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*ACTA BIOLOGICA CRACOVIENSIA Series Botanica* 64/1: 7–14, 2022
DOI: 10.24425/abcsb.2021.136703

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ODDZIAŁ W KRAKOWIE

# SEASONALITY STUDY OF EXTRACTS FROM LEAVES OF *THUJA OCCIDENTALIS* L.

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Received August 21, 2021; revision accepted December 20, 2021

The market value of essential oils is steadily increasing every year. They are mainly used as aromas and preservatives in food and cosmetics industries. The content and yield of the obtained extracts change across the seasons of the year. The knowledge of these differences is important for essential oils industry to gain the best quality and quantity of products. In this study *Thuja occidentalis* L. (Eng. northern white cedar) extract was obtained from leaves by maceration in dichloromethane. Plant material was collected during each season of the year and the finished products were thoroughly examined through GC-MS analysis. The seasonal variations of volatile compound composition showed that the most adequate period to obtain *T. occidentalis* extract on an industrial scale is spring. This is due to the best efficiency (almost 10%) and the highest content of monoterpenes (67%).

**Keywords:** extract, GC-MS, *Thuja occidentalis*, volatile compounds.

## INTRODUCTION

Essential oils are a mixture of volatile organic compounds synthesized in plant cells. Volatile compound synthesis involves mostly mono- and sesquiterpenes with admixture of ketones, aldehydes and alcohols (Adams, 2007). This mixture may be obtained in many ways, mostly by hydrodistillation and steam distillation but also by extraction from an organic solvent or, in particular cases when the plant material is fragile, by the enfleurage method. In the past essential oils were believed to be only unnecessary, secondary metabolites, however nowadays it is known that plants use these compounds to lure insects, protect them from parasites and heal wounds (Magalhães et al., 2020). All this is possible due to volatility of semiochemicals and antimicrobial properties of bioactive compounds and in this sense they are designated as specialized metabolites (Pichersky et al., 2006; Pichersky and Lewinsohn, 2011). In every essence there is usually one compound that prevails, for example menthol (43.7-78.2%) in *Mentha arvensis* L. (Rajeswara Rao et al., 1999) or 1,8-cineole (47.5%) in *Salvia fructicosa* L. (Sivropoulou et al., 1997).

However, the composition of volatile compounds varies depending on many aspects: the season in which plant material is collected, the time of the day and also the weather. The changing composition of volatile compounds is determined by the temperature, quantity of oxygen and exposure to light. These factors cause a lot of reactions, such as isomerization, oxidation, polymerization, thermal rearrangement and dehydrogenation, which essential oils component are prone to due to reactive heteroatomic groups. As a result, quality, organoleptic properties and bioactivity can be different in each essence (Turek and Stintzing, 2013; Vallejo et al., 2005).

Relatively few plants (about 2000 species) are classified as aromatic, that is, containing more than 0.01% of an essential oil (Kohlmünzer, 1998). There are a few plant families, for instance Myrtaceae, Pinophyta and Apiaceae that store a significant amount of essential oils in their cells. Additionally,

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PL ISSN 1898-0295

the Cupressaceae family, which *T. occidentalis* belongs to, is also a group of aromatic plants (Moghaddam and Mehdizadeh, 2017). Essential oils can be obtained from every plant structure: flowers, leaves, roots, stems (also modified stalk, e.g., a rhizome), wood and fruits. The extraordinary composition of essential oils may have a big impact on human health. Used for inhalation or rubbed, essential oils are helpful in reducing stress, improving sexual activity or curing anxiety and depression (Park et al., 2015; Worwood, 2010). Besides therapeutic properties, essential oils are also germicidal, fungicidal, antiviral and demulcent (Fletcher and Riley, 1999; Worwood, 2010). It is confirmed that metabolites contained in *T. occidentalis* leaves have an antibacterial activity against both Gram-positive and Gram-negative bacteria (Mehta, 2015). Moreover, black pepper (*Piper nigrum* L.) that has been used in folk medicine to cure diabetes and hypertension, can be mentioned as a proof of health-promoting effects of essential oils (Takooree et al., 2019). Nowadays, it is known that essential oil made from *P. nigrum* seeds and leaves that contains pinene and 1,8-cineol has radical scavenger abilities and ferric-reducing antioxidant activity which help treating these diseases (Buchbauer and Wallner, 2016).

