Comparative Anatomical Studies on Three *Tanacetum* L. Taxa (Asteraceae) Growing in Turkey

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Geliş / Received: 01/10/2018, Kabul / Accepted: 08/03/2018

Abstract

Tanacetum L. (Asteraceae) is a complex genus represented by 160 species in the world and 47 species in Turkey. *Tanacetum parthenium* (L.) Sch.Bip., among the species of this genus which have been used in traditional treatments, is an officinal species registered in many pharmacopoeia and monographs due to its parthenolide content. Studies have shown that subspecies of *T. argenteum* (Lam.) Willd. also contain parthenolide as the major active constituent. In this study, besides the well known *T. parthenium*, the anatomical characteristics of parthenolide-containing taxa with therapeutic potential; *T. argenteum* (Lam.) Willd. ssp. *canum* (K.Koch) Grierson var. *pumilum* Grierson and *T. argenteum* (Lam.) Willd. ssp. *flabellifolium* (Boiss. & Heldr.) Grierson were also investigated for the first time. The transverse and superficial sections taken from the stems and leaves of the taxa were examined by light microscope and specific anatomical features were defined. Uniserial, multicellular covering hairs on stem and leaf surface and bifacial leaf structure are the most prominent characters that distinguish *T. parthenium* from other studied taxa. There was no significant anatomical difference found between the two infraspecific taxa of *T. argenteum*.

Keywords: Tanacetum, Stem, Leaf, Plant anatomy, Asteraceae

Türkiye'de Yetişen Üç *Tanacetum* L. Taksonu (Asteraceae) Üzerinde Karşılaştırmalı Anatomik Çalışmalar

Öz

Tanacetum L. (Asteraceae) dünyada 160 kadar, Türkiye'de ise 47 tür ile temsil edilen kompleks bir cinstir. Geleneksel tedavide kullanılan birçok türe sahip bu cinsin türleri arasında yer alan *Tanacetum parthenium* (L.) Sch.Bip., içeriğinde bulunan partenolit nedeniyle birçok farmakope ve monografta kayıtlı ofisinal bir türdür. Yapılan çalışmalar, *T. argenteum* (Lam.) Willd.'in alttürlerinde de temel aktif bileşik olarak partenolit olduğunu göstermiştir. Bu çalışmada, yaygın olarak bilinen *T. parthenium* yanında, partenolit içeriği nedeniyle tıbbi bitki potansiyeline sahip olan ve daha önce üzerinde anatomik bir çalışmaya rastlamadığımız *T. argenteum* (Lam.) Willd. ssp. *canum* (K.Koch) Grierson var. *pumilum* Grierson ve *T. argenteum* (Lam.) Willd. ssp. *flabellifolium* (Boiss. & Heldr.) Grierson taksonlarının anatomik özellikleri araştırılmıştır. Taksonların gövde ve yapraklarından alınan enine ve yüzeysel kesitler ışık mikroskobu ile incelenmiş ve anatomik karakterler tanımlanmıştır. Gövde ve yaprak yüzeyinde bulunan tek sıralı, çok hücreli örtü tüyleri ile bifasyal yaprakları *T. parthenium*'u çalışılan diğer taksonlardan ayıran en belirgin anatomik karakterlerdir. *T. argenteum*'un iki alt türü arasında anatomik olarak belirgin bir fark olmadığı tespit edilmiştir.

Anahtar Kelimeler: Tanacetum, Gövde, Yaprak, Bitki anatomisi, Asteraceae

1. Introduction

Asteraceae is the largest family of flowering plants with more than 1600 genus and 23000

species (Rahman et al., 2008). The *Tanacetum* L. (Syn. *Balsamita* Mill., *Chrysanthemum* L. pro parte, *Hemipappus*

