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Chemical and Molecular Characterization of Three Plant Species from Lamiaceae that Grow in Syria

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Abstract. The chemical composition of the essential oils extracted from three medicinal plant species that grow in Syria, namely *Thymus syriacus* Boiss., *Origanum syriacum* L. and *Thymbra spicata* L., was identified by GC-MS. Results showed that *T. syriacus* oil contained 27 compounds, *T. spicata* oil contained 39 compounds and *O. syriacum* oil contained 33 compounds. Thymol was the most abundant component in the three species. Monterpenes and sesquiterpes were the chemical markers for those species and may be considered as general chemical markers for all species belonging to Lamiaceae family regardless of their genus. Inter-Simple Sequence Repeat (ISSR) analysis used to evaluate the genetic variation between the three species showed a close genetic relationship between *T. syriacus* and *T. spicata*. Overall, results, indicated that it is possible to discriminate between the three plant species based on both of their chemical composition and ISSR markers.

Keywords: essential oil, GC-MS, ISSR, *Origanum syriacum*, *Thymbra spicata*, *Thymus syriacus*

Introduction

In recent years, application of medicinal plants has increased due to their effects, availability, low cost and patients' compliance to these medications and taking into consideration the known side effects of the synthetic drugs (Arzani *et al*., 2013). Essential oils and various extracts obtained from these plants are gaining a growing popularity and scientific interest. They have been used as functional ingredients in food, drugs and perfumery (Magwa *et al*., 2005; Tepe *et al*., 2005). These essential oils play an important role in protecting plants against viruses, bacteria, fungi and insects and against herbivores by reducing their appetite for such plants or by repelling undesirable others (Poma *et al*., 2018). Globally, many studies have been conducted to elucidate the chemical structure of plant species that contain such oils and the differences between them in relation to environmental factors and location of growth.

To date, many studies have described the chemical composition and pharmacological activity of a variety of *Lamiaceae* species (syn. Labiatae, the mint family) (Lukas *et al*., 2009; Özgüven *et al*., 2006; Hajhashemi *et al*., 2003; Milos *et al*., 2000). Within this family, the three species *Thymus syriacus* Boiss., *Thymbra spicata* L. and *Origanum syriacum* L., are among the most consumed plants in eastern Mediterranean region. These species have the same vernacular name "Za'ter" (Lukas *et al*., 2009; Fleisher and Fleisher, 1988) and they are more important than other species in the family due to the secondary metabolites and phytochemical compounds they contain. The results generated by Al Hafi *et al*. (2017) presented scientific evidence for the implementation of *T. syriacus* and *T. spicata* in the Lebanese folk medicine and lent support to use them as natural alternatives for synthetic antimicrobials. The aerial parts and volatile constituents of thyme plants are used as a medicinal material (Sáez and Stahl-Biskup, 2002).

Thyme is widely used all over the world and is mentioned in many drug constitutions (European Pharmacopoeia, 2002; British Pharmacopoeia, 2001). *T. syriacus* is a wide bush that grows in Syria. It is used as herbal tea and condiment. The fresh leaves of this plant are used for aromatization of home-made jams, candies and similar confections. *T. syriacus* is also known to have positive results for coughs and other respiratory complaints, as well as some cases of gastrointestinal disorders (Al-mariri *et al*., 2013).

T. spicata (common name: Mediterranean thyme "za'atar"), is also an important aromatic medicinal plant species that grows wild in various parts of Syria. It is used as a spice as well as a preservation agent in the food industry in most of the Mediterranean region

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(İnan *et al*., 2011; Akin *et al*., 2010). The dried plant is also used for treating asthma, colic, bronchitis and coughs (İnan *et al*., 2011; Baytop, 1984). The essential oils and leaves of *T. spicata* have different industrial uses for flavouring several kinds of food products, liqueur production, perfumery, herbal teas, antiseptic and antimicrobial agent in medicine.