To show how the composition of volatile compounds changes, an analysis of *T. occidentalis* leaf extract was carried out. The plant's leaves were collected in every season: at the end of January, April, July and October, and were used to obtain four extracts. Thereafter, using the GC-MS apparatus it was possible to see all changes in the compositions of each extract, even in compounds found in trace amounts.

The knowledge of the changes caused by the season and vegetative period can indicate when the extracts have the best quality and hence, curative and therapeutic properties. Such results can be useful for companies that produce essential oils and help them decide when is the best time to obtain the biggest amount of essential oil of the best quality.

## MATERIALS AND METHODS

### PLANT MATERIAL

*T. occidentalis* leaves were collected in the middle of every season of the year: on 25 January 2020 at 3 PM (completely cloudy, 5°C), 25 April 2020 at 5 PM (partly cloudy, 11°C), 25 July 2020 at 10 AM (sunny, 20°C) and 25 October 2020 at 3 PM (sunny, 14°C). The gymnosperm tree that was used for the purposes of this study grows in northern Poland, near the Baltic Sea (geographic coordinates: 54°28'18.4"N 18°28'13.3"E), in warm temperate transient climate. After collecting, the plant material (40 g per each sample) was tightly closed and stored in a freezer (temperature -7°C) until the maceration.

### EXTRACTION

The extract from *T. occidentalis* leaves (three samples each month) was obtained by maceration in an organic solvent, specifically in dichloromethane (AR quality, Stanlab, Poland), in 200 mL conical flasks. The volume of dichloromethane used for maceration was 100 mL per each sample. The weight of the plant material was about 10 g and was not pretreated. The extraction lasted for seven days in a dry and dark place at temperature 23°C. The obtained extract was filtered on fluted filter paper (Eurochem, Poland) and afterwards dichloromethane was evaporated from the mixture in a vacuum rotary evaporator. The temperature of the process was 30°C and revolutions intensity 180 rpm. When almost whole organic solvent was removed from the mixture, the extract was transferred from the flask to four milliliters vials (Alwasci Technologies, Anchem, Poland). In these vessels the rest of dichloromethane was evaporated through a nitrogen gas stream. The obtained pure extract was stored in the vials (2 mL) (Alwasci Technologies, Anchem, Poland) in a dark cold place (4°C) till the GC-MS analysis. Every sample was extracted in three replicates.

### EXAMINATION OF PHYSICAL PROPERTIES

In this study six physical properties were examined: color, smell, viscosity, yield, refractive index and specific gravity. Color, smell and viscosity were organoleptically assessed. The yield was calculated according to the formula: v/w(%) = 100% × amount of obtained extract (g) /amount of plant material (g). The refractive index was measured with a refractometer at 20°C. Specific gravity was measured using a densitometer at 20°C.

### CHROMATOGRAPHIC CONDITIONS OF GC-MS MEASUREMENTS

In the GC-MS analysis (GCMS-QP2010SE, Shimadzu) the carrier gas was helium passing the column under pressure 100 kPa. The chro-

matographic column was a capillary column ZB-5MSi made by Zebron, 30 m long, with a diameter of 0.25 mm and the stationary phase film 0.25 μm thick. The injection temperature was 250°C. The oven temperature was 50°C, held for 3 minutes and increased to 250°C at 5°C/min. The interface temperature was 250°C and the electron source temperature in mass spectrometry was 200°C.

The first analyzed sample was a mixture of aliphatic alkanes (containing from 8 to 24 carbon atoms in a molecule). Based on the results, it was possible to designate the Kovats Index (KI) of the extract compounds. The next four samples were *T. occidentalis* extracts from each season of the year. Every sample was analyzed three times to obtain a measure of error; the results were averaged and the standard deviation was calculated. The compounds of the extracts were identified on the basis of mass spectra (intensity of ions and *m/z* (mass-to-charge *ratio*)) and the Kovats Index by comparison with data in the book "Identification of essential oil components by gas chromatography / mass spectrometry, 4th Edition" by Robert P. Adams, PhD. and by comparison with a computer library (NIST_54k). The obtained data were statistically processed using the t-pairwise test to determine significant differences in the terpenes and other compounds contents in the extracts from each season of the year.