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K.Koch, Pyrethrum Medikus.) comprising of nearly 160 species worldwide is the third largest genus in the tribe Anthemideae of this family. Majority of the Tanacetum species are distributed in Mediterranean basin, northern America, central and eastern Asia (Sonboli and Oberprieler, 2012). Tanacetum genus is represented by 61 taxa in Turkey, 27 of which are endemic (Güner et al., 2012; Korkmaz et al., 2015). Several species of the genus Tanacetum have been used as folk medicine for pain. fever. migraine, arthritis, inflammation, respiratory and gastrointestinal disorders remedy. The members of this genus contain mainly sesquiterpenes, essential oils, and phenolic compounds. Thanks to their phytochemical contents, possess they numerous pharmacological activities including cytotoxic, antiinflammatory, antifeedant, insecticidal, antimicrobial etc. (Abad et al., 1995; Gören et al., 2002). Especially sesquiterpene lactones have diverse biological activities and comprise a large array of secondary metabolites. More than 5000 structural variants have been identified mostly from Asteraceae taxa (Kreuger et al., 2012).T. parthenium (L.) Sch.Bip. (i.e. feverfew) is the most significant species of this genus relative to its migrane preventive effect (Pareek et al., 2011). Parthenolide was indicated as its major compound by many As researchers. a naturally occuring germacranolide type sesquiterpene lactone, parthenolide has methylene-y-lactone and epoxide groups with nucleophilic habit and by the help of these groups it easily interacts with biological sites. These interactions were found to be the explanation for its anticancer, pro-apoptic characteristics besides for many other activities (Mathema et al., 2012). As well as occuring in feverfew, parthenolide has also been reported in other Tanacetum species including T. argenteum (Lam.)

Willd. which contains three infraspecific taxa; ssp. argenteum, ssp. canum and ssp. flabellifolium (Grierson, 1975).Recently, Erdoğan Orhan et al. (2015) investigated total parthenolide quantities (%) of several Tanacetum taxa -including three subspecies of T. argenteum- by LC-MS and their results showed that the leaf of *T. argenteum* (Lam.) Willd. ssp. argenteum possessed the richest amount. European Pharmacopoeia requires minimum 0.2% parthenolide content for T. parthenium and this work also showed that T. argenteum ssp. argenteum, T. argenteum (Lam.) Willd. ssp. canum (K.Koch) Grierson var. canum and T. argenteum (Lam.) Willd. ssp. flabellifolium (Boiss. & Heldr.) Grierson had high amount of parthenolide over 1% in total. Also in our previous study, we examined the parthenolide contents of 8 taxa of Tanacetum genus (T. argenteum (Lam.) Willd. ssp. canum (K.Koch) Grierson var. *pumilum* Grierson, Τ. argenteum ssp. flabellifolium, T. armenum (DC.) Sch.Bip., T. cadmeum (Boiss.) Heywood ssp. cadmeum, T. cilicicum (Boiss.) Grierson, T. parthenium, T. praeteritum (Horw.) Heywood ssp. massicyticum Heywood, T. praeteritum (Horw.) Heywood ssp. praeteritum) growing Antalya province by Thin Layer in Chromatography (TLC) and High Pressure Liquid Chromatography (HPLC) methods. The results of our studies showed that T. parthenium, T. argenteum ssp. canum var. pumilum and T. argenteum ssp. flabellifolium had higher amount of parthenolide than 0.2% (Arıtuluk, 2016). Due to higher active ingredient contents. utilization of miscellaneous extract is preferred to the powder of a crude herb for preparation of plant-based products. However, pharmaceutical manufacturers often obtain T. *parthenium*-derived products from the powdered aboveground part of the plant in capsule form (Chaves and Da Costa, 2008).

To ascertain that medicinal plants such as feverfew are safe and effective, the plant material must be subjected to quality assessment for ensuring the correct botanical identification of the plant taxa. The morphology and anatomy of Tanacetum species are studied in only a limited number of researches. Tekin and Kartal (2016) investigated anatomical features of six endemic taxa from Turkey including T. argenteum ssp. argenteum. Pollen morphology of several taxa was also examined with light microscope (LM) and electron scanning microscope (SEM) (Özmen et al., 2009; Kodak et al., 2012). The similarity of Tanacetum species to other members of the Asteraceae family, especially Matricaria chamomilla L. with flower morphology, has led to the misidentification

and adulteration of herbal products (Simmons et al., 2002). For this reason in this study, especially the leaf and the stem structure have used been for the characterization of three phytochemically important taxa of this genus and the anatomical features of T. argenteum ssp. canum var. pumilum, T. argenteum ssp. and T. parthenium flabellifolium are described in detail, their important diagnostic characters are comparatively pointed out. The investigated T. argenteum taxa are endemic for Turkish flora.

