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*Original scientific article*

# COMPOSITIONAL DIVERSITY IN ESSENTIAL OIL OF *Ziziphora tenuior* L. ECOTYPES

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Present study reports a comparative screening of three ecotypes of *Ziziphora tenuior* L., sampled from three region with different elevation in middle and northeastern Turkey, based on their essential oil composition, total phenolic content and antioxidant capacities. *Z. tenuior* ecotypes showed twenty compounds, representing 89.18-91.44% of the essential oil (EOs) identified by GC-MS. The major components of EOs in ecotypes were pulegone (37.23-49.12%), and followed by 1,8-Cineole (2.26-7.78%), limonene (4.20-5.44%), *β*-Caryophyllene (3.88-5.11%) and thymol (2.68-4.11%), respectively. Ecotypes showed variable pulegone content and it was increased with elevation increase. The total phenolic contents varied among ecotypes ranged from 21.13 to 27.50 mg of gallic acid equivalent/g EOs. Antioxidant capacity was determined *in vitro* using DPPH radical-scavenging and FRAP (Ferric reducing power) assays and expressed as concentration of each extract required to inhibit radical by 50% ($IC_{50}$ and $EC_{50}$) values that ranged from 0.80 to 0.97 mg/ml in DPPH and 0.42 to 0.55 mg/ml in FRAP assays, respectively. Our results indicated that antioxidant capacity changed among ecotypes. Taking into account the essential oil content, it seems that to select ecotype was the most appropriate to obtain more pulegone for this plant from its wild habitat.

*Keyword:* Essential oils, genetic background, ecotype, biological activity, *Ziziphora tenuior* L.

## INTRODUCTION

Plants are very diverse group and are adding value of earth's diversity and fundamental to all life. They include high content of non-nutritive, nutritive, and bioactive compounds (ZIA-UL-HAQ *et al*., 2013; GUNDOGDU *et al*., 2014; ENGIN and MERT, 2020; SUBASI, 2020).

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Recently, there have been renewed interests in traditional medicine using natural products due to their availability, as well as better biodegradability compared to the synthetic agents (LOIZZO *et al*., 2013). In addition, there has been increased interest looking at biological activities of essential oils of aromatics and medicinal plants (SIENKIEWICZ *et al*., 2012; SHAHBAZI, 2017). All parts of aromatic and medicinal plants may contain essential oils. It is worthy to develop a better understanding of their chemistry and the biological properties of these extracts and their individual components for new and valuable applications in human health, agriculture, and the environment (SERCE *et al*., 2010; ALJAIYASH *et al*., 2018).

Plant essential oils (EOs) are mixture of chemical constituents, which have less molecular weight substances, such as alcohols, polyphenols, terpenoids, carbonyl compounds, and aliphatic compounds which provide smell and possess biological properties. Various plant parts produce Essential oils (EOs), subtle, aromatic, and volatile liquids, as secondary metabolites (COSTA *et al*., 2015). Plant secondary metabolites play important ecological and biological roles and are not only a useful array of natural products but also an important part of plant defense system against pathogenic attacks and environmental stresses as they often contain antimicrobial and antioxidative properties (NAJAFIAN, 2014).

EOs has been used as folk medicine throughout the history in all parts of the world. EOs has been widely employed for centuries in the pharmaceutical, agricultural, hygienic, cosmetic and food industries due to their antibacterial, antifungal, antiviral, antiparasitical, antidiabetic, anticancer (cytotoxic), insect repellent, food industry (flavoring), aromatherapy, antioxidant, perfume, and cosmetic properties (ZHANG *et el*., 2015). They are extracted from such plant materials as flowers, leaves, roots, barks, seeds, roots, fruit, and wood (NAJAFIAN, 2014). EOs may be affected by a variety of factors including genetics, nutrition, solar radiation, temperature, humidity, location and harvesting time (LOZIONE and VENSKUTONIS, 2005).

Ecotype, wild plant, refers to a locally adapted population or populations and the local adaptation is assumed to be a result of the action of natural selection. They are historically occurred in one area, and over time have become adapted to that special area (soil type, climate, altitude etc.). It is important to determine suitable ecotypes for commercial use (ERCISLI *et al*., 2003; ALP *et al*., 2016).

