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Chemotaxonomy and flavonoid profiling of *Torilis* species by HPLC/ESI/MS^2

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ABSTRACT

The flavonoid profiles of Turkish *Torilis* Gaertn. (Apiaceae) species were studied by TLC, HPLC-UV and HPLC/ESI/MS^2 (negative mode). *O*-glycosides of luteolin, apigenin and chrysoeriol were identified from crude extracts with the help of mass spectra in different MS/MS modes, such as full scan, precursor ion scan and product ion scan. Luteolin-7-*O*-glucoside and luteolin-7-*O*-rutinoside were common to all species. Flavonoid profiles usually differ from one species to another and can be put to use for a genus such as *Torilis* which has been little studied. By the help of different flavonoid profiles, it is concluded that, the plants, which are recognised as less rayed subspecies of *Torilis arvensis* (Huds.) Link. in various floras including Turkish one, must be classified in species category as *Torilis chrysocarpa* and *Torilis purpurea*. Flavonoid profiles seem to be in relation with evolutionary biogeography of the species. Because the most isolated species of the genus, endemic *Torilis triradiata*, has the most different flavonoid pattern. Moreover, geographically isolated species, *T. triradiata* and *Torilis leptocarpa*, do not share any flavonoid except for the two which are common to all species.

1. Introduction

Torilis Gaertn. (Apiaceae) is a genus of potential use in medicine because of interesting anti-infectious and anticancer properties (Kim et al., 1998a, 1998b, 2000, 2001; Park et al., 2003; Youn et al., 2004). It is comprises ca. 13 species centred mainly in the Mediterranean area but distributed also in South and Central Asia. Anatolia is thought to be the main diversity centre for the genus because 11 of these 13 species and also the only endemic species of the genus are found in Anatolia (Güzel, 2009). Torilis has been described as a "heterogeneous genus in need of further study" in the Flora of Turkey (Cullen, 1972). Indeed, members of this genus display intraspecific variations like heterocarpy and heterophylly as well as interspecific similarities, thus complicating their classification. There is often confusion between different samples of this genus in herbariums and so the aim of this study is to develop a method to find characters providing data of both taxonomical and pharmaceutical use.

Most phytochemical studies on *Torilis* have focused on terpenoids (Halim et al., 1990; Saad et al., 1994, 1995; Park et al., 2003; Bigdeli et al., 2004) so data on flavonoids is limited. Crowden et al. (1969) studied leaf flavonoids of approximately 300 Apiaceae species and found only luteolin-7-glucoside in some taxa of *Torilis*. Harborne and Williams (1972) studied the

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fruit flavonoids of approximately 100 Apiaceae species including 5 species of *Torilis*. They found luteolin-7-glucoside and luteolin-7-rutinoside in all of these *Torilis* species. Halim et al. (1990) reported the presence of luteolin and apigenin glycosides (glucoside or glucuronide) in *Torilis arvensis.*

In our study, the phytochemical investigation of *Torilis webbii*, *Torilis leptocarpa*, *Torilis chrysocarpa, Torilis purpurea* and endemic *Torilis triradiata* was undertaken for the first time.

Mass spectrometry coupled with liquid chromatography (LC/MS) and tandem mass spectrometry (MS/MS) are effective tools for identifying flavonoids in plant extracts. They are frequently used in combination with electrospray ionisation (ESI) to identify flavonoid content even in raw extracts (Ferreres et al., 2008) when trace amounts of flavonoid glucosides are present (Ma et al., 1997) and in the absence of standards (Sánchez-Rabaneda et al., 2004; Lhuillier et al., 2007).

2. Experimental

The following experimental procedures were performed: (1) methanolic extraction, (2) TLC of acid-hydrolysed extracts to ascertain the aglycon content of plants, (3) validation of aglycon content with HPLC, (4) LC/MS/MS/ESI (−) experiments to identify all flavonoid content with or without the use of standards. (5) TLC of methanolic extracts with available standards for validation.