## RESULTS AND DISCUSSION

The extract from *T. occidentalis* leaves was dark yellow, thin liquid, with a fresh, woody scent. The amount of the obtained extracts and selected physical properties are reported in Table 1. The best ratio of extract to plant material is in spring (9.9%). In this season, due to longer exposure to sunlight and a higher temperature, plants start to intensify photosynthesis and hence elongate and develop generative organs (Wiese and Heber, 2001). Also the best yield of volatile compounds is obtained in the vegetative cycle when the plant is forming reproductive structures (cones) (Moldão-Martins et al., 1999). Therefore, there are a lot of chemical reactions including the mevalonate pathway, which create components of essential oil (Kohlmünzer, 1998). The lowest yield of extract was in winter (3.3%). In this season the metabolism of plants decreases and so is the creation of volatile compound constituents. Overall amount of the obtained extract was relatively big, as compared to Tsiri et al. (2009), a study were the yield varied between 0.58% and 0.87%. This difference may be due to the type of the method used. As can be seen, extraction is a more efficient method than hydrodistillation.

The results of GC-MS analysis are given in Table 2. Seventy compounds were identified in the summer extract. The number of identified compounds is bigger than in previous reports, e.g., 36 compounds were identified in *T. occidentalis* (Szołyga et al., 2014). In every sample monoterpenes were the prevailing class of compounds, ranging between 51.7% in winter to 67.3% in spring. Diterpenes were also found in large amounts. In spring and summer it was about 25% of the whole extract, however in autumn and winter it was 37.6% and 37.7%, respectively. Sesquiterpenes are in minority and the content varies between 3.4% in spring and 7.7% in summer. According to the obtained results, the amount of diterpenes increased during colder seasons (autumn and winter), while the quantity of monoterpenes, which have much smaller molecular weight, decreased. In warmer seasons (spring and summer) the situation was reverse (Figure 1). The component with the highest molecular weight found in the extract was n-pentatriacontane. In each season there are three components that dominate in the extract composition: *cis*-thujone, *trans*-thujone and *trans*-ferruginol. The content of *cis*-thujone varies between 19.7% in summer and 36.4% in spring, *trans*-thujone 6.8% in spring and 10.6% in summer

TABLE 1. Quantity of obtained *T. occidentalis* extracts.

| **Month** | **April** | **July** | **October** | **January** |
|---|---|---|---|---|
| Plant material (g) | 9.8 | 11.5 | 10.4 | 11.2 |
| Extract (mg) | 964.5 | 411.3 | 735.1 | 372.0 |
| Yield (%) | 9.9 | 3.6 | 7.0 | 3.3 |
| Refractive Index (at 20°C) | 1.475 | 1.469 | 1.470 | 1.473 |
| Specific Gravity (g/cm$^3$) | 0.904 | 0.883 | 0.890 | 0.894 |

TABLE 2. Chemical composition of *T. occidentalis* extracts during harvests.