2. Material ve Method

The plant samples were collected in flowering time from different areas of Antalya province of Turkey. The species were identified by authors using Flora of Turkey (Davis, 1975). Voucher specimens have been deposited in the HUEF (Herbarium of Hacettepe University, Faculty of Pharmacy, Ankara). Collection data and herbarium numbers of the investigated plants are given in Table 1.

Table 1. Collection data and herbarium numbers

Plant taxa	Collection data	HUEF numbers
<i>T. argenteum</i> (Lam.) Willd. ssp. <i>canum</i> (K.Koch) Grierson var. <i>pumilum</i> Grierson	C3 Antalya: Alanya, 40 km from Alanya to Taşkent, 1381 m, 12.07.2013.	13026
<i>T. argenteum</i> (Lam.) Willd. ssp. <i>flabellifolium</i> (Boiss. & Heldr.) Grierson	C4 Antalya: Gazipaşa, Plateau of Çobanlar town, 2010 m, 9.7.2013.	13024
<i>T. parthenium</i> (L.) Sch.Bip.	C3 Antalya: Kaş, Gömbe, 1221 m, 25.06.2014.	14062

For anatomical studies, the fresh leaf and stem samples of plant materials were preserved in 70% ethanol. Transversal and superficial sections were taken by hand using a sharp razor-blade, stained and mounted in Sartur Reagent (Çelebioğlu and Baytop, 1949) and chloralhydrate (50%) solution. The specimens with very dense hairy surface were slightly scratched with a blade to get proper cross-sections. The characteristic elements were determined by Leica DM500 light microscope and their microphotographs were taken by Leica ICC50 camera attached

to the light microscope. Some literature sources also contributed to the interpretation of anatomical data we obtained during our study (Metcalfe and Chalk, 1950; Upton et al., 2011; European Pharmacopoeia, 2014; Tekin and Kartal, 2016).

3. Findings

3.1. Stem anatomy

3.1.1. Transverse sections

Transverse sections from middle of the stems of three *Tanacetum* taxa revealed the following elements (Figure 1, Figure 2, Figure 3: A, B, C, D, E, F): The stem is more or less round or ridged in transversal section (Figure 1, Figure 2, Figure 3: A) . Epidermal cells are single-layered, square, polygonal or slightly rounded in shape and covered with a thin cuticle (Figure 1, Figure 2, Figure 3: B).



Figure 1. Stem anatomy of *T. parthenium*: A, B, C, D, E, F. Transverse sections. G, H, I. Superficial sections. c: cuticle, ca: calcium oxalate crystals, co: collenchyma, ct: covering

trichome, **e:** epidermis, **en:** endoderm, **gt:** glandular trichome, **p:** pith, **pa:** parenchyma, **pe:** pericycle, **ph:** phloem, **s:** stomata, **x:** xylem.

Cortex situated beneath the epidermis is thin and composed of flat or rounded parenchyma cells which are 3-6 layers in *T. parthenium* and 3-5 layers in *T. argenteum* ssp. *canum* var. *pumilum* and *T. argenteum* ssp. *flabellifolium*. Collenchyma cells with unevenly thickened walls are located beneath the epidermis of the stem ridges as 4-9 layers in *T. parthenium*, 1-8 layers in *T. argenteum* ssp. *canum* var. *pumilum* and 6-12 layers in *T. argenteum* ssp. *flabellifolium* (Figure 1, Figure 2, Figure 3: C).



Figure 2. Stem anatomy of *T. argenteum* ssp. *canum* var. *pumilum*: A, B, C, D, E, F. Transverse sections. G, H, I. Superficial sections. c: cuticle, ca: calcium oxalate crystals, co: collenchyma, ct: covering trichome, e: epidermis, en: endoderm, gt: glandular trichome, p: pith, pa: parenchyma, pe: pericycle, ph: phloem, s: stomata, x: xylem.