2.1. Chemicals

Methanol, chlorhydric acid, ethyl acetate, acetic acid, formic acid, toluene, aceton, petroleum ether, 2-aminoethyl diphenylborate, polyethyleneglycol and acetonitrile (HPLC grade) were purchased from Sigma Chemical Co. (USA). Flavonoid standards – luteolin, apigenin, chrysoeriol, quercetin, kaempferol, myricetin, chrysin, genistein, hyperoside, isorhamnetin, rutin, luteolin-7-O-glucoside, orientin (luteolin-8-C-glucoside), homoorientin (luteolin-6-C-glucoside), apigenin-7-O-glucoside, apiin (apigenin-7-O-apiosylglycoside) and vitexin (apigenin-8-C-glucoside) were purchased from Extrasynthese (France).

2.2. Plant material and extraction

Aerial parts of all plants were collected from natural populations. For each species at least two populations from distant habitats were studied. Voucher specimens were deposited in the herbarium of the Biology Department at Mustafa Kemal University. List of all species with complete binomials, voucher numbers, collection localities, collector, collection dates and GPS coordinates of localities are given in Table 1. Room-dried aerial parts of plants were ground. Species size differed from small, with an approximate height of 30 cm and stem width 1 mm such as *Torilis tenella*, to large, with an approximate height of 150 cm and stem width 5 mm like *Torilis japonica*. Therefore, amounts of dried and ground samples ranged from 2 g to 50 g. These plant powders were extracted with methanol at room temperature for 24 h. After filtration, the extract was evaporated at 40 °C under reduced pressure. Plant powders yielded approximately 1/5 (m/m) crude extracts which were used directly in all experiments.

For acid hydrolysis, 50 mg of crude extract was hydrolysed with 10 ml HCl 2 N at 100 °C for 1 h. The acidic solution was partitioned with 10 ml ethyl acetate after cooling. Ethyl acetate phase was dried with Na_2SO_4 and evaporated to obtain hydrolysed extract.

2.3. TLC

TLC analyses were performed with silica coated aluminium plates (Sigma). Ethyl acetate/petroleum ether (1:1, v/v) was used for the hydrolysed extracts. Ethyl acetate/acetic acid/formic acid/water (100:11:11:13, v/v/v/v) and toluene/acetone/

Table 1
list of all species with complete binomials, voucher numbers, localities, collector, collection dates and GPS coordinates of localities.

Species	Voucher No	Locality	Collector	Date of collection	GPS coordinates
Torilis leptophylla (L.) Rchb.	MKUF-172	Hatay, Altınözü, between Hanyolu-Yiğityolu villages,	Y. GÜZEL	29-4-2006	N 36° 01 933–E 036° 12 069
Torilis tenella (Delile) Rchb.	MKUF-204	Mersin. Taşucu, Boğsak	Y. GÜZEL	23-4-2006	N 36° 12 652–E 036° 10 699
Torilis chrysocarpa Boiss. & Blanche	MKUF-451	Mersin, Erdemli, Limonlu Lemas Valley	Y. GÜZEL	15-5-2005	N 36° 59 303–E0 34° 34 316
Torilis purpurea (Ten.) Guss.	MKUF-265	Mersin, Erdemli, Limonlu Lemas Valley	Y. GÜZEL	15-5-2005	N 36° 59 303–E0 34° 34 316
Torilis nodosa (L.) Gaertn.	MKUF-306	Mersin, Gözne	Y. GÜZEL	14-6-2005	N 36° 58 923– E 034° 34 378
Torilis webbii Jury	MKUF-118	Antalya, Akseki	Y. GÜZEL	10-5-2006	N 36° 53 477 –E 031° 47 726
Torilis triradiata Boiss. & Heldr.	MKUF-134	Antalya, Akseki	Y. GÜZEL	10-5-2006	N 36° 53 477–E 031° 47 726
Torilis arvensis (Huds.) Link	MKUF-236	Adıyaman, Çatalağaç	Y. GÜZEL	17-7-2006	N 37° 37 360–E 037° 30 245
Torilis leptocarpa (Hochst.) C.C.Towns.	MKUF-345	Elazığ, near the Kömürhan Bridge	Y. GÜZEL	27-7-2006	N 38° 26 550–E 038° 50 187
Torilis japonica (Houtt.) DC.	MKUF-404	Ankara, Kızılcahamam	Y. GÜZEL	9-8-2005	N 40° 27 780–E 032° 37 422
Torilis ucranica Spreng.	MKUF-249	Ankara, Kızılcahamam	Y. GÜZEL	9-8-2005	N 40° 28 122–E 032° 38 045′

formic acid (3:3:1, v/v/v) solvent systems were used for methanolic extract. After drying, TLC plates were observed under 366 nm UV light before and after spraying with NEU's reagent (1% of 100:5, w/w 2-aminoethyl diphenylborate/polyethyleneglycol in methanol).