| Compounds | | $IK_{EXP}$ | Content [%] | | | |
|---|---|---|---|---|---|---|
| | | | April | July | October | January |
| 1. | Ethyl 2-methylbutanoate | 842 | <0.1 | <0.1 | <0.1 | <0.1 |
| 2. | *trans*-Hex-2-enal | 857 | 0.1±0.0 | 0.1±0.0 | <0.1 | <0.1 |
| 3. | Tricyclene | 924 | <0.1 | <0.1 | <0.1 | <0.1 |
| 4. | α-Thujene | 928 | 0.2±0.0 | 0.4±0.0 | 0.3±0.0 | 0.3±0.0 |
| 5. | α-Pinene | 939 | 1.1±0.0 | 1.3±0.0 | 0.8±0.2 | 0.7±0.0 |
| 6. | α-Fenchene | 950 | 0.6±0.0 | 0.5±0.0 | 0.4±0.1 | 0.4±0.0 |
| 7. | Camphene | 954 | 0.5±0.0 | 0.4±0.0 | 0.3±0.1 | <0.1 |
| 8. | Sabinene | 977 | 4.1±0.1 | 6.2±0.1 | 4.1±0.7 | 3.3±0.1 |
| 9. | β-Pinene | 980 | 0.1±0.0 | 0.1±0.0 | <0.1 | 0.1±0.0 |
| 10. | Myrcene | 992 | 0.8±0.0 | 1.7±0.0 | 0.7±0.2 | 0.6±0.0 |
| 11. | γ-Car-2-ene | 1000 | - | <0.1 | <0.1 | <0.1 |
| 12. | α-Phellandrene | 1004 | - | <0.1 | <0.1 | - |
| 13. | α-Terpinene | 1016 | <0.1 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 |
| 14. | p-Cymene | 1025 | 0.2±0.0 | 0.1±0.0 | 0.2±0.0 | 0.2±0.0 |
| 15. | Limonene | 1031 | 0.9±0.0 | 1.3±0.0 | 0.8±0.2 | 0.7±0.0 |
| 16. | γ-Terpinene | 1061 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 |
| 17. | *cis*-Sabinene hydrate | 1072 | 0.5±0.0 | 0.5±0.0 | 0.4±0.1 | 0.4±0.0 |
| 18. | Fenchone | 1088 | 6.3±0.1 | 6.4±0.1 | 4.9±0.6 | 4.7±0.1 |
| 19. | *cis*-Thujone | 1103 | 36.4±0.5 | 19.7±0.4 | 24.1±2.3 | 24.7±0.5 |
| 20. | *trans*-Thujone | 1115 | 6.8±0.1 | 10.6±0.2 | 7.4±0.9 | 7.2±0.1 |
| 21. | *trans*-p-Menth-2-en-1-ol | 1123 | 0.1±0.0 | 0.1±0.0 | 0.1±0.1 | 0.1±0.0 |
| 22. | Camphor | 1145 | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | 0.1±0.0 |
| 23. | Camphene hydrate | 1147 | 0.1±0.0 | 0.2±0.0 | 0.1±0.0 | 0.1±0.0 |
| 24. | Sabina ketone | 1157 | 0.2±0.0 | 0.1±0.0 | 0.2±0.0 | 0.2±0.0 |
| 25. | Borneol | 1169 | - | 0.1±0.0 | - | 0.1±0.1 |
| 26. | *trans*-p-Menth-8-en-3-one | 1174 | 0.1±0.0 | 0.2±0.0 | 0.1±0.0 | 0.1±0.0 |
| 27. | Terpinen-4-ol | 1177 | 0.3±0.0 | 0.2±0.0 | 0.3±0.1 | 0.3±0.0 |
| 28. | p-Cymen-8-ol | 1180 | 0.1±0.0 | 0.1±0.0 | 0.1±0.1 | 0.1±0.0 |
| 29. | α-Terpineol | 1189 | 0.1±0.0 | 0.1±0.0 | 0.1±0.1 | 0.1±0.0 |
| 30. | Myrtenol | 1197 | - | 0.1±0.0 | <0.1 | <0.1 |
| 31. | Verbenone | 1206 | 0.1±0.0 | <0.1 | <0.1 | <0.1 |
| 32. | *endo*-Fenchyl acetate | 1220 | 0.3±0.0 | 0.4±0.1 | 0.3±0.0 | 0.3±0.0 |
| 33. | Thymol methyl ether | 1233 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 |
| 34. | Pulegone | 1235 | <0.1 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 |
| 35. | Carvacrol methyl ether | 1244 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 |
| 36. | *trans*-Sabinene hydrate acetate | 1254 | 1.3±0.0 | 2.4±0.0 | 0.9±0.1 | 0.8±0.0 |
| 37. | *iso*-Thujan-3-ol acetate | 1265 | 0.4±0.0 | 0.5±0.0 | 0.3±0.0 | 0.3±0.0 |