The large, rectangular or oval endodermal cells forming the innermost layer of the cortex constitute a distinct clear-cut ring framing the stele in *T. parthenium* and *T. argenteum* ssp. *canum* var. *pumilum*. However, the endodermal cells of *T*.

argenteum ssp. flabellifolium become discontinuous in some parts of the ring. The pericycle is composed of sclerenchyma cells lying just under the endodermis. The pericyclic sclerenchyma cells are 1-12 layers in T. parthenium, 1-9 layers in T. argenteum ssp. canum var. pumilum and 1-10 layers in T. argenteum ssp. flabellifolium (Figure 1, Figure 2, Figure 3: B, C, D, E). Vascular bundles in the stem are also arranged as a ring. The arrangement of vascular tissues within the bundle is collateral (Figure 1, Figure 2, Figure 3: A). Phloem is composed of squashed irregularly shaped cells. They

are 3-8 layered in T. parthenium, 2-7 layered in T. argenteum ssp. canum var. pumilum and generally 3-6 layered and interrupted in T. argenteum ssp. flabellifolium. Cambium is indistinct in all investigated taxa. Xylem consists of distinct tracheas and tracheids. Vascular bundles are surrounded bv sclerenchymatous cells (Figure 1, Figure 2, Figure 3: D). The pith is composed of polygonal, thin-walled, large parenchymatic cells. Calcium oxalate crystals are observed in this region (Figure 1, Figure 2, Figure 3: **F**).



Figure 3. Stem anatomy of *T. argenteum* ssp. *flabellifolium*: A, B, C, D, E, F. Transverse sections. G, H, I. Superficial sections. c: cuticle, ca: calcium oxalate crystals, co: collenchyma, ct: covering trichome, e: epidermis, en: endoderm, gt: glandular trichome, p: pith, pa: parenchyma, pe: pericycle, ph: phloem, s: stomata, x: xylem.

3.1.2. Superficial sections

Superficial stem sections exhibited the following elements (Figure 1, Figure 2, Figure 3: G, H, I): Epidermis has anomocytic (irregular) stomata. The stomata remain surrounded by 3-7 subsidiary cells in T. parthenium, by 3-6 subsidiary cells in T. argenteum ssp. canum var. pumilum and by 4-5 subsidiary cells in T. argenteum ssp. flabellifolium which are very similar to adjacent epidermal cells (Figure 1, Figure 2, Figure 3: I). Glandular and covering trichomes occur on the stem as epidermal outgrowths (Figure 1, Figure 2, Figure 3: G, H). Both of T. argenteum taxa have very dense indumentum consisting of T-shaped trichomes (Figure 2, Figure 3: G). In contrast, T. parthenium has a sparse indumentum with uniseriate and multicellular covering trichomes: The stalk consisting of a large conical basal cell followed by several small rectangular cells which are ended with an elongated, plane, slightly convex terminal cell (Figure 1: G). The glandular trichomes of all taxa are the most common type described in Asteraceae species. The biseriate and multicellular glandular trichome has a characteristically large head with the cuticle forming a bladder-like covering around the secretory cells (Figure 1, Figure 2, Figure 3: H).

3.2. Leaf anatomy

3.2.1. Transverse sections

Transverse sections of the lamina midrib and around of three *Tanacetum* taxa exhibited the following elements (Figure 4): The lower and upper epidermises consist of rounded, square, rectangular or angular single layered cells and are covered with thin cuticle. The leaf of *T. parthenium* is bifacial in transverse section (Figure 4: A, Β, C). The palisade parenchyma which is only one layer thick and located beneath the upper epidermis (adaxial) consists of compactly arranged and anticlinally elongated cells. The spongy parenchyma located in the lower part of the leaf (abaxial) consists of 3-4 seriate, rounded cells with large intercellular spaces (Figure 4: C). In contrast to T. parthenium, the leaves of Τ. argenteum taxa are monofacial in transverse section (Figure 4: D, E, F, G, H, I). Palisade and spongy parenchyma are 1-2 seriate in T. argenteum ssp. canum var. pumilum and 1-3 seriate in T. argenteum ssp. flabellifolium (Figure 4: F, I). The midrib is very prominent on the lower surface of the blade in T. parthenium (Figure 4: A, B) and T. argenteum ssp. canum var. pumilum (Figure 4: D, E). this region, In irregularly thickened collenchyma cells are observed just above the lower epidermis. But in T. argenteum ssp. flabellifolium, the midrib is prominent on both surfaces of the blade and irregularly thickened collenchyma cells are observed bordering the upper and lower epidermises (Figure 4: G, H). In all taxa, xylem and phloem are differentiated on the adaxial side and on the abaxial side, respectively. The collenchyma cells are also found neighboring the veins. They are 1-5 layered in T. parthenium and T. argenteum ssp. canum var. pumilum, 1-8 layered in T. argenteum ssp. flabellifolium (Figure 4: B, E, H). In T. argenteum ssp. canum var. pumilum and T. argenteum ssp. flabellifolium 1-4 layered sclerenchyma cells may be observed instead of collenchyma cells. In T. argenteum ssp. flabellifolium, the vascular bundle is surrounded by a single layer of large parenchyma cells to form bundle sheath (Figure 4: H). In T. argenteum ssp. canum var. pumilum, the bundle sheath consists of smaller parenchyma cells (Figure 4: E)