The species of *O. syriacum* (syn. *Amaracus syriacus* L., *Majorana crassa*, *Origanum maru* L.) are all aromatic, herbaceous and perennial plant that also grows wild in Syria (Lukas *et al*., 2009). It is also known as Syrian oregano and white oregano. In Syria, it is commonly known as "Soba'a" or "Za'atar khalil". It has been used for ages in traditional medicine mainly in Lebanon and the Arab world (Farhat *et al*., 2012). It has antiseptic properties and has the ability to relieve stomach and intestinal pain. It is also used to treat heart problems, cough, toothaches (Gardner, 1989), cold, anxiety and wounds (Chandler-Ezell, 2004). The volatile phenolic oil has been reported to be among the top 10 essential oils (Letchamo *et al*., 1995), showing anti-bacterial, antimycotic, antioxidative, natural food preservative and mammalian age delaying properties (Letchamo *et al*., 1995; Jackson and Hay, 1994). Because *T. syriacus, T. spicata* and *O. syriacum* are important medicinal plants that are actually in danger of extinction and because the knowledge of the chemical composition of their essential oils and genetic phylogeny is an essential factor in their conservation, we conducted this study to analyze the chemical composition of their essential oils and to study the genetic relationships among those plant species that grow in Syria. We used GC-MS to analyze the chemical composition of the essential oils of the three species and we applied the Inter-Simple Sequence Repeat (ISSR) analysis to study the genetic relationships among those species.

Materials and Methods

Plant materials. The seedlings of wild *T. syriacus, T. spicata* and *O. syriacum* were collected from three different populations in Syria, (Soujeh (Damascus suburb), Beit Yashout and Wata Al-Khan (Lattakia)) and replanted in one location (Deir Al-Hajar, Damascus suburb, Syria). Vouchers of these species were deposited in the herbarium of plant biotechnology department at the Atomic Energy Commission of Syria (AECS). Leaves were collected from representatives of the three species at the flowering stage in May 2016.

Isolation of essential oils. Leaf samples were first air-dried at room temperature for 6 days until they were crisp and then powdered. Oil was extracted from those samples by hydrodistillation for 3 h using a Clevenger-type apparatus (Clevenger, 1928). Oil yields were then estimated on the basis of the dry weight of the plant material. The hydrodistillated mass was about 100 g DW.

Gas chromatography–mass spectrometry (GC/MS) analysis. GC-MS analysis was performed using Agilent GC-MS model GC-6890 and mass selective detector 5973 inert. The capillary column was DB-35 (30 x 0.2 mm, film thickness 0.25 μm). The operating materials and conditions were as follows; carrier gas, helium, with a flow rate of 1 mL/min; volume injected 1 μL of the essential oil and ionization potential 70 ev. The initial temperature of the column was 50 °C (hold time is 2 min). The column was heated to 1700 °C at a rate of 2 °C/min (held 7 min), and then to 250 °C at a rate 4 °C/min (hold time is 10 min).

The compounds were identified by comparison of their relative retention times and the mass spectra with those of authentic reference compounds given in literature (Adams, 2007).

Genetic analyses. *DNA extraction*. DNA was isolated from the leaf samples of the three species targeted according to the method of the Dorokhov and Klocke (1997). The DNA was also isolated from fresh leaves of one sample of sage (*Salvia oficinalis* L., Lamiaceae) to be used as an outgroup in the cluster analyis of species studied. Recovered DNA pellets were dried under the laminar flow and then resuspended in 150 mL of doubled sterile distilled water. DNA was quantified using Gene Quant Spectrometer (Amersham Biosciences) and the concentration of all samples was set at 10 ng/mL.

***ISSR analysis*.** Using 19 selected primers (Table 1), ISSR analysis (Bornet and Branchard, 2001) was carried out on samples targeted. The amplification was carried out in a 25 μL reaction volume containing 7.5 mM Tris-HCl (pH 9 at 25 °C), 50 mM KCl, 2 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.6 mM of each dNTP (Roche), 1.5 U of Taq DNA polymerase (Eurobio), 30 ng of genomic DNA and 100 μM of each primer (Invitrogen) (Table 1).

Using an Eppendrof Cycler (Germany), these reactions were subjected to a cycle of 5 min at 94 °C followed by 40 cycles, each of which consisted of 10 s at 94 °C, 10 s at 50 °C and 10 s at 72 °C. A final extension cycle

was performed at 72 °C for 7 min. Amplification products were stored at 4 °C until visualization on agarose gel electrophoresis. At least two replicates were prepared for each PCR reaction.