Located between junction of 3 gene centers Turkey is rich for genus *Ziziphora* L. (Lamiaceae) and the country is represented by six Ziziphora taxa including *Z. clinopodioides, Ziziphora capitata, Ziziphora persica, Ziziphora tenuior, Ziziphora taurica* subsp. *taurica* and *Ziziphora taurica* subsp. *cleonioides*. All Ziziphora taxa are characterized by strong aroma thus used as herbal teas and spices for centuries (ALP *et al*., 2016). In Turkish folk medicine, *Ziziphora* species have been used as infusion for various purposes such as antimicrobial, antiseptic, sedative and carminative effects as well as to treat stomach ache and wound healing (KILIC and BAGCI, 2013; CELIK *et al*., 2016).

*Ziziphora tenuior* L. is one of four species belonging to the genus Ziziphora (Lamiaceae). It is distributed in steppe, rocky slopes, uncultivated fields, sandy and gravelly coasts between 0-1750 m. Additionally, this plant grows in Mediterranean and Iran Turonian zones, including some parts of Iran, Afghanistan, Iraq and Azerbaijan. *Z. tenuior* is also distributed in Russia, Turkmenistan, Afghanistan, Pakistan, Caucasus and Siberia (RECHINGER, 1982; PIRBALOUTI *et al*., 2013).

Among Ziziphora taxa, previous studies mostly concentrated on *Z. clinopodioides* to determine its content and biological activity (ALIAKBARLU and SHAMELI, 2013; MARAL *et al*., 2015; GHANBARIAN *et al*., 2017; EROGLU OZKAN *et al*., 2019) and studies on *Z. tenuior* is scarce.

To our knowledge, no documented reports on diversity of chemical composition, total phenolic content and antioxidant capacity of the essential oils of various populations of *Z. tenuior* in terrestrial regions in Turkey are available. The aim of this study was to determine the variation of chemical constitutes and biological activity of different ecotypes of *Z. tenuior* collected from the eastern and northeastern Anatolia of Turkey and to assess the relationships between variations of chemical composition contents and the genetic and environmental factors involved in different geo-ecological regions.

## MATERIAL AND METHODS

*Plant materia.*

*Z. tenuior* are grown different parts of Turkey as populations (Figure 1). In this study *Z. tenuior* ecotypes were collected in 2018 from Artvin (975 m), Gumushane (1235 m) and Sivas (1650 m) provinces of Turkey (Table 1) in middle and last week of June in 2018. Plant material was identified by M. Sengul, Department of Biology, Ataturk University.

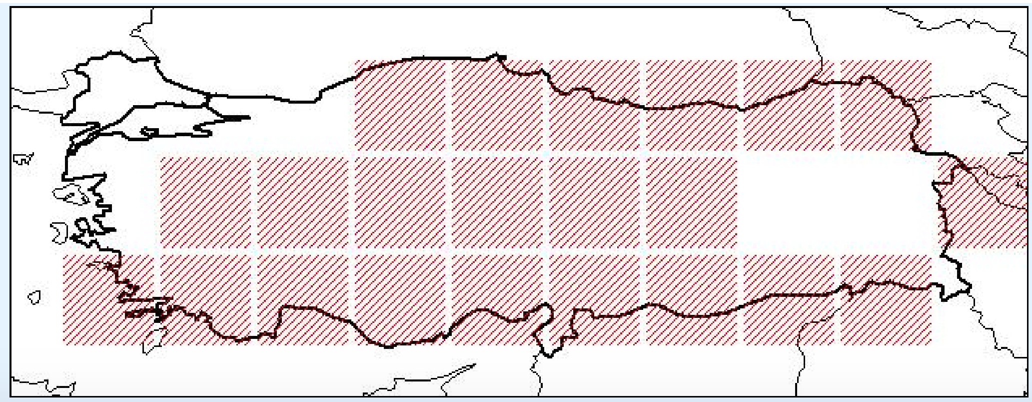

Figure 1. Distribution map of *Z. tenuior* in Turkey

*Table 1. Collection sites of Z. tenuior ecotypes*

| Collection site | Elevation (m) | Latitude | Longitude |
|---|---|---|---|
| Artvin (975 m) | 975 | 40°7833$^0$N | 41°4029$^0$E |
| Gumushane (1235 m) | 1235 | 40°4709$^0$N | 39°4713$^0$E |
| Sivas (1650 m) | 1650 | 39°8893$^0$N | 37°9586$^0$E |