| Compounds | | $IK_{EXP}$ | Content [%] | | | |
|---|---|---|---|---|---|---|
| | | | April | July | October | January |
| 38. | *neo*-Thujan-3-ol acetate | 1272 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 |
| 39. | *neoiso*-Thujan-3-ol acetate | 1279 | <0.1 | 0.1±0.0 | - | <0.1 |
| 40. | *iso*-Bornyl acetate | 1287 | 3.7±0.0 | 4.5±0.1 | 3.4±0.4 | 3.3±0.0 |
| 41. | α-Terpineol acetate | 1292 | 0.2±0.0 | 0.4±0.0 | 0.2±0.0 | 0.2±0.0 |
| 42. | *iso*-Menthyl acetate | 1304 | 0.2±0.0 | 0.3±0.0 | 0.1±0.0 | 0.1±0.0 |
| 43. | σ-Terpinyl acetate | 1318 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 |
| 44. | *trans*-Piperitol acetate | 1343 | 1.2±0.1 | 2±0.0 | 1.2±0.1 | 1.2±0.0 |
| 45. | α-Terpinyl acetate | 1346 | 0.1±0.0 | 0.1±0.0 | <0.1 | 0.1±0.0 |
| 46. | Thymol acetate | 1349 | <0.1 | 0.1±0.0 | <0.1 | 0.1±0.0 |
| 47. | Geranyl acetate | 1381 | 0.2±0.1 | 0.3±0.0 | 0.2±0.0 | 0.2±0.0 |
| 48. | β-Elemene | 1390 | 0.1±0.0 | 0.2±0.0 | - | 0.1±0.0 |
| 49. | Vanillin | 1396 | <0.1 | <0.1 | <0.1 | <0.1 |
| 50. | *trans*-beta-Caryophyllene | 1419 | 0.1±0.0 | 0.3±0.0 | 0.1±0.0 | 0.1±0.0 |
| 51. | β-Copaene | 1430 | 0.1±0.0 | <0.1 | - | <0.1 |
| 52. | *trans*-Cinnamyl acetate | 1446 | 0.4±0.0 | 1.1±0.0 | 0.5±0.0 | 0.5±0.0 |
| 53. | Sesquisabinene | 1459 | 0.1±0.0 | 0.4±0.0 | 0.1±0.0 | 0.1±0.0 |
| 54. | Tridecan-2-one | 1494 | 0.1±0.0 | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 |
| 55. | *trans*-β-Guaiene | 1500 | - | 0.2±0.0 | <0.1 | <0.1 |
| 56. | γ-Cadinene | 1513 | - | <0.1 | <0.1 | <0.1 |
| 57. | σ-Cadinene | 1523 | - | 0.1±0.0 | <0.1 | <0.1 |
| 58. | Elemol | 1547 | - | 0.3±0.1 | - | <0.1 |
| 59. | Raspberry ketone | 1549 | 0.2±0.0 | 0.4±0.0 | 0.3±0.1 | 0.4±0.0 |
| 60. | D-Germacrene-4-ol | 1574 | 0.1±0.0 | 2.2±0.2 | 0.2±0.0 | 0.3±0.0 |
| 61. | 3,4,5-Trimethoxy- benzaldehyde | 1602 | - | <0.1 | <0.1 | <0.1 |
| 62. | Pentadecan-2-one | 1700 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.2±0.0 |
| 63. | Oplopanone | 1739 | 2.8±0.1 | 3.7±0.1 | 3.9±0.4 | 4.5±0.1 |
| 64. | Rimuene | 1896 | 2.6±0.1 | 3.7±0.1 | 3.7±0.2 | 4.2±0.1 |
| 65. | Beyerene | 1931 | 5.5±0.2 | 5.6±0.2 | 7.3±0.7 | 8.4±0.1 |
| 66. | Kaurene | 2045 | 0.3±0.0 | 0.4±0.0 | 0.4±0.0 | 0.4±0.0 |
| 67. | Abieta-8,11,13-triene | 2066 | 0.5±0.0 | 0.6±0.0 | 0.7±0.1 | 0.8±0.0 |
| 68. | Abieta-7,13-diene | 2093 | 1.3±0.1 | 3.3±0.0 | 1.6±0.2 | 1.7±0.0 |
| 69. | *trans*-Ferruginol | 2330 | 14.8±1.0 | 9.8±0.3 | 24.0±1.6 | 22.3±0.2 |
| 70. | Pentatriacontane | 3317 | 3.1±0.1 | 4.8±0.1 | 4.3±0.4 | 3.9±0.1 |
| | Summary: | | | | | |
| | Total monoterpenes [%] | | 67.4 | 61.7 | 52.4 | 51.7 |
| | Total sesquiterpenes [%] | | 3.5 | 7.7 | 4.6 | 5.5 |
| | Total diterpenes [%] | | 25.1 | 23.6 | 37.6 | 37.7 |
| | Other compounds [%] | | 4.0 | 7.0 | 5.4 | 5.1 |

