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Assurance and contrasting of the fundamental oil segments in wild and developed populaces of *Thymus kotschyanus* Boiss. and Hohen

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Abstract

Thymus kotschyanus* Boiss. & Hohen. is a grassy, permanent herb which belongs to *Labiatae* family and grows wild in some regions of Iran including West Azarbaijan province. In this study, variations in the quantity and quality of the essential oils of wild and cultivated populations of *T. kotschyanus* are reported. The aerial parts of *T. Kotschyanus* at the beginning of flowering stage (20% flowering) were collected from provenance and agriculture research field. The essential oils were extracted in a Clevenger apparatus by using hydro-distillation method. Results show that the essential oil in the provenance and cultivated *Thymus* were obtained by hydro-distillation at yield of 0.91, 1.43 and 1.72% (based on v/w), respectively. The essential oils were analyzed by GC/MS. In the provenance plants (T.k-W_1), (T.k-W_2) 20 components, making up 99.49 and 96.69% of the oils respectively, were identified. The major components of the essential oils in the plants T.k-W_1 were Thymol (43.83), Carvacrol (22.11), and Terpinene (19.73%). And in T.k-W_2, major components of the essential oil were carvacrol (29.39), cineol (11.95), thymol (8.48) and α-terpieol (6.29%). In the essential oils of the cultivated *Thymus*, the main components were Thymol (51.79), Terpinene (12.31), Comphene (7.06), α-terpieol (6.83) and Carvacrol (6.69%). Also, in the present study, the antioxidant activity of *T. kotschyanus* essential oils was determined using DPPH radical scavenging assay.

Keywords: *Thymus kotschyanus*, wild and cultivated, thymol and carvacrol.

INTRODUCTION

About 350 species of *thymus* are found worldwide and 14 to 16 species of which are reported in Iran which are widely spread in North and West of the country. There are 10 species in Northern provinces (Gorgan, Gilan, and Mazandaran), 11 species in western provinces (Azarbayjan, Bakhtaran, Hamedan, Kordestan, Lorestan, Chaharmahal-o-Bakhtiari, Kohkilooye-o-Boyerahmad, and Isfahan), 7 species in central provinces (Tehran, Semnan, Qazvin, Arak, and Yazd), 1 type of species in Fars and 2 species in Kerman (Some of the species mentioned above are repeated in different regions of Iran). The following are the species found in Iran:

(1) *T. persicus* (Ronniger ex Rechinger) Jalas (2) *T. daenensis* Celak. ssp. Daenensis (3) *T. Fallax*fisch. and C.A. Mey. (4) *T. Transcaueasicus* Ronniger (5) *T. kotschyanus* Boiss. & Hohen. (6) *T. fedtschenkoi* Ronniger. (7) *T. migricus* Klokov. & Desj- shost. (8) *T. trautvetteri* Klokov. & Desj- shost. (9) *T. pubescens* Boiss. & Kotschy ex Celak. (10) *T. caucasicus* wild ex Ronnigerssp. *grossheimii* (Ronniger) Jalas. (11) *T. eriocalyx* (Roommonlyonniger) Jalas.

Such genus of plants are commonly woody, aromatic, durable and evergreen subshrubs, mostly found in calcic soils and grassfields throughout Europe and Asia. One of its aromatic herbaceous species is *Thymus kotschyanus*

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Table 1. Specifications of provenances of *T. kutschyanus*Boiss. & Hohen. plants.

Provenance	Height (m)	Species density (%)	Cover crown (cm^2)	Slope (%)	Slope direction
1. Qoushchi Mountain Pass (T.k-W_1)	1650	20	4	35	Northeast
2. Urumia Martyrs Valley (T.k-W_2)	1550	5	2	40	Western