*Isolation of essential oil*

Fresh aerial parts from the three ecotypes of *Z. tenuior* were cleaned and dried for six days at room temperature (24±2°C) and powdered using mechanical grinder. The essential oil was extracted from 40 g of ground tissue in 1 L of water contained in a 2 L flask and heated by heating jacket at 100°C for 3 h in a Clevenger–type apparatus, according to producers outlined in the British Pharmacopoeia. The averages determined with 3 replications. The collected essential oil was dried over anhydrous sodium sulphate into bottles for GC/MS analysis and stored at +4°C until analyzed.

*GC-MS Analysis*

GC-MS analysis of these extracts performed with GC clarus 500 Perkin Elmer system and Gas chromatograph interfaced to a Mass spectrometer (GC-MS) equipped with an Elite-1 fused silica capillary column (30 mm x 0.25 mm 1D x 1 um df, composed of 100% Dimethyl poly siloxane). The relative % amount of each component was calculated by comparing its average peak area with the total areas, software adopted to handle mass spectra and chromatograms was a turbo mass. All peaks of the chromatograms were analyzed using - NIST-Mass Spectral Library in order to identify the corresponding compounds.

*Determination of total phenolic content in the essential oils*

Total phenolic content of the phenolic compounds in the essential oils (EOs) were determined by Folin- Ciocalteu's method as gallic acid equivalents (GAE) (SINGLETON *et al.*, 1999). Total phenolic contents were expressed as mg of gallic acid equivalent per g of the essential oil. All tests carried out in triplicate.

*1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay*

The capacity of the essential oils to donate a hydrogen atom or electron and scavenge DPPH radical was evaluated. Briefly, 50 µl of the different concentrations (2.5, 5 and 10 µl/ml) of essential oils in ethanol was mixed with 2 ml of ethanol solution of DPPH (24 µg/ml). The mixture was incubated at room temperature for 60 min in the dark. Then, the absorbance was measured against a blank at 517 nm with a UV/Vis spectrophotometer. All experiments were carried out in triplicate and results were reported as mg/ml (MOLYNEUX, 2004).

*Ferric reducing power (FRAP)*

Ferric reducing power of the EOs of *Z. tenuior* ecotypes were determined according to the previously method described by BENZIE and STRAIN (1996). The reducing power of the *Z. tenuior* EOs was assessed at 690 nm in the Microplate Reader. Results expressed as mg/ml.

*Statistical analysis*

All experiments were repeated three times. The statistical analysis was performed using SPSS 16.0 software program (SPSS, Chicago, IL, USA). Statistical significance levels used was $p< 0.05$.

## RESULTS AND DISCUSSION

### *GC-MS Analysis*

Essential oil composition of the *Z. tenuior* ecotypes was identified by GC–MS. It is obvious from data in Table 2 that the oils were made largely up of monoterpenes. Other minor groups in the ecotypes were sesquiterpenes. Twenty compounds representing 89.18%, 91.12% and 91.4% of the oil were identified, respectively. Variable ratio of pulegone (37.23-49.12%), 1,8-Cineole (2.26-7.78%), limonene (4.20-5.44%), *β*-Caryophyllene (3.88-5.11%) and thymol (2.68-411%) were identified the major components of *Z. tenuior* ecotypes (Table 2).