Amplified DNA fragments were visualized by electrophoresis on 1.8% agarose (Q-BIOgene) gel to which ethidium bromide (Fluka) was added using a 0.5X Tris Borate EDTA (TBE) buffer. Electrophoresis was performed in 1x TAE buffer at 100 V for 2.5 h. A 100 bp ladder (Vivantis) was used to estimate the approximate molecular weight of amplification products. Amplification profiles generated were visualized and photographed under UV light using Gel Documentation System (GDS8000, UVP). Since, more than one replicate was prepared for each primer used, only bright and reproducible bands were scored as present (1) or absent (0). The un-weighted pair group method with arithmetic averages (UPGMA) and percent disagreement values (PDV) of the STATISTICA program were used to construct the matrices and the dendrograms (Grzesiak *et al*., 2003).

Results and Discussion

Chemical composition of essential oils. Syrian flora is well known for its richness and diversity and it contains numerous species of medicinal use (Zayzafoon *et al*., 2012) such as *T. syriacus*, *T. spicata* and *O. syriacum*. Bozkurt *et al*. (2020) found that the essential oils of those species have a promising potential to be used as antibacterial agents. *O. syriacum* is one of the most important stable herbs in the Arab region due to its pleasant flavor, low cost and diversity of culinary applications (Dbaibo *et al*., 2020). Alwafa *et al*. (2021) reviewed *O. syriacum*`s taxonomy, morphology and distribution. The authors collected available data regarding the species cultivation, production and processing. They also provided information about environmental and processing conditions that can influence the composition of the essential oil of this species. According to Zayzafoon *et al*. (2012), *T. syriacus* is commonly used as a flavouring agent, herbal tea, condiments and spices. Using photochemiluminescence assay, the authors determined the integral antioxidant capacity of *T. syriacus* samples collected from different Syrian locations. They also analyzed the composition of this species essential oil by GC-MS. The antioxidative properties of the volatile oils of *T. syriacus* and *T. spicata* that grow wild in Kurdistan-Iraq were assessed.

In this study, we used GC-MS to analyze the chemical composition of the essential oils of *T. syriacus*, *T. spicata* and *O. syriacum* because it is the most precise technique to identify chemical constituents in essential

Table 1. Names and sequences of primers used for ISSR and number of polymorphic lines and bands generated

Primer name	Sequence (5′-3′)	Total no. of lines	Total no. of polymorphic lines	% polymorphic lines	No. of fragments amplified
A1	CACACACACACARR	20	20	100	29
A8	CACACACACACARM	17	16	94.1	25
A16	CACACACACACAR	8	8	100	10
A26	CACACACACACAK	14	14	100	22
A30	AGCAGCAGCAGCR	9	8	88.9	18
A41	AGCAGCAGCAGCK	8	8	100	14
B3	CTCTCTCTCTCTCTCTTG	8	8	100	11
B5	CACACACACACAGG	11	11	100	17
D4	GATAGATAGATAGATA	11	11	100	24
C25	GTGGTGGTGGC	7	7	100	12
C31	AGAGAGAGAGAGAGAGT	6	4	66.7	15
UBC850	GTGTGTGTGTGTGTGTCTC	4	4	100	7
UBC855	ACACACACACACACACCTT	7	7	100	11
UBC857C	ACACACACACACACACCTGC	7	7	100	11
UBC857G	ACACACACACACACACCTGG	7	7	100	11
IG-09	AGAGAGAGAGAGAGAGC	8	8	100	14
IG-12	GAGAGAGAGAGAGAGAC	6	6	100	9
UBC825	ACACACACACACACACT	5	3	60.0	13
UBC826	ACACACACACACACACC	4	4	100	7
Sun		167	161	96.4	280

oils (Lukas *et al*., 2009; Tayoub *et al*., 2006). Essential oils extractions provided high yields: 2.5, 3.4 and 1.25% (v/w) for *T. syriacus*, *T. spicata* and *O. syriacum* oils, respectively. Results of chemical analyses of the essential oils are shown in Table 2. The number of components identified in *T. syriacus*, *T. spicata* and *O. syriacum* oils were 29, 42 and 33, respectively. Based on GC–MS investigations, the oils were complex mixtures of monoterpenes, sesquiterpenes and non-terpenes. Five compounds were common among the three essential oils. The monoterpenes compounds represented 95.63% in *T. syriacus* oil, 92.97% in *T. spicata* oil and 93.83% in *O. syriacum* oil with dominance of oxygenated forms. The content of sesquiterpenes in *T. syriacus*, *T. spicata* and *O. syriacum* oils were 2.06%, 4.67% and 3.76%, respectively. Other common constituents were non-terpene derivatives such as aromatic and aliphatic compounds (Table 3).