which belongs to *thymus* L. genus of the *Lamiaceae* family (Ghahreman,1994; Mehrgan et al., 2008; Mozaffarian,1996; Rechinger, 1982; NaghdiAbadi et al., 2003). The essential oil is thymus active ingredient. *Thymus* essential oil is yellow or reddish brown with a pleasant, strong smell and a strong, stable and cooling taste which is extracted by distilling its leaves and flower branches. Thymus twigs contain essential oil, tannins, bitter substances, saponins, and herbal antiseptic (Aeineh-chi, 1986; Moameni and Shahrokhi, 1991; Zargari, 1989; Volak and Stodola, 2003). All thymuses are rich in volatile compounds (essential oils) and often contain phenolic compounds which are strong antiseptics (NaghdiBadi et al., 2003; Sefidkon et al., 2001). As aroma compounds, essential oils are extracted from different parts of woody and grassy plants (flowers, buds, leaves, seeds, fruits, roots, branches, and plants' 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 (Burt, 2004; Cassiano, 2007; Holley and Patel, 2005). The components of the areal parts of *Thymus kotschyanus* and *Thymus pubescences* essential oil collected from in full flowering stage have been investigated. Pulegoun (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 paracymen (6.1%) reported as those of *T.pubescens* essential oil (MortezaSemnani et al., 2006). Antimicrobial activity of *Thymus* essential oil against helicobacter pylori (the bacteria responsible for stomach inflammation) has also been demonstrated (Ghannadi et al., 2004). Further, antimicrobial activity of *Thymus 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 et al., 2002).

Also, some studies have been carried out on the effect of harvesting stage and distillation method on the quantity and quality of essential oils of various aromatic plants. The following is a couple of these studies. Sefidkon et al. (2009) investigated the effect of harvesting time and extraction methods of essential oil on the quantity and quality of essential oil of *Thymus vulgaris* L. Overall, according with the results of this study, early flowering stage can be generally considered as the best time for harvesting of *thymus* and hydro-distillation as the best extracting method. So far, there is no report on comparison of the yield and composition of the essential oil of cultivated and growing wild *T. kotschyanus* populations in literatures. Accordingly, in the present study the aerial parts of *T. kotschyanus* plants (different ecotypes) were collected from two habitats, Qoushchi Mountain Pass and Urmia Martyrs Valley (having different ecological conditions) and Urumia agriculture research field and in early flowering stage (20% flowering) to compare their essential oils composition and antioxidant activity together and with cultivated plants oils. The essential oil was extracted using hydro-distillation method and its components and the antioxidant activity of the essential oil of aforesaid plants were analyzed and compared. Three chemotypes of essential oils were identified. The essential oils of the Thymol and Carvacrol main chemotypes were found in the plants growing wild and cultivated populations and all of extracts manifested almost the same pattern of antioxidant activity as ascorbic acid (vitamin C).

MATERIALS AND METHODS

Plant samples

In June 2009, the aerial parts of these plants at the beginning of flowering stage (20% flowering) were collected from Qoushchi Mountain Pass and the heights of Urmia Martyrs Valley (protected area of natural resources) in different ecological conditions indicated in Table 1 and Urmia agriculture research field, located in West Azerbaijan province, Iran.

Essential oil extraction

The areal parts of the collected plants from provenance and research fields were dried in the laboratory conditions. The samples were then powdered by a mill and the essential oil was extracted from 30 g of the resulting powder using Clevenger apparatus and hydro-distillation (3 times). After being dehydrated by sodium sulfate anhydrous, the essential oils were preserved in sealed