*Table 2. Essential oil rates (%) and components obtained from Z. tenuior ecotypes collected from different altitudes*

| No | Compounds | Relative Retention Index | Locations | | |
|---|---|---|---|---|---|
| | | | Artvin | Gumushane | Sivas |
| | | | Relative (%) | | |
| 1 | *α*- Pinene | 1014 | 2.22 | 1.97 | 1.83 |
| 2 | Sabinene | 1032 | 1.98 | 1.07 | 1.25 |
| 3 | *β*-Pinene | 1038 | 1.14 | 1.26 | 1.44 |
| 4 | *β*-Myrcene | 1049 | 1.35 | 1.45 | 1.54 |
| 5 | Limonene | 1072 | 5.44 | 4.67 | 4.20 |
| *6* | 1,8- Cineole | 1094 | 7.78 | 3.02 | 2.26 |
| 7 | Camphor | 1139 | 0.82 | 0.70 | 0.63 |
| 8 | Cyclohexanone | 1145 | 2.14 | 2.35 | 2.28 |
| 9 | *α*-Terpinolene | 1156 | 0.67 | 0.73 | 0.60 |
| 10 | Pulegone | 1183 | 37.23 | 46.81 | 49.12 |
| 11 | Piperitone | 1192 | 3.36 | 2.98 | 2.70 |
| 12 | Methyl acetate | 1204 | 3.02 | 3.33 | 3.25 |
| 13 | *Cis*- piperitone oxide | 1220 | 2.95 | 3.12 | 3.01 |
| 14 | Thymol | 1235 | 4.11 | 2.68 | 2.93 |
| 15 | Cyclohexane | 1250 | 1.44 | 1.30 | 1.34 |
| 16 | *β*-Caryophyllene | 1263 | 5.11 | 4.56 | 3.88 |
| 17 | Germacrene D | 1274 | 2.14 | 2.44 | 2.30 |
| 18 | Bicyclogermacrene | 1284 | 3.02 | 3.27 | 3.20 |
| 19 | Caryophylle oxide | 1295 | 2.90 | 3.11 | 3.35 |
| 20 | *α*-Farnesene | 1309 | 0.36 | 0.30 | 0.33 |
| | Total | | 89.18 | 91.12 | 91.44 |

Previous studies reported that EOs of species and subspecies of *Ziziphora* in general had pulegone as the major constituent, with the exception of a report by AGHAJANI *et al*. (2008) that indicated that Germacrene D was the main compound in *Z. capitata* oil. This chemical differentiation was in agreement with previous studies indicating genetic background strongly effect composition of *Ziziphora* (PIRBALOUTI *et al*., 2010; ALP *et al*., 2016; SHAHBAZI *et al*., 2017). PIRBALOUTI *et al*. (2013) studied on essential oil composition of wild populations of *Ziziphora tenuior* in Iran and GC-MS analyses revealed 16 compounds, constituting 89-97% of the essential oils. Strong chemical variability, depending on the origin of the samples, was observed. The main constituents of the essential oils were pulegone (71.2 to 85.3%), limonene (0.51 to 7.8%), thymol (1.0 to 4.3%), and menthone (0.01 to 3.7%). The chemical composition of our samples was in accordance with above studies. The same configuration was obtained by NAJAFI *et al*. (2011) in North Iran and they reported the dominant presence of pulegone (85.0%) and limonene (5.1%) in essential oil of *Z. tenuior*.

Pulegone was the main component for all 3 locations having almost 41.75% in Artvin location, 51.37% in Gumushane location and 53.72% in Sivas location of the essential oil composition in *Z. tenuior*. Pulegone content increased from 37.23% lower elevation (Artvin location, 975 m) to 49.12% in higher elevation (Sivas location, 1650 m). As the altitude increased, the rates of some components in essential oil, such as limonene, 1,8-cineole and *β*-caryophyllene content decreased and thymol content first decreased according to elevation increase but later increased with elevation increase in contrast to the other major components such as pulegone (Table 2). EBRAHIMI *et al*. (2012) studied on the chemical composition of *Z. clinopodioides* oil of two regions from the Golestan Province with different elevation revealed that in general, that there are some differences in the major components and their relative concentrations. They reported higher pulegone content at higher elevations. They also indicated that genetic background also effect composition of different ecotypes within species. Previously a number of studies showed the oil of some *Ziziphora* species are rich in pulegone (SALEHI *et al*., 2005; OZTURK and ERCISLI, 2006; OZTURK and ERCISLI, 2007). The major constituent of *Z. tenuior* L. oil has been reported to be pulegone (87.1%) (SEZIK *et al*., 1991). The essential oil of Turkish endemic *Z. taurica* subsp. *clenioides* contains pulegone (81.9%), limonene (4.5%) and piperitenone (2.3%) (MERAL *et al*., 2002). ALP *et al*. (2016) studied on *Z. clinopodioides* ecotypes and they found that genetic background was the main factor for chemical diversity and the main components of all samples include pulegone (40.13-51.13%), and followed by 1,8-cineole, limonene, menthol, *β*–pinene, menthone, piperitenone and piperitone. KILIC and BAGCI (2013) found that the main compounds of *Z. tebuior* essential oil were pulegone and 1,8-cineole. They also showed that in respect to the major components *Ziziphora* taxa were chemically similar except *Z. taurica* subsp. *taurica*.