3.2.2. Superficial sections

Surface views of both abaxial and adaxial epidermis revealed the following elements (Figure 5): Upper and lower epidermises have anomocytic stomata. The stomata are surrounded by 4-6 subsidiary cells on both epidermises in *T. parthenium* (Figure 5: C). In *T. argenteum* ssp. *canum* var. *pumilum*, the stomata have 3-7 subsidiary cells on the lower epidermis and 4-5 subsidiary cells on the upper epidermis (Figure 5: F). In *T.*

argenteum ssp. *flabellifolium*, the stomata have 3-7 subsidiary cells on the lower epidermis and 4-6 subsidiary cells on the upper epidermis (Figure 5: I). Cuticular striations are also found particularly around stomata. Covering and glandular trichomes are present on both surfaces of the leaves and similar epidermal extensions can be observed on the stems (Figure 5: A, B, D, E, G, H).



Figure 4. Leaf Anatomy of *Tanacetum* taxa: A, B, C. Transverse sections of *T. parthenium*. D, E, F. Transverse sections of *T. argenteum* ssp. *canum* var. *pumilum*. G, H, I. Transverse sections of *T. argenteum* ssp. *flabellifolium*. bs: bundle sheath, c: cuticle, ca: calcium oxalate chrystals, co: collenchyma, ct: covering trichome, gt: glandular trichome, le: lower epidermis, pp: palisade parenchyma, ph: phloem, s: stomata, sp: spongy parenchyma, ue: upper epidermis, x: xylem, vb: vascular bundle



Figure 5. Leaf Anatomy of *Tanacetum* taxa: A, B, C. Superficial sections of *T. parthenium*. D, E, F. Superficial sections of *T. argenteum* ssp. *canum* var. *pumilum*. G, H, I. Superficial sections of *T. argenteum* ssp. *flabellifolium*. ct: covering trichome, gt: glandular trichome, le: lower epidermis, s: stomata, ue: upper epidermis.

4. Result and Discussion

Within the scope of our study, the anatomical characters of stem and leaf of the taxa; *T. parthenium*, *T. argenteum* ssp. *canum* var. *pumilum* and *T. argenteum* ssp. *flabellifolium*

were examined in detail and the prominent features were presented in Table 2 and Table 3, comparatively.

	<i>T. argenteum</i> ssp. <i>canum</i> var. <i>pumilum</i>	T. argenteum ssp. flabellifolium	T. parthenium
Covering trichome	T-shaped	T-shaped	uniseriate and multicellular
Glandular trichome	Asteraceae type, biseriate	Asteraceae type, biseriate	Asteraceae type, biseriate
	and multicellular	and multicellular	and multicellular
Stoma	Anomocytic, with 3-6	Anomocytic, with 4-5	Anomocytic, with 3-7
	subsidiary cells	subsidiary cells	subsidiary cells

Table 2. Comparison of stem anatomic features of investigated *Tanacetum* taxa

Epidermis	Single layer, square or rounded	Single layer, square or rounded	Single layer, polygonal or slightly rounded
Cortex parenchyma	3-5 layers	3-5 layers	3-6 layers
Collenchyma	1-8 layers	6-12 layers	4-9 layers
Endoderm	distinct	intermittent	distinct
Pericycle	1-9 layers	1-10 layers	1-12 layers
Vascular bundles	collateral	collateral	collateral
Phloem	2-7 layers	3-6 layers, intermittent	3-8 layers