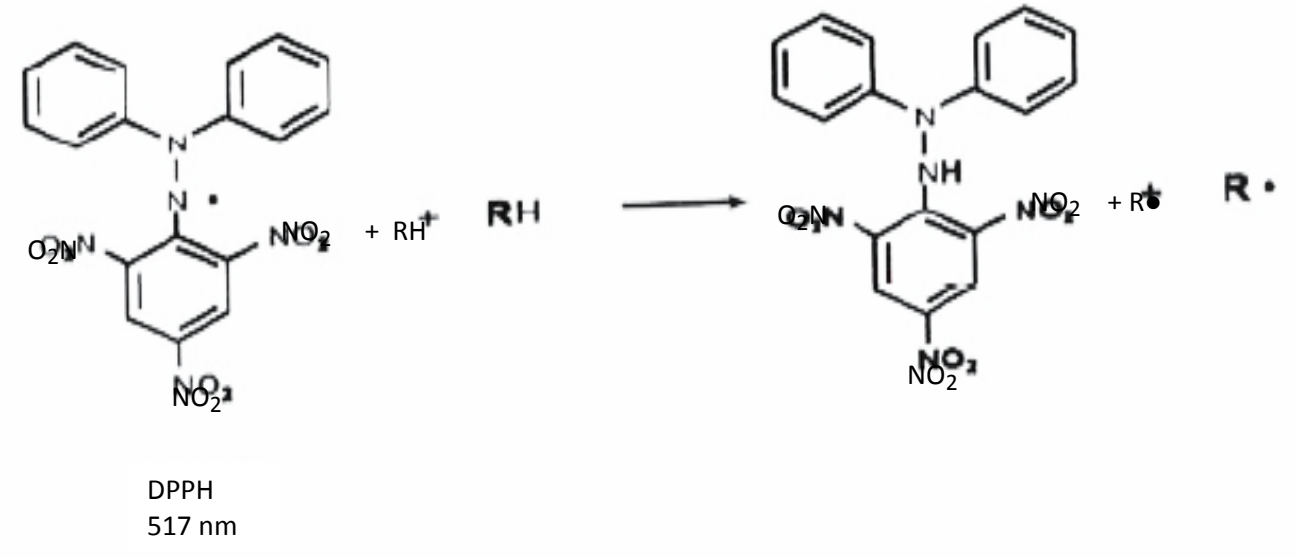

Figure 1. The reaction of DPPH radical scavenging by essential oils.

sterile glass vials in the dark at 4°C for quantitative and qualitative analyses (British Pharm.,1988).

Procedure

The essential oils were analyzed by gas chromatography- mass spectrometry (GC/MS). Thermo Finnigan Trace 2000 GC/MS, made in the USA, was employed with a HP-5MS capillary column (30 m long and 0.25 mm wide, and a 0.25 µm of film thickness) at a 250°C of injector chamber. The initial column temperature was at 120°C for 5 min then raised to 280°C at the rate of 10°C/min. Helium was used as a carrier gas at a rate of 35 ml/min. MS parameters were as follows: ionization energy, 70eV; ion source temperature, 200°C; voltage, 3000 v; and mass range, 30 to 600. The compositions of the essential oil were identified by comparison of their retention indexes (RI), retention times (RT) and mass spectra with those of authentic samples in Wiley library (Adams, 2001).

Measuring free radical scavenging activity by essential oils

Stable free radical DPPH (2-2-diphenyl, 1-picrylhydrazyl) from Sigma-Aldrich Co, code D9132, was used to measure free radical scavenging activity (Ebrahimzadeh et al., 2008a, b). DPPH is a stable nitrogen-centered free radical, the color of which changes from violet to yellow upon reduction by either the process of accepting hydrogen- or electron (Figure 1). Substances which are able to perform this reaction can be considered as antioxidants (Brand-Williams et al., 1995). To prepare 0.004% methanol solution of DPPH, 0.01 g DPPH powder (black) was dissolved in 250 cc methanol which resulted in a purple solution. The resulting solution was used as control at 517 nm using a spectrophotometer.50 ml of each extract was mixed with 5 ml of 0.004% methanol solution of DPPH. The mixtures were shaken and incubated for 30 min in the dark. Their absorbances were then measured at 517 nm using a spectrophotometer (Biowave S2100, made in England). The mixtures' absorbance at 517 nm was determined against a blank solution. The experiments repeated three times and the result were reported. According to the results, the IC_{50} value for each extract was calculated from the inhibition curve against extract concentration.