Thymol (77.32%), Isoborneol (6.48%), Ç-Terpinen (4.87%), *p*-Cymene (4.15%) were the most abundant components in *T. syriacus* essential oil, while the major components in *T. spicata* oil were Thymol (51.00%), Carvacrol (23.68%), Ç-Terpinen (9.65%) and *O*-Cymene (4.22%). The major components in the essential oil of *O. syriacum* were Thymol (52.04%), *p*-Cymene-2-ol (17.97%), Ç-Terpinen (10.12%), and *p*-Cymene (5.05%).

The variations in components percentages among the studied essential oils of the cultivated three wild species may be due to changes in environmental conditions (i.e., rainfall, temperature, humidity and altitude above sea level in addition to the new soil conditions) between the regions where those species grow wild and the cultivation station region. The physiological dissimilarities among the studied plant species and their different climate adaptation behaviour may also contributed in differences in contents seen among the analyzed essential oils. The interactions of environmental conditions with genetic factors was confirmed by different studies (Andiego *et al*., 2019; Tayoub *et al*., 2006; Akgül *et al*., 1999; Barbosa and Wagnger, 1998; Cañigueral *et al*., 1994).

We used the ISSR technique for the genetic characterization of the three species targeted in this study because this technique proved efficient for revealing genetic relationships between *Triticum* L. and *Aegilops* L. (Haider *et al*., 2010) and within Orchidaceae family (Haider *et al*., 2012), as well as for several aromatic and medicinal plant groupings (Farajpour *et al*., 2011; Manica-Cattani *et al*., 2009; Pezhmanmehr *et al*., 2009; Suárez González *et al*., 2007; Fracaro *et al*., 2005). The ISSR technique can be the marker of choice as believed by Sarwat (2012) for revealing genetic polymorphism among plant species due to its reliability, simplicity, cost effectiveness, speed and its effectiveness in assessing genetic diversity among closely related species.

It is worth noting here that this study is considered the first attempt to reveal genetic relationships among *T. spicata*, *O. syriacum* and *T. syriacus*. When ISSR was performed on the aforementioned species using 19 primers (ranging between 11 and 20 bases), 167 band (fragment) lines were obtained out of which 161 lines were polymorphic (81.7%). Out of the 19 primers used, 15 primers generated polymorphism in all lines of bands (100%). The primer with the highest number of bands amplified was A1 (29 bands), while the lowest number of bands was observed for ISSR primers UBC850 and UBC826 (7 bands each). Fig. 1, 2 and 3 show the amplification products resultant from the use of primer A30, A8 and C31 on the studied samples, respectively. Clustering analysis of *T. spicata*, *O. syriacum* and *T. syriacus* based on the data generated from ISSR using 19 primers clearly distinguished all the three tested species (Table 1). In the phylogenetic tree constructed,

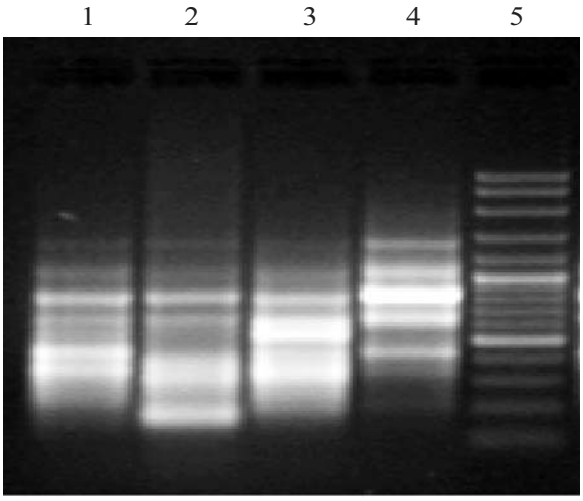

Fig. 1. Polymorphism generated from the use of ISSR primer A30 on species studied. Lane 1, *O. syriacum*; lane 2, *T. spicata*; lane 3, *T. syriacus*; lane 4, *S. officinalis*; lane 5, 100 bp DNA ladder.

