (17.8%) thymol (14.9%), cineol (9%), piperitenone (6.3%), and carvacrol (5.5%) have been reported as major components of *T. kotschyanus* essential oils and carvacrol (32.1%), thymol (19.1%), alpha-terpineol (14.6%), and para-cymene (6.1%) reported as those of *T. pubescens* essential oil.

Antimicrobial activity of *Thymus* essential oil against *Helicobacter Pylori* (the bacteria responsible for stomach inflammation) has also been proved (Ghannadi et al, 2004). Further, antimicrobial activity of *T. pubescens* methanolic extractions using disc diffusion method against Gram-negative and Gram-positive bacteria has been studied. Such studies have demonstrated that antibacterial activity of essential oils is mostly due to phenolic compounds (thymol and carvacrol) found in essential oils which affect the bacterial cell membrane, disrupt its permeability to ATP and potassium ions and bring about cell death (Mehrgan et al., 2008; Nejad et al, 2008; Rasooli and Mirmostafa, 2002).

Sefidkon et al. (2009) studied the effect of harvesting time and extraction methods of essential oil on the quantity and quality of *Thymus vulgaris* L. essential oil. Comparison of the treatments revealed that the highest essential oil yield of (1.18%) was in early flowering stage which was a significant difference compared to others. The difference in the amount of thymol in the essential oil in the early flowering and full flowering stages was small but the amount was lower before the flowering stage. Generally speaking, given the results of the present study, early flowering stage can be generally considered as the best time for harvesting *Thymus* and hydro-distillation as the best extracting method.

Accordingly, in the present study the aerial parts of *T. pubescens* plants (different ecotypes) were collected from two natural provenances or habitats, with different ecological conditions and field in early flowering stage. The essential oil was extracted using hydro-distillation method and Clevenger apparatus and composition and the antioxidant activity of the essential oil of aforesaid plants were analyzed and compared.

MATERIALS AND METHODS

Plant materials

In June, 2009, the aerial parts of these plants at the beginning of flowering stage (20% flowering) from two different habitats, Shahindezh (*T. pub*-w_1) and Salmas (*T. pub*-w_2) mountains having different ecological conditions indicated in Table.1 and Urmia Agriculture Research field (*T. pub*-F), located in West Azarbaijan province, Iran.

The taxonomic identification of plant materials was confirmed by a senior plant taxonomist, in Agricultural Researchs Center,West Azarbaijan, Urmia, Iran. Collected plant materials were dried in shadow at room temperature for 5 days, and the air-dried aerial parts of plant (the leaves with young stems) were powdered in a grinder with a 2 mm diameter mesh. A voucher specimen of each population has been deposited at the Herbarium of the Agricultural Research Center, Urmia, Iran.

Isolation of the essential oils

The *Thymus* samples of at least 30 g of the air-dried aerial parts of plants collected were hydro-distilled for 2 h (3 times) using a Clevenger-type apparatus (Britania Farmacopeia Model), (yield ranging from 0.43 to 0.73% v/w). The obtained essential oils (EO) were dehydrated over anhydrous sodium sulphate and after filtration, stored at +4◦C until tested and analyzed (Sahin and Gulluce, 2004).

The analysis of the essential oil was performed using a Thermo Finnigan Trace MS2000 GC- MS (made in USA designe: Italy),

$$DPPH^{\bullet} + A\text{-}H = DPPH\text{-}H + A^{\bullet}$$

$$DPPH^{\bullet} + R^{\bullet} = DPPH\text{-}R$$

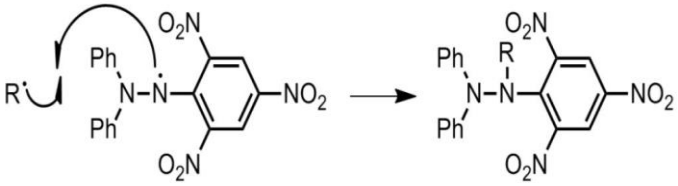

Figure 1. DPPH radical scavenging reaction.

GC–MS analysis conditions

equipped with a HP-5 MS capillary column (30 m, 0.25 mm i.d, film thickness 0.25 µm). For GC–MS detection an electron ionization system with ionization energy of 70 eV was used. Helium was the carrier gas, at a flow rate of 35 ml/min. Injector and detector temperatures were 200 and 250°C, respectively. Column temperature was initially kept at 120°C for 5 min, then gradually increased to 260°C at a 10°C/min rate. The components were identified based on the comparison of their relative retention time and retention indexes with Wiley Library data of the GC–MS system and literature data (Adams, 2001).