RESULTS AND DISCUSSION

The essential oils in the provenance and cultivated *Thymus* plants were obtained by hydro-distillation at yield of (0.91 and 1.43%) and 1.72% (based on v/w), respectively. 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 (Qoushchi Mountain Pass and Urmia Martyrs Valley) and at yield of 0.91 and 1.43% and field at yield of 1.72%. After analyzing the essential oils by gas chromatography-mass spectrometry (GC/MS), 20 compounds (99.49%) were identified in the essential oils of plants from Qoushchi Mountain Pass (T.k-W1) and 20 compounds (96.69%) were identified from Urmia Martyrs Valley (T.k-W2). Thymol (43.83), carvacrol (22.11), and gamma-terpinene (19.73%) were the major compounds of T.k-W1 plants essential oil and carvacrol (29.39%), cineol (11.95%), thymol (8.48%), and alpha-terpineol (6.29%) were the major compounds of T.k-W2plants essential oil. In the *Thymus* essential oil cultivated in the field (T.k-F), 20 compounds (97.35%) were identified as the following: thymol (51.79%), gamma-terpinene (12.31%), comphene (7.06%), alpha-terpineol (6.83%), and carvacrol (6.69%) were the major compounds of the plant essential oil (Table 2). 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: (Zijia et al., 2009).

$$(\%)\ \text{inhibition} = \left[\frac{A_0-(A_S-A_2)}{A_0}\right] \times 100$$

Table 2. The composition of the essential oils (%) of *T. kutschyanus* Boiss. & Hohen.plants*, a field & wild (2 various habitats) in Urmiadistrict, Iran.

NO	Composition	RI	Components (%)		
			Research field * Provenances		
			T.k -W_1	T.k-W_2	T.k-F
1	Comphene	943	0.163.667.06		
2	ϒ-Terpinen	998	19.73	–	12.31
3	Cineole	1019	1.7011.95	-	
4	Linalool	1082	–	5.57	–
5	Borneol	1088	–		2/061.91
6	(-)-Camphor	1121	0.373.04		–
7	α-Terpieol	1143	–		6.296.83
8	Thymol	1262	40.388.4851.	79	
9	Carvacrol	1263	22.1129.396.	69	
10	Geranyl acetate	1352	–	–	2.61
11	ϒ - Elemene	1431	1.99	-	-
12	ϒ-Muurolene	1435			0.512.071.22
13	ϒ-Cadinene	1440			2.331.540.54
14	Caryophyllen	1494			2.185.440.22
15	Caryophyllene oxide	1507	0.98	–	1.41
16	Elemol	1522		1.911.91	–
17	β-Guaiene	1523	0.13	–	–
18	Ent-Spathulenol	1536			0.974.800.97
19	Humulene	1579			1.901.901.00
20	Cubenol	1580			0.95 0.570.30
21	tau-Cadinol	1580			1.013.101.33
22	α-Cadinol	1580		0.282.450.16	
23	Bisabolol	1625	–	–	0.24
24	Hexahydrofarnesyl acetone	1754	0.160.27		–
25	Cedrane-8,13-diol	1786	–	–	0.28
26	Phytol	2045	0.43	–	0.13
27	Retinoic acid	2352	0.840.210.35		
	SUM		49/99	69/96	35/97
	Monoterpene hydrocarbons		19.89	3.36	19.37
	Oxygenated monoterpenes		62.02	66.78	67.22
	Sesquiterpene hydrocarbons		7.56	12.94	2.98
	Oxygenated sesquiterpene		7.19	12.83	4.88
	Others		2.83	0.48	2.90

W_1 - Qoushchi Mountain Pass, W_2 -Urmia Martyrs Valley, F- Urmia agriculture research field.

Where A_0 was the absorbance of the control, A_S was the absorbance of solutions contacting the extracts, and $A_1$was the absorbance of blank solution without DPPH.

A_O (Control) = 50 µl (methanol) + 5 ml (DPPH)
A_S (Extract sample) = 50 µl (extract) + 5 ml (DPPH)
A_1 (Blank) = 50 µl (extract) + 5 ml (methanol)

The IC_{50} values for the extracts of wild samples T.k-W1 and T.k-W2 were 5.27 ± 0.15 µg/ml and 5.20 ± 0.23 µg/ml respectively, and for those of field samples 5.20 ± 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. The results of the study confirmed the findings of the studies on the essential oil of *T. carnasus, T. pubescens, T. serpylum*, and *T .persicus* which posited that in all of the essential oils phenolic compounds (thymol and carvacrol), gamma-terpinene and paracymen were the major components (Sefidkon and Askari, 2005). Furthermore, these results pointed out the effect of the place of growing and probably the growing season and the time of collecting the plant on the major components

Table 3. The main chemotypes and their components of the known essential oils of *T. Kutschyanus* Boiss. & Hohen. plants under study in Iran.