Table 2. Essential oil composition of *T. spicata*, *O. syriacus* and *T. syriacum* leaves collected in Damascus, Syria

Compounds	RT	*T. spicata* (%)	*O. syriacus* (%)	*T. syriacum* (%)
3-Thujene	3.95	0.4	0.4	-
α-Pinene	4.16	-	0.2	-
Camphene	4.75	-	tr.	-
β-Pinene	5.64	-	0.1	-
2-Thujene	5.8	0.9	1.1	1.0
Amyl vinyl carbinol	6.25	0.4	1.3	-
α-phellandrene	6.61	0.4	-	-
3-Octanol	6.68	-	1.2	-
(+)-4-Carene	7.06	1.7	-	1.1
D-Limonene	7.53	0.2	0.2	-
α-phellandrene	7.88	0.2	0.2	-
O-Cymene	8.29	4.2	-	-
p-Cymene	8.38	-	5.1	4.2
Ç-Terpinen	9.43	9.7	10.1	4.9
Terpineol	10.32	0.1	0.2	-
Terpinolen	10.65	0.1	2.4	-
α-Linalool	11.9	0.1	0.2	-
Cis-alpha-Terpineol	12.51	-	tr.	-
Styrene	12.66	0.1	-	0.3
α,*p*-Dimethylstyrene	12.69	-	0.1	-
Borneol	17.78	0.1	0.2	0.7
Terpinen-4-ol	18.3	0.9	1.6	-
p-Menth-1-en-8-ol	19.96	0.1	-	-
Isoborneol	21.06	-	-	6.5
Methy salicylate	22.41	0.1	-	-
Anisole	23.25	0.1	-	-
Carvone	23.97	0.1	-	-
Trans-dihydrocarvone	24.55	-	0.1	-
Cyclohexanone	25.01	0.1	-	-
p-Isopropylanisole	28.54	0.1	-	-
Carvacrol	29.72	23.7	1.0	tr
Thymol	31.73	51.0	52.0	77.3
p-Cymen-7-ol	33.64	0.1	18.0	
Caryophyllene	34.02	2.6	2.8	-
Eudesma-3, 7(11)-diene	35.02	0.1	-	-
(+)-Aromadendrene	35.69	0.4	-	-
Eugenol	36.14	0.4	0.1	-
α-Caryophyllene	37.4	0.1	0.3	0.2
Aromadendrene	37.85	tr.	-	-
Ç-Muurolene	37.9	-	-	0.2
Neoisolongifolene	38.76	tr.	-	-
(+)-Ledene	38.84	-	-	0.3
α-Amorphene	39.33	tr.	-	-
α-Muurolene	39.51	-	-	0.2
Germacrene D	40.01	-	0.2	-
(+)-Ledene	40.48	0.3	-	-
Cubenene	41.16	-	-	0.3
α-Bisabolene	41.19	-	0.3	-
Germacrene B	41.33	0.1	-	-
L-calamenene	42.72	-	-	0.1
α-Amorphene	42.96	tr.	-	-
α-Cadinene	43.25	0.2	0.1	-
β-Guaiene	44.62	-	-	tr.
α-Calacorene	45.16	-	-	0.1
Globulol	50.08	0.1	-	-
(-)-Spathulenol	50.57	0.3	-	-
Caryophyllene oxide	50.9	0.4	0.3	0.7
Isoarmadendrene epoxide	55.27	-	-	0.1
Total identified		99.7	99.4	98

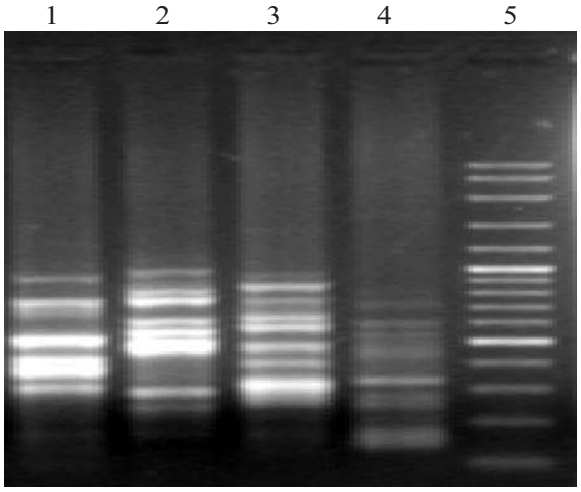

Fig. 2. Polymorphism generated from the use of ISSR primer A8 on species studied. Lane 1, *O. syriacum*; lane 2, *T. spicata*; lane 3, *T. syriacus*; lane 4, *S. officinalis*; lane 5, 100 bp DNA ladder.