Antioxidant activity (DPPH assay)

The hydrogen atoms or electrons donation ability of the essential oils was measured from the bleaching of purple coloured methanol solution of DPPH (Figure 1).

DPPH radical scavenging activity was determined as described by Zijia et al. (2009) and Pirigharnaei et al. (2011) with a slight modification. Fifty microliter of various concentrations of the extracts in methanol was added to 5 ml of a 0.004 $mg.ml^{-1}$ methanol solution of DPPH. After gentle mixing and 30 min incubation period at room temperature the absorbance of the resulting solutions was measured at 517 nm using a Biowave S2100 England spectrophotometer. The percent DPPH inhibition by each sample oil was calculated.

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentration. Synthetic antioxidant reagent ascorbic acid (vit C) was used as positive control and all tests were carried out in triplicate.

RESULTS

By distilling 100 g of areal parts of the plant at the beginning of flowering stage (20% flowering), the *Thymus* essential oil was obtained from provenance Shahindezh (*T. pub*-w_1) and Salmas (*T. pub*-w_2) and at yield of 0.73 and 0.67% and from field plant (*T. pub*-F) at yield of 0.43%. After analyzing the essential oils by GC/MS, 20 compounds (99.49%) and 23 compounds (98.96%) were identified in the essential oils of plants from *T. pub*-w_1 and *T. pub*-w_2, respectively. The major components of their essential oils in % were carvacrol (33.49), (30.16), thymol (14.19), (15.16), linalool (15.76), (13.14), geranyl acetate (9.59 (9.08), respectively. And in the field *Thymus* plants (*T. pub*-F), 24 constituents, representing 96.35% of the oil, were identified. Major components of the essential oil in % were carvacrol (34.37), thymol (13.48), linalool (6.4) borneol (10.17), 1,3,8-p-menthatriene (8.87), pinene (8.48). Two chemotypes of essential oils were identified. The main chemotype all of them was carvacrol (Tables 2, 3).

The essential oils from *T. pubescens* Boiss. & Kotschy ex Celak. plants collected in 3 localities in West Azarbaijan (Iran) contained carvacrol (ranging from 30.16 to 34.37%) as the main constituent that the highest percentage of carvacrol (34.37%) in essential oils was related to *T. pub*-F (Table 2 and Figure 3). The two major and important constituents (carvacrol, thymol) of essential oils were almost the same in all samples from 3 localities (Table 2 and Figure 3). The oxygenated and hydrocarbon monoterpenes contributed 66.08% and 17.35% in oil sample collected from field or cultivated plants (*T. pub*-F) followed by sesquiterpene hydrocarbons and oxygenated sesquiterpenes (3.89 and 8.09%), respectively. carvacrol (34.37%), borneol (10.17%) and thymol (13.48%) were the major oxygenated monoterpenoids and 1,3,8-p-menthatriene (8.87%) and β-pinene (8.48%) were the major monoterpene hydrocarbons. Of the 11.98% sesquiterpenes, tau-cadinol (3.08%), was the major component of this fraction. In the sample population of Shahindezh (*T. pub*-w_1), monoterpenes fraction consisted the highest proportion (85.19%) of the oil, of which oxygenated monoterpenes accounted for the 83.07%, with carvacrol (33.49%), linalool (15.76%) and thymol (14.19%), being the major components of this fraction. Also caryophyllene oxide (6.74%) was the main component of oxygenated sesquiterpenoids. In the Salmas mountain collection (*T. pub*-w_2), the mono- and sesquiterpenoids accounted for 85.19% and 12.03%, respectively. carvacrol was recognized as the most abundant oxygenated monoterpene. (30.16%) together with thymol (15.16%), linalool (13.14%) and borneol (8.13%).

This study revealed that free radical scavenging activity increased by the increase in concentration in each extract. The percentage of inhibition (I%) was calculated as:

$$(\%I) = [A_o - (A_s - A_1)] / A_o \times 100$$

Where A_0 was the absorbance of the control, A_s was the absorbance of solutions containing the extracts, and A_1 was the absorbance of blank solution without DPPH (Figure 2).

The IC_{50} values for the extracts of wild samples *T .pub*-w_1 and *T. pub*-w_2 were 5.5 ± 0.15 µg/ml and 5.46 ±0.23µg/ml respectively and for those of *T. pub*-F samples

Table 2. The composition of the essential oils (%) of *T. pubescens* Boiss. & Kotschy ex Celak. plants*, cultivated at the field and growing wild (2 various habitats) in Urmia district, Iran.