2.4. HPLC

HPLC analyses were performed with Shimadzu HPLC system which consisted of valve injector, SCL-10A VP model system controller, SPD-10A model UV/VIS detector and LC-10AS model pump. The chromatographic column was Novapak C18 (4 μm, 150 × 4 mm i.d.) (Waters). The mobile phase was 0.05% formic acid in water (A) and acetonitrile (B). Isocratic elution with 40% A and 60% B for 30 min at room temperature was used for analysis of aglycons in hydrolysed extracts. Total flow was 0.6 mL/min and injection volume was 20 μL. UV detection was performed at 254 and 360 nm. Concentrations of injected solutions were 3 mg/mL crude extract and 1 mg/10 mL pure standard in methanol/water (3:1, v/v).

2.5. LC-MS/MS ESI(−)

LC-MS/MS experiments were performed with Applied Biosystems 140B HPLC coupled with a Perkin–Elmer Sciex triple quadrupole spectrometer equipped with an electrospray ionisation interface. Experimental conditions were as follows: Temp.: 500 °C, flow: 600 mL/min, nebuliser gas and curtain gas: N_2, injection vol.: 50 mL, CE (collusion energy): −35 V, DP (declustering potential): −60 V. An isocratic elution with 80% A (0.05% formic acid in water) and 20% B (acetonitrile) was used on methanolic extracts for 30 min.

Fragmented ions of flavonoids designated according to ion nomenclature proposed by Ma et al. (1997) Y_0^- ion corresponds to the aglycon part of *O*-glycosides and Y_1^- corresponds to the monoglycosidic part of a diglucoside after the loss of one sugar unit.

2.6. Grouping analysis

Multivariate analysis, using the species analysed as Operational Taxonomic Units and the flavonoids as characters, was carried out with MVSP ver. 3.2 (Kovach, 2007). The resulting UPGMA dendrogram is shown in Fig. 1

3. Results and discussion

3.1. Aglycon identification

The TLC of the hydrolysed extracts gave 1 yellow and 2 green spots after spraying with NEU. When compared with authentic standards (luteolin, apigenin, chrysoeriol, quercetin, kaempferol, myricetin, chrysin, genistein, hyperoside, isorhamnetin and rutin), the yellow spot was seen to relate to a luteolin spot, green spots to apigenin and chrysoeriol spots according to Rf values and colours (absorbances). Complete hydrolysing indicated that all glycosides were *O*-glycoside type.

These results were validated with HPLC and LC/MS/MS ESI (−). Luteolin, apigenin and chrysoeriol peaks were present in all HPLC chromatograms.

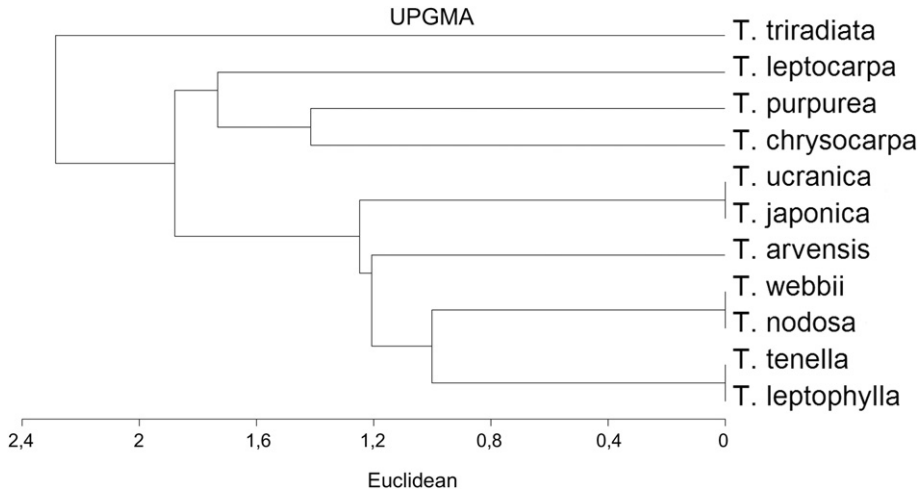

Fig. 1. Dendrogram of *Torilis* sp. based on flavonoid distributions and UPGMA clustering method.