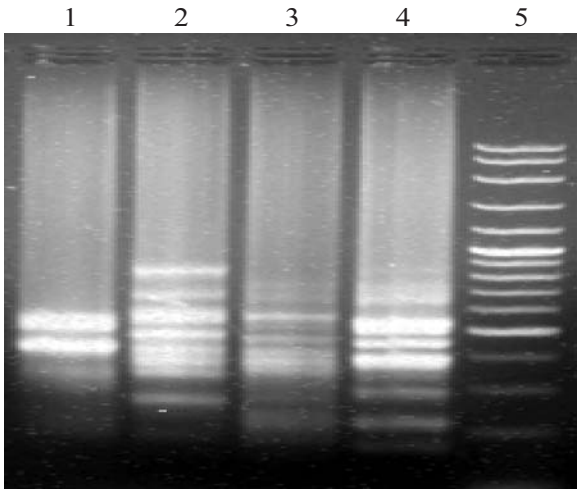

Fig. 3. Polymorphism generated from the use of ISSR primer C31 on species studied. Lane 1, *O. syriacum*; lane 2, *T. spicata*; lane 3, *T. syriacus*; lane 4, *S. officinalis*; lane 5, 100 bp DNA ladder.

a very close genetic relationship between *T. syriacus* and *T. spicata* was observed since both species appeared as sister species. The two species had the same genetic distance from *O. syriacum*, which was the closest to the reference group *S. oficinalis* (Fig. 4).

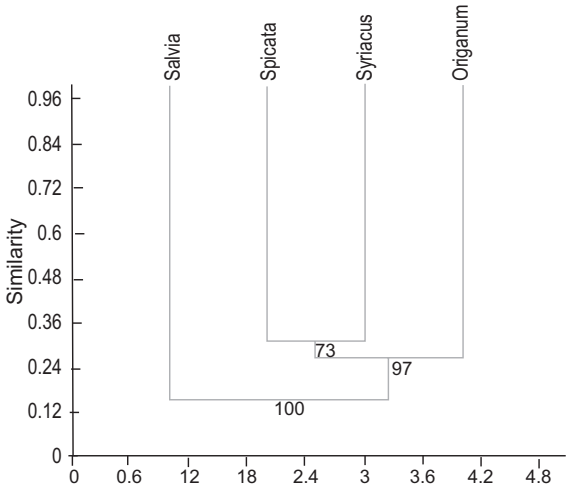

Fig. 4. Dendrogram showing the degree of relatedness among the studied species based on ISSR data.

Table 3. Main chemical components in the essential oils of *T. syriacus, T. spicata* and *O. syriacum*

	T. spicata	*O. syriaucm*	*T. syriacus*
Monoterpines	93.88	92.97	95.63
Sesquiterpines	4.67	3.76	2.06
Other	1.11	2.78	0.45

The addition of ISSR analysis that was performed to reveal the genetic relationships among the three species targeted to the chemical analysis results (mono- and sesquiterpenes) provided additional information on the similarity among the targeted species. We suggest that other species in the same grouping may have similar routes of biosynthesizing of secondary compounds, which result in the activation of similar genes. However, the presence or absence of insects and other parasites in addition to the environmental factors could affect the activated metabolic routes (Paolini *et al*., 2010; Pichersky and Gershenzon, 2002) which must be taken into consideration when making associations between genes and component concentration. Thus, further research is necessary to evaluate more thoroughly the genetic and chemical correlations regarding the essential oil production and its components in medicinal plant species.

Conclusion

Here, we present the first report that combines the study of the chemical properties of *T. syriacus*, *O. syriacum* and *T. spicata* and their genetic relationships. ISSR

analysis allowed us to detect the affinity among the studied species at the molecular level. Results generated from ISSR confirm the observations based on the analysis of the chemical composition (mono and sesquiterpenes) of essential oils in these species. Based on the combined results of chemical and molecular analyses, a very close relationship was revealed between *T. syriacus* and *T. spicata*, stressing the role of environment conditions on oil characteristics quality.

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Conflict of Interest. The authors declare that they have no conflict of interest.

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