NO	Composition	RI	Components (%)		
			* Field	* Provenances	
			T. pub-F	*T. pub*-W_1	*T. pub*-W_2
1	B-Pinene	943	8.48	2.69	2.03
2	1R-α-Pinene	948	---	0.43	0.23
3	1,3,8-p-Menthatriene	1029	8.87	---	1.1
4	Linalool	1082	6.4	15.76	13.14
5	Borneol	1088	10.17	7.09	8.13
6	Terpineol	1143	0.35	2.76	5.72
7	Thymol	1262	13.48	14.19	15.16
8	Carvacrol	1263	34.37	33.49	30.16
9	Linalyl formate	1270	---	0.19	0.22
10	Geranyl acetate	1352	1.31	9.59	9.08
11	Cadinene	1440	1.55	---	---
12	Caryophyllen	1493	0.29	2.29	3.10
13	β-Bisabolene	1500	0.88	0.73	0.63
14	Caryophyllene oxide	1507	2.24	6.74	5.63
15	Nerolidol	1563	0.38	---	---
16	Humulene	1579	1.17	1.17	1.03
17	Isoaromadendrene epoxide	1579	0.62	---	0.70
18	Cubenol	1580	0.62	---	0.23
19	tau-Cadinol	1580	3.08	0.60	1.04
20	Nerolidol acetate	1754	0.46	---	0.17
21	Hexahydrofarnesyl acetone	1754	0.30	---	---
22	3-Deoxyestradiol	1949	0.18	---	---
23	Hexadecanoic acid	1968	0.21	0.31	0.22
24	Phytol	2045	0.48	0.28	0.36
25	n-Heptacosane	2705	0.04	0.05	0.05
26	Spinacene	2914	---	0.03	---
27	1-Heptatriacontanol	3942	0.28	0.33	0.32
28	n-Tetratetracontane	4395	0.14	0.06	0.05
SUM (%)			96.35	98.78	98.96
Monoterpene hydrocarbons (%)			17.35	3.12	3.36
Oxygenated monoterpens (%)			66.08	83.07	81.83
Sesquiterpene hydrocarbons (%)			3.89	4.19	4.76
Oxygenated sesquiterpene (%)			8.09	7.65	7.27
Others (%)			0.94	0.75	1.74

*F- Urmia Agriculture Research Field, W_1 – Shahindezh mountains (Kurdkandi Village), W_2 - Salmas mountains (Broshkhoran Village).

5.37 ± 0.13 µg/ml which were so similar to the control sample of vitamin C (ascorbic acid) that is, 5.05 ± 0.12 µg/ml.

DISCUSSION

The results of this study on *T.pubescens* plants collected from forenamed localities showed that oils in all of them contained the highest amount of carvacrol (ranging from 30.16 to 34.37%). Two main chemotypes of essential oils were identified: 1- Carvacrol- thymol- borneol, for (*T. pub*-F) and 2- Carvacrol- linalool- thymol for (*T. pub*-w_1), (*T. pub*-w_2) that the main chemotype was Carvacrol (Table 3).

The study of (Sefidkon et al., 2002), on the essential oils of *T. subescens* has shown that the percentage of carvacrol (64.8 and 48.8%) and thymol (11.9 and 13.9%),

Table 3. The main chemotypes and their components of the known essential oils of *T. pubescens* Boiss. & Kotschy ex Celak. plants under study in Iran.

Plants*	Chemotypes	% Components
(I)-T. pub-F	Carvacrol	34.37
	Thymol	13.48
	Borneol	10.17
(II)-T. pub-W1	Carvacrol	33.49
	Linalool	15.76
	Thymol	14.19
(III)-T. pub -W2	Carvacrol	30.16
	Thymol	15.16
	Linalool	13.14