Aglycon standard mixtures and hydrolysed extracts were also analysed with LC/MS/MS ESI (−). There were 3 masses related to flavonoid aglycons in chromatograms of hydrolysed extracts. These masses and their MS/MS fragments were identical to those of reference standards.

3.2. Flavonoid glycoside identification

After ascertaining that derivatives of luteolin, apigenin and chrysoeriol were present in all of the species through TLC, HPLC and LC/MS/MS/ESI (−) analyses of the hydrolysed extracts, methanolic extracts were analysed using the precursor ion scan approach to find precursor ions (i.e., derivative masses) releasing fragmented ion with the mass of the related aglycon. Thereafter, precursor ion identities were validated by product ion scan approach according to fragmentation patterns. All data on the identified compounds is given in Table 2. Distribution of these compounds to species is indicated in Table 3.

According to some recent studies (Cuyckens and Claeys, 2005; Petsalo et al., 2006; Lhuillier et al., 2007) radical aglycon ion $[Y_0 - H]^{\bullet -}$ has very low relative intensity in MS/MS spectra of 7-*O*-glucosides, in comparison with non-radical deprotonated aglycon ion Y_0^-. It may even not be visible. Our results were compatible with these studies. As in the MS/MS spectrum of luteolin-7-*O*-glucoside standard, it had to increase as collusion energy increased (Petsalo et al., 2006) and yet there was no peak of radical aglycon ion $[Y_0 - H]^{\bullet -}$ even with −40 V collusion energy. Besides standards, all compounds which were compared with standards or not, gave deprotonated aglycon ion Y_0^- in their MS/MS spectra. On the other hand, none gave radical aglycon ion $[Y_0 - H]^{\bullet -}$. It was therefore assumed that all identified compounds were 7-*O*-positioned.

$[M - H]^-$ ions *m/z* 577, 593 and 607, gave $[M - H - 308]^-$ ion which also corresponds to deprotonated aglycon Y_0^-. Loss of 308 u implies a rhamnoglucoside moiety like rutinoside (1–6 linkage) or neohesperidoside (1–2 linkage). Cuyckens et al. (2001), Sánchez-Rabaneda et al. (2003) and Shi et al. (2007) indicated that fragmentation patterns of these substances are discriminative in their identification. The main difference noted by all of these authors was that the fragmentation pattern of flavonoid *O*-neohesperidosides was more pronounced than that of their rutinoside analogues. Cuyckens et al. (2001) also indicated a relatively greater abundance of Y_1^- ions and Shi et al. (2007) emphasised the existence of $[M - H - 120]^-$ ions in the MS/MS spectrum of flavonoid *O*-neohesperidosides. In our study, only *m/z* 607 out of the above-mentioned $[M - H]^-$ ions belonging to flavonoid-diglucosides had a very pronounced product ion mass spectrum with *m/z* 607, 489, 447, 425, 393, 341, 329, 327 and 299 (Y_0^-) ions. Although there were no specific Y_1^- or $[M - H - 120]^-$ ions in the spectrum, *m/z* 489 $[M - H - 118]^-$ and *m/z* 447 ($Y_1^- + 2$) were probably related fragments. So, *m/z* 607 was assumed to be the *O*-neohesperidoside of chrysoeriol. $[M - H]^-$ ions *m/z* 577 and *m/z* 593 were assumed to be *O*-rutinosides of apigenin and luteolin respectively, because of less pronounced mass spectra. As seen in the product ion mass spectrum of *m/z* 593, there were only (Y_0^-) fragments except for the $[M - H]^-$ ion.

A luteolin-*O*-pentoside which has $[M - H]^-$ ion at *m/z* 417 was identified by the loss of 132 u and its intense *m/z* 285 (Y_0^-) peak.

In precursor ion scans of *T. triradiata*, *Torilis nodosa* and *T. webbii*, two $[M - H]^-$ ions at *m/z* 593 were found. One of them was a precursor of luteolin identified as luteolin-*O*-rutinoside and the other a precursor of chrysoeriol. Product ion mass spectrum of this chrysoeriol derivative gave intense *m/z* 299 (Y_0^-) ions with a loss of 294 u suggesting the presence of 162 u (hexoside) and 132 u (pentoside) moieties. So, this *m/z* 593 was assumed to be a chrysoeriol-*O*-pentoside-hexoside.