Plants*	Chemotypes	Composition
(I)- T.k-F	Thymol	51.79
	γ-Terpinene	12.31
	Comphene	7.06
	α-Terpieol	6.83
	Carvacrol	6.69
(II)- T.k-W_1	Thymol	43.83
	Carvacrol	22.11
	γ-Terpinene	19.73
(III)- T.k -W_2	Carvacrol	29.39
	Cineole	11.95
	Thymol	8.48
	α-Terpieol	6.29

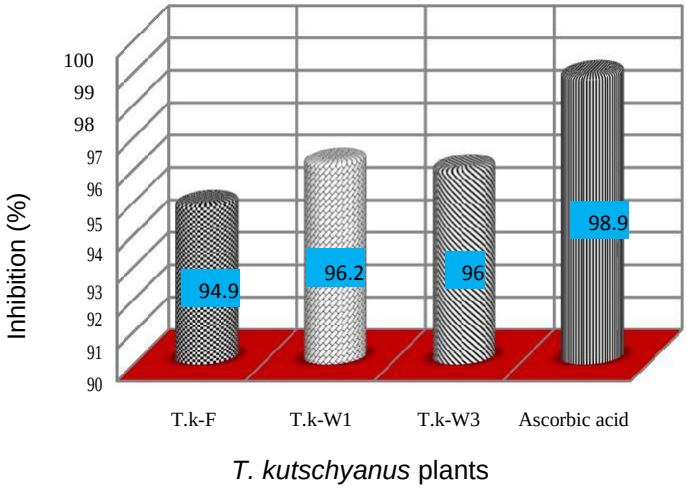

Figure 2. Comparison of the antioxidant activity of essential oil in *T. kutschyanus* plants in the provenance (W) and field (F).

of volatile oil. By quantitative and qualitative comparison of the components of the essential oil of plant *T. kotschyanus* Figure extracted 2-Comparison from habitats of and the agriculture antioxidant filed, it is revealed that essential oil obtained in T.k-W1 (Qoushchi in the Mountain provenance Pass) at (W) yield and of 1. 43% field and (F) in. T.k-F (agriculture field) 1.72% which are more than 0.91% in T.k-W2 (Urmia Martyrs Valley) and that the components of the essential oil in T.k-W1(99.49%) and T.k-F (97.35%) are more than T.k-W2 (96.69%) (Table 2). Considering the degree of the major components in essential oils, the three chemotypes are identified: 1) - wild plants of T.k-W1: thymol-carvacrol– gamma-terpinenechemotype, 2) - wild plants of T.k-W2: carvacrol-cineol-thymol-alpha-terpineolchemotype and 3) - field plants of T.k-F: thymol-gamma- terpinene, terpieol-carvacrol (Table 3). Table 2 indicates that the components of the essential oil in all three chemotypes, are in four groups of hydrocarbonated and oxygenated monoterpenes or hydrocarbonated and activity oxygenated sesquiterpenoids in which the highest degrees of components in all three chemotypes go to oxygenated monoterpenes (62.02, 68.78, and 67.22%). The results denote that the two major phenolic compounds (thymol and carvacrol) are common in all three chemotypes as to which in plants T.k-F and T.k-W1, thymol (51.79 and 43.83% respectively) has the highest concentration and the highest concentration in plants T.k-W2 goes to carvacrol (29.39%) (Table 3, Figure 3). Considering the antioxidant activity of the essential oil, all chemotypes (wild and cultivated) have, in a sense, showed a similar pattern of antioxidant activity of ascorbic acid (Table 2, Figure 2). The results indicate that the field plants (T.k-F) have the highest percentage