Table 3. Comparison of leaf anatomic features of investigated Tanacetum taxa

	T. argenteum ssp. canum var. pumilum	T. argenteum ssp. flabellifolium	T. parthenium
Covering trichome	T-shaped	T-shaped	Uniseriate and multicellular
Glandular trichome	Asteraceae type, biseriate and multicellular	Asteraceae type, biseriate and multicellular	Asteraceae type, biseriate and multicellular
Stoma	Anomocytic, with 4-5 subsidiary cells on upper epidermis and with 3-7 on lower epidermis	Anomocytic, with 4-6 subsidiary cells on upper epidermis and with 3-7 on lower epidermis	Anomocytic, with 4-6 subsidiary cells on upper and lower epidermises
Epidermis	Single layer, square rectangular or rounded	Single layer, square rectangular or rounded	Single layer, angular or rounded
Collenchyma	Above the lower epidermis	Bordering the upper and lower epidermis	Above the lower epidermis
Mesophyll	Monofacial	Monofacial	Bifacial
Palisade parenchyma	1-2 layers	1-2 layers	Single layer
Spongy parenchyma	1-2 layers	1-3 layers	3-4 layers
Midrib	1-5 layered collenchyma cells, bundle sheath present	1-8 layered collenchyma cells, bundle sheath present	1-5 layered collenchyma cells, bundle sheath absent

In Asteraceae family members, both nonglandular and glandular hairs are present (Metcalfe and Chalk, 1950). While nonglandular hairs are observed in numerous morphological types, the glandular hairs are more homogenous in shape. The latter are usually composed of a stalk and a head of one or many cells (Ciccarelli et al., 2007). In our observations with light microscope, three *Tanacetum* taxa also show clearly that characteristic trichomes of stem and leaf surface are essential for establishing a more precise discrimination. Uniserial and multicellular covering hair which is composed of numerous small, square or rectangular cells on a conical basement and ended with a relatively long, flat, slightly convex cell at the top was the most important anatomical character that distinguishes T. parthenium from the other two taxa. On the other hand, in T. argenteum ssp. canum var. pumilum and T. argenteum ssp. flabellifolium the dense leaf indumentum is mostly composed of T-shaped trichomes. Additionally, biseriate glandular hairs are the common characters found in all three taxa. Besides the presence of anomocytic stomata on the stem and leaf surfaces of all taxa studied, there is no distinctive difference between the taxa in terms of numbers of subsidiary cells which are very similar to the other adjacent epidermal cells. The stems are more or less protruding in transverse section. Collenchyma cells with irregular cell walls are well developed in these protruding ribs and are 4-9 lines in T. parthenium, 1-8 lines in T. argenteum ssp. canum var. pumilum, 6-12 lines in T. argenteum ssp. flabellifolium. Endoderm is an easily visible ring in T. parthenium and T. argenteum ssp. canum var. pumilum stems, whereas it is not a complete circle in T. argenteum ssp. Pericycle and collateral flabellifolium. vascular bundles which are generally observed in Asteraceae family members, are common features seen in all three taxa (Metcalfe and Chalk, 1950).Leaf cross section clearly reveals a bifacial structure in T. parthenium and monofacial structure in T. argenteum ssp. canum var. pumilum ve T. ssp. flabellifolium. Another argenteum distinctive feature for T. argenteum ssp. canum var. pumilum and T. argenteum ssp. flabellifolium is that the occurrence of "bundle sheath" which is composed of tightly circled, starch rich parenchymatic cells. The vascular tissue is composed of bundles, with xylem towards the upper side and phloem at

the abaxial surface in all three taxa observed. Tekin and Kartal (2016) investigated anatomical features of six endemic taxa from Turkey including T. albipannosum Hub.-Mor. & Grierson, T. argenteum ssp. argenteum, T. cappadocicum (DC.) Sch.Bip., T. densum (Labill.) Sch.Bip. ssp. sivasicum Hub.-Mor. & Grierson, T. haussknechtii (Bornm.) Grierson, and T. heterotomum (Bornm.) Grierson; observed that leaf cross sections of all the examined taxa are monofacial and the vascular bundles of each are surrounded by a bundle sheath.Due to the fact that it is a well known and widely used official species, the anatomical features of T. parthenium are included in many monographs and pharmacopoeias (including European Pharmacopoeia) and all characters handled in these official publications are compatible with our findings (Rahfeld, 2009; Upton et al., 2011; European Pharmacopoeia, 2014). In this study as a pharmacopoeial contribution to be, the anatomical characters of T. argenteum ssp. canum var. pumilum and T. argenteum ssp. flabellifolium, two other taxa with therapeutic potential due to their high parthenolide content were demonstrated comprehensively for the first time.

5. Thanking

Many thanks to Ankara University Scientific Research Projects Foundation for its financial support (Project no: 13L3336007).

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