One of the precursor ions of apigenin, which has a $[M - H]^-$ ion at *m/z* 563 was identified as apigenin 7-*O*-apiosylglucoside (apiin) through its product ion mass spectrum which gave intense *m/z* 269 (Y_0^-) fragments and by comparing with standard.

There was an unidentified apigenin precursor ion of 450 u (apigenin+180) molecular mass with $[M - H]^-$ at *m/z* 449 in most of the plants. This substance gave a very pronounced product ion mass spectrum with *m/z* 449, 287 ($Y_0^- + H_2O$), 285

Table 2
A list of the identified compounds and their LC/MS/MS/ESI (−) data.

Compound number	Compound	t_r (min)	MW	$[M - H]^-$ (Precursor ion)	MS/MS fragments of $[M - H]^-$	Comparison with standard
1	Apigenin	18.6	270	269	269, 151, 117, 107, 35	Yes
2	Chrysoeriol	14.8	300	299	299, 284, 261, 256, 35	Yes
3	Luteolin	19.5	286	285	285, 175, 151, 133, 107, 35	Yes
4	Apigenin 7-O-glucoside	13.2	432	431	431, 269	Yes
5	Apigenin 7-O-apiosylgucoside (apiin)	10.5	564	563	563, 269	Yes
6	Apigenin 7-O-rutinoside	8.51	578	577	577, 269	No
7	Apigenin derivative (Apigenin + 180)	7.22	450	449	449, 287, 285, 269, 180, 150	No
8	Luteolin derivative (Luteolin−0−glucosideisomer)	4.12	448	447	447 285	No
9	Luteolin 7-O-glucoside	12.52	448	447	447, 285	Yes
10	Luteolin 7-O-rutinoside	8.46	594	593	593, 285	No
11	Luteolin-O-pentoside	17.56	418	417	417, 285	No
12	Chrysoeriol 7-O-glucoside	10.77	462	461	461, 299 181	No
13	Chrysoeriol 7-O-neohesperidoside	7.48	608	607	607, 489, 447, 425, 393, 341, 329, 327 and 299	No
14	Chrysoeriol-O-pentoside hexoside	7.96	594	593	593, 299, 191	No

Table 3
Distribution of identified flavonoids to the species.

Species	Compounds													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Torilis leptophylla (L.) Rchb.	+	+	+	+	+	+	+		+	+		+	+	
Torilis tenella (Delile) Rchb.	+	+	+	+	+	+	+		+	+		+	+	
Torilis chrysocarpa Boiss. & Blanche	+	+	+	+		+	+	+	+	+	+	+	+	
Torilis purpurea (Ten.) Guss.	+	+	+	+			+	+	+	+		+	+	
Torilis nodosa (L.) Gaertn.	+	+	+	+	+	+	+		+	+		+	+	+
Torilis webbii Jury	+	+	+	+	+	+	+		+	+		+	+	+
Torilis triradiata Boiss. & Heldr.	+	+	+	+	+				+	+				+
Torilis arvensis (Huds.) Link	+	+	+	+	+	+	+	+	+	+		+	+	
Torilis leptocarpa (Hochst.) C.C.Towns.	+	+	+			+	+	+	+	+			+	
Torilis japonica (Houtt.) DC.	+	+	+	+	+	+	+		+	+			+	
Torilis ucranica Spreng.	+	+	+	+	+	+	+		+	+			+	

($[Y_0 - 2H]^{-2} + H_2O$), 269 (Y_0^-), 180 and 150 ions. It was also found by Hwattum (2002) in rose hip (*Rosa canina* L. fruits) with similar fragments (269, 180, 151). Although the fragmentation pattern of *m/z* 449 might cause confusion with eriodictyol-glucoside because of intense *m/z* 287 fragments, it was straightforward to reach a conclusion thanks to TLC results and comparison with fragmentation patterns of the apigenin standard. There was no spot corresponding to the bright red one of the eriodictyol standard in the TLC of the hydrolysed plant extracts. As the *m/z* 287 ion was also present in the product ion mass spectrum of apigenin standard, the product was identified as water-bound apigenin rather than eriodictyol.