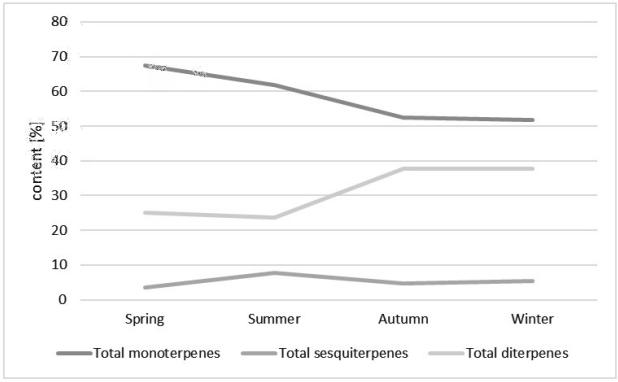


**Fig. 1.** Differences among terpenes content (%) in *Thuja occidentalis* extract during harvest time.

and *trans*-ferruginol 9.8% in summer and 24.0% in autumn. Figure 2 shows that in spring the *cis*-thujone content increases significantly and the content of *trans*-thujone and *trans*-ferruginol decreases. The same relationship occurs in summer, autumn and winter. In summer the *trans*-thujone content increases, while *cis*-thujone and *trans*-ferruginol decrease. In colder seasons the *trans*-ferruginol content is higher, while the content of other main constituents is relatively low. Additionally, sabinene, fenchone, iso-bornyl acetate, oplopanone, rimuene, beyerene and pentatriacontane were found in a relatively big quantity in the extract (more than 2.5%). Significant differences in the compounds contents between spring and winter, summer and autumn and also between autumn and winter were confirmed using a pairwise *t*-test ($t = 2.4$, $t = 2.1$ and $t = 2.8$, respectively). However, based on this test, there were no significant differences between spring and summer, spring and autumn, and also between summer and winter ($t = 1.2$, $t = 1.4$ and $t = 0.5$, respectively).

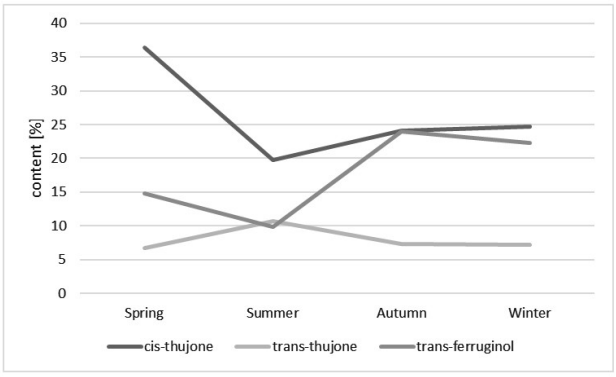


**Fig. 2.** Differences among main compounds content (%) in *Thuja occidentalis* extract during harvest time.

A previous report on *T. occidentalis*, also collected in Poland (17°02'E, 51°07'N) in August and November 2011 (Szołyga et al., 2014), showed that the main components of essential oil were mostly the same as reported here, however instead of diterpenoid *trans*-ferruginol, which was not identified in this research, the