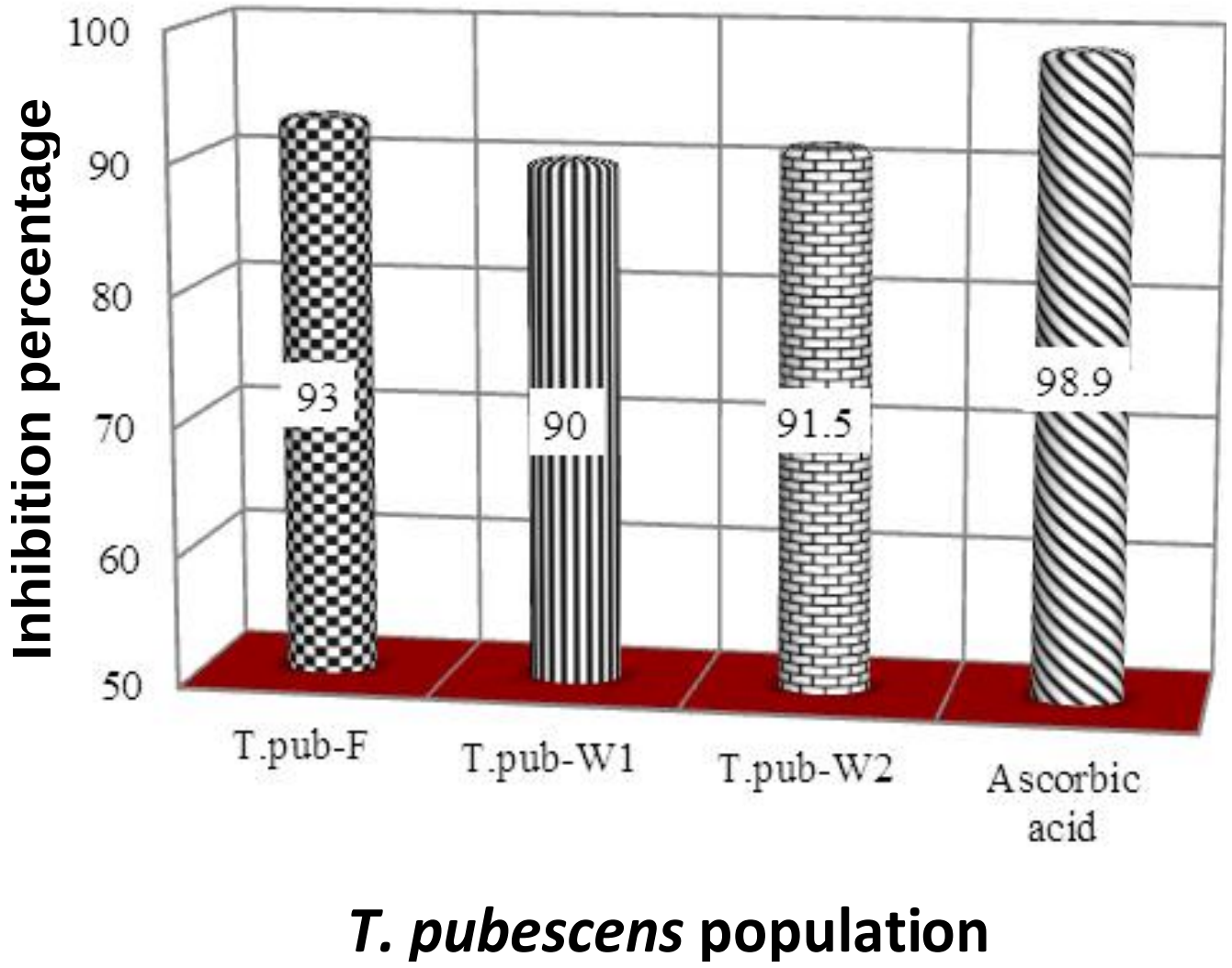

Figure 2. Comparison of the antioxidant activity of essential oil in *Thymus pubescens* populations in the wild (W) and in the cultivated (F). T. pub-W1 = Shahindezh (Kurdkandi Village), T. pub-W2 = Salmas (Broshkhoran Village), and T. pub-F = Urmia Agriculture Research.