Analysing methanolic plant extracts by LC/MS/MS/ESI (−) with precursor ion scan and product ion scan procedures after identifying aglycon content from hydrolysed extracts by TLC and HPLC provides certain and valuable data even when there is little or no information about flavonoid content. Another advantage in such phytochemical analysis is that only small amounts of plant tissue are required – only 50–60 mg of crude extract.

3.3. Chemotaxonomic and ecological significance

This study confirmed that flavonoid content is a useful marker in *Torilis* taxonomy. Luteolin, apigenin and chrysoeriol derivatives exist in all species. Luteolin-7-*O*-glucoside and luteolin-7-*O*-rutinoside are found in all species. We studied fruit methanolic extracts with TLC and also noted the presence of luteolin-7-*O*-glucoside and luteolin-7-*O*-rutinoside spots, as previously reported (Harborne and Williams, 1972). In fact the TLC pattern of each fruit was quite similar to that of aerial parts, which means that either fruit or aerial parts can be used for chemotaxonomic purposes. Flavonoid profiles are highly constant within species but while there are some interspecific similarities between close species like *T. nodosa* and *T. webbii*, flavonoid profiles of the species are generally different. As seen in Table 3, distribution characters of derivatives, other than two one which are common to all species, are of importance for classifying the species. For example, chrysoeriol-*O*-pentoside-hexoside is found only in *T. triradiata*, *T. webbii* and *T. nodosa* and luteolin-*O*-pentoside is a marker for *T. chrysocarpa*.

While the dendrogram is a phenogram rather than a cladogram it reflects the phylogenetic relationships accurately and indicates the usefulness of flavonoid profiles in *Torilis* phylogeny (Fig. 1). Morphologically similar and taxonomically closer species have similar even same flavonoid profiles. So, they clustered together in the dendrogram. For example, *Torilis ucranica* and *T. japonica* are the only bracted species of the genus and they clustered together as a result of the same flavonoid profiles. These two species clustered near to *T. arvensis* which shares 7 flavonoids with them in addition to similar morphological characters like radiant petals, hermaphrodite and male flowers in umbels and elongation of style during anthesis. These shared morphological characters are also indicators of the outcrossing. All of these features indicate close phylogenetic relationship between these species and flavonoid profiles are congruent with them.

In our revision study of the Turkish *Torilis* species (Güzel, 2009), homophyllous and heterophyllous plants, which are recognised as less rayed subspecies of *Torilis arvensis* (Huds.) Link. in various floras including Turkish one, were rearranged according to characters of homocarpy-heterocarpy, the presence of carpophore cleft and flowering times. Flavonoid profiles also support the difference between the two groups. Heterocarpic, carpophore cleft-absent group contains Apigenin 7-O-rutinoside and Luteolin-O-pentoside unlike homocarpic, carpophore cleft present plants. So the first group was collected under the name of *T. chrysocarpa* Boiss. & Blanche and the second group was collected under the name of *T. purpurea* (Ten.) Guss.

The most remarkable result of our study was seen in the flavonoid profile of endemic *T. triradiata*. Comparison with others indicates the importance of flavonoid profiles in explaining evolutional relationships. *T. triradiata* is the only endemic and most isolated species of the genus with a narrow distribution area compared to the others. It is known from 4 localities situated at high altitudes of C_3 and C_4 squares of the grid system of Davis (Davis, 1965). This isolated species has the most different flavonoid pattern. As seen in Fig. 1 it occupied a separate branch in the dendrogram. The most significant comparison is between *T. triradiata* and *T. leptocarpa* which do not share any flavonoid except for the two that are common to all species and are geographically the most distant species. *T. leptocarpa* is only to be found in the east of the Anatolian Diagonal (Davis, 1971) and so does not have any contact with *T. triradiata,* but some varying contacts with other species. So while it occupied

another separate branch in the dendrogram, it is closer to other species than *T. triradiata*. (Fig. 1) These observations are surely indicators of common or different evolutionary pathways: species like *T. triradiata* and *T. leptocarpa* which are geographically isolated from each other have evolved along different evolutionary pathways, while others have shared the same pathways and probably the same ancestor for a long time.

Finding compounds which enable the study of secondary metabolism in terms of biosynthetic pathways and evolutionary significance at the genus level is very useful for the characterisation and conservation of biodiversity in countries such as Turkey that has a very high number of endemic species and frequent variations at the subspecific level.

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