Pulegone is the major constituent of *Ziziphora* species oil and is used as fragrance and flavor in the cosmetic, perfume, drug and food industries. Pulegone and pulegone-derived lactones have also been known anti-feedant, antibacterial, antifungal and insecticide activity. The difference in EOs composition affected by the species, ecotypes, stage of the plant growth, genetic background, elevation and preparation process (BURT *et al*., 2007).

*Total phenolic content and antioxidant activity*

Table 3 shows total phenolic content of *Z. tenuior* ecotypes wildly grown on different elevations (Artvin location, 975 m), (Gumushane location, 1235 m) and (Sivas location, 1650 m). Ecotypes and locations with different elevation had a significant effect on the total phenolic content ($p<0.05$). *Z. tenuior* wildly grown in Sivas location (1650 m) gave higher total phenolic content (27.50 mg GAE/g EOs) than Gumushane (23.28 mg GAE/g EOs) and Artvin locations (21.13 mg GAE/g EOs), respectively. The results suggesting that despite large adaptation ability of *Z. tenuior* (Figure 1), the plant may have different ecotypes with diverse genetic composition and also require specific environmental conditions to accumulate higher total phenolic content. ALIAKBARLU and SHAMELI (2013) reported that total phenolic content of EOs of *Ziziphora* species were between 17 mg GAE/g (*Z. tenuoir*) and 114 mg GAE/g EOs (*Z. clinopodioides*). SARIKURKCU *et al*., (2019) used different extraction solvents and reported that *Ziziphora taurica* subsp. *cleonioides* had total phenolic content between 18.19 and 37.45 mg GAE/g. Our literature review revealed that most previous research evaluated total phenolic content of only one ecotype of *Z. tenuior* without effect of genetic and elevation. The present study, however, conducted a comprehensive comparison among three ecotypes indigenous to Turkey, which is expected to provide new insights useful for food or pharmaceutical applications. Phenolic compounds are important for defense mechanisms and contribute significantly to the taste, color, flavor and odor (SANBOLI *et al*., 2010).

*Table 3. Total phenolic content, antioxidant capacity and aerial dry yield of Z. tenuior ecotypes*

| Collection site | Total phenolic content (mg GAE/g EOs) | DPPH radical-scavenging activity ($IC_{50}$; mg/ml) | Ferric reducing power ($EC_{50}$; mg/ml) | Aerial dry yield (g/plant) |
|---|---|---|---|---|
| Artvin | 21.13c | 0.97a | 0.55a | 10.12b |
| Gumushane | 23.28b | 0.91b | 0.51b | 10.55b |
| Sivas | 27.50a | 0.80c | 0.42c | 11.94a |

Means in same column with different letter denote significant difference ($p<0.05$)

Antioxidant capacity, which determined by DPPH and FRAP assays of *Z. tenuior* ecotypes and results are given in Table 3. The ecotypes differed significantly each other in terms of antioxidant capacity for both DPPH and FRAP assays ($p<0.05$).