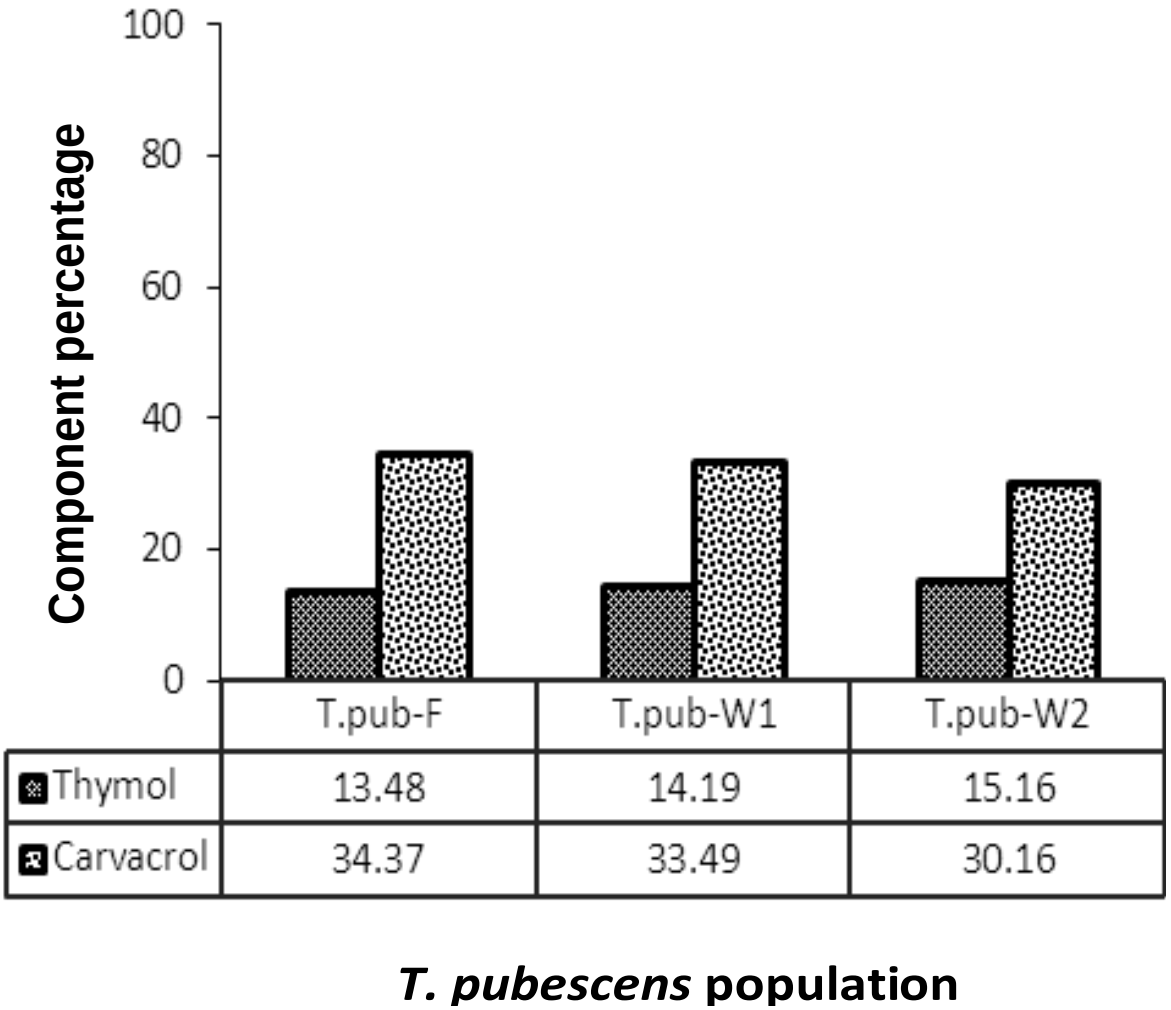

Figure 3. Comparison of two major componenets of thymol and carvacrol in *T. pubescens* plants.

were remarkably high in before flowering and full flowering stages, respectively. Also in the research of Abousaber (2002), on the oils composition of aforesaid species from two different localities in Iran demonstrated that the oils of *T. pubescens* contained thymol (63.4%, 11.1%), α-terpineol (19.2%), limonene (1.2%, 8.8%), p-cymene (4.0%, 5.0%) and carvacrol (3.7%, 0.4%). These results showed that variation of ecological conditions, the period of plant growth and deferent methods of distillation may affect on the quantity and the quality of the essential oil components. Our findings on the chemical composition of the essential oils of this specimen in various habitats are in accordance with previous reports.

DPPH radical scavenging activity of the oils was very high, and this was obviously related to its chemical composition. In several reports, thymol and carvacrol, in particular, were found to be the main antioxidant constituents of the oils isolated from several *Origanum* species (Milos et al., 2000; Barrata et al., 1998; Ruberto et al., 2002). The results of the current study on radical scavenging activity of the essential oil of *T. pubescens* species are in accordance with these reports as, the percentage of carvacrol (30.16 to 34.37%) and thymol (13.48 to 15.16%) were remarkably high in all of essential oils (Figure 3).

Our findings have demonstrated that the chemical composition of the essential oils of *T. pubescens* varies with geographical location of collection site, climate and other ecological factors, which suggest both intrinsic/ genetic and extrinsic/environmental factors (like altitude, edaphic, temperature, slope percentage, humidity and climate, etc.), play a role in determining of the oils composition and DPPH scavenging capacity.

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