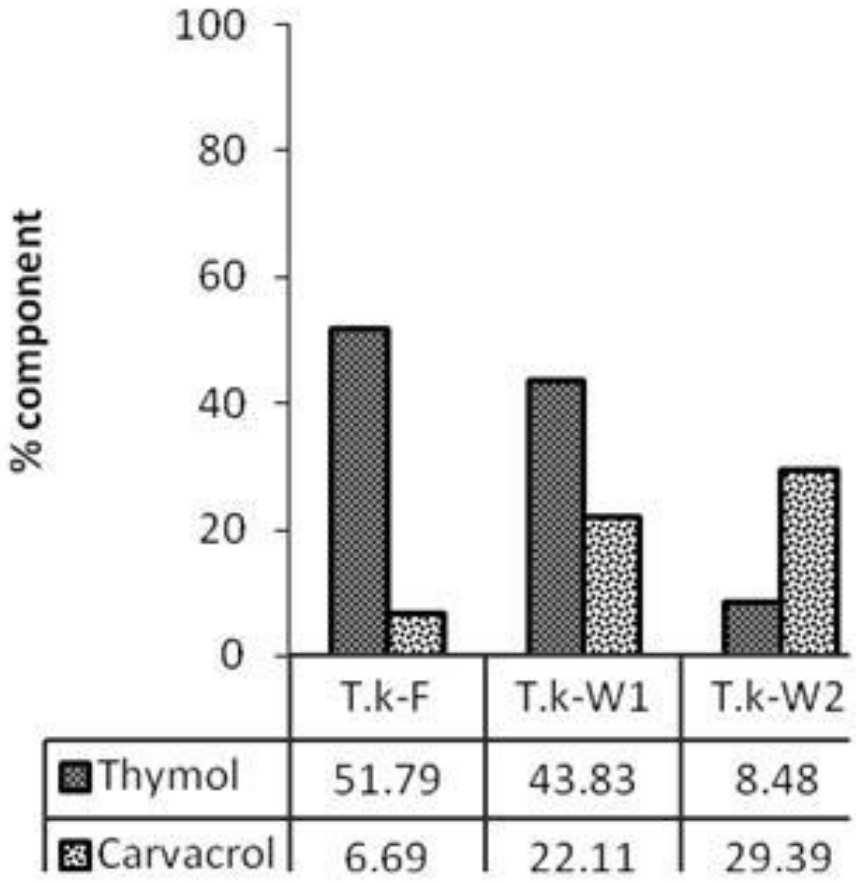

Figure 3. Comparison of two major componenets of thymol and carvacrol in*T. kutschyanus* plants.

of thymol (51.79%) while wild plants (T.k-W1 and T.k-W2) have the highest percentage of carvacrol (22.11% and 29.39% respectively) (Table 3, Figure 3). Considering the dominance of phenolic compounds (carvacrol and thymol) in all collected samples of *T.kotschyanus,* the samples are expexted to show antimicrobial activity. The findings of Mehregan et al. (2008) confirmed the antibacterial properties of *T. pubescens*. With the therapeutic effects of carvacrol in oral care products in mind, the wild samples (T.k-W1 and T.k-W2) can be useful for dental purposes. Further, according to carvacrol's higher toxicity of compared with thymol (Stammati et al., 1999), the essential oil of the field sample T.k-F (due to having less carvacrol) appears to be more useful for edible and medical purposes.

Conclusion

In our previous studies and others, it has been demonstrated that the chemical composition of the essential oil of *O. vulgare* L. varies with geographical location of collection site, climate and other ecological conditioning factors (Pirigharnaei et al., 2011; Kumar et al., 2007; Mockute et al., 2001). Also, our findings in the chemical composition of the essential oils of this species in varios habitats are in accordance with these reports.

In this study, interestingly, there were significant differences (quantitative and qualitative) in the oils composition in all collections, which suggest extrinsic/environment (like altitude, edaphic, temperature, humidity and climate …) and intrinsic/genetic factors play a role in determining the oils composition.

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