FRAP assay measures the reducing power of a potential antioxidant, which reduces the ferric ion ($Fe^{3+}$) to the ferrous ion ($Fe^{2+}$) leading to the formation of a deep blue complex ($Fe^{2+}$/TPTZ). Aerial part of *Z. tenuior* from higher elevation location (Sivas) exhibited significantly ($p<0.05$) higher antioxidant capacity (0.42 mg/ml) than the two other locations Gumushane (0.51 mg/ml) and Artvin (0.55 mg/ml), respectively. Aerial plant samples of *Z. tenuior* from the Gumushane also had higher antioxidant capacity than Artvin in their FRAP values ($p < 0.05$) (Table 3).

Considering results of DPPH assay, similar trend were also evident. In this assay lower amount required to give $IC_{50}$ indicates higher free radical scavenging activity. *Z. tenuior* sample from Sivas had a higher DPPH radical scavenging values (0.80 mg/ml) and followed by Gumushane (0.51 mg/ml) and Artvin (0.55 mg/ml) (Table 3). There were statistically significant differences among three ecotypes ($p>0.05$) (Table 3).

In literature there were a few studies on antioxidant capacity of *Z. tenuior* EOs, which highlighting importance of this study. Previously SHAHBAZI (2017) used *Z. clinopodioides* ecotypes to determine antioxidant capacities (DPPH and FRAP assays) and reported that there were significant differences among ecotypes collected from different parts of Iran. They found DPPH and FRAP values between 0.30-0.56 mg/ml in DPPH and 0.40-0.91 in FRAP assays. ALIAKBARLU and SHAMELI (2013) studied on essential oil of *Ziziphora* species and they found that the antioxidant capacities of essential oils can be attributed to their phenolic contents. The results obtained in this study are in agreement to a certain degree with the traditional uses of *Z. tenuior* as a valuable source of antioxidant drugs.

## CONCLUSIONS

The results indicate that there were differences among *Z. tenuior* ecotypes in terms of chemical composition, total phenolic content and antioxidant capacity that could be results of genetic background of ecotypes and also environmental conditions. In conclusion, *Z. tenuior* found to be rich raw source of pulegone, which has been widely used in food and drug industries. The two antioxidant determining assays, DPPH and FRAP, clearly indicated that the studied *Z. tenuior* ecotypes possess variable but considerable antioxidant. In future more detailed studies needs to determine the effects of genetic background and environmental on essential oil composition and biological activity of *Z. tenuior* plants.

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**RAZLIČITOST U SASTAVU ETERIČNIH ULJA EKOTIPOVA**
***Ziziphora tenuior* L.**

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Izvod

Ovaj rad prikazuje uporedni skrining tri ekotipa *Ziziphora tenuior* L., uzorkovanih iz tri regiona sa različitim nadmorskim visinama u srednjoj i severoistočnoj Turskoj, na osnovu njihovog sastava esencijalnog ulja, ukupnog sadržaja fenola i antioksidativnih kapaciteta. *Z. tenuior* ekotipovi su pokazali dvadeset jedinjenja koja predstavljaju 89,18-91,44% esencijalnog ulja (EO) identifikovanog pomoću GC-MS. Glavne komponente EO u ekotipovima bili su pulegon (37,23-49,12%), a zatim 1,8-cineol (2,26-7,78%), limonen (4,20-5,44%), b-kariofilen (3,88-5,11%) i timol (2,68-411%). Ekotipovi su pokazali promenljiv sadržaj pulegona i on je povećan sa porastom nadmorske visine. Ukupan sadržaj fenola varirao je među ekotipovima u rasponu od 21,13 do 27,50 mg ekvivalenta galne kiseline / g EO. Antioksidativni kapacitet je određen *in vitro* pomoću DPPH testova za uklanjanje radikala i FRAP (*Ferric Reducing power*) testova i izražen kao koncentracija svakog ekstrakta potrebnog za inhibiciju radikala za 50% (IC50 i EC50) vrednosti koje su se kretale od 0,80 do 0,97 mg / ml u DPPH i 0,42 do 0,55 mg / ml u FRAP testovima, respektivno. Naši rezultati su pokazali da se antioksidativni kapacitet promenio među ekotipovima. Uzimajući u obzir sadržaj esencijalnog ulja, čini se da je odabir ekotipa najprikladniji za dobijanje više pulegona iz ove biljke iz njenog divljeg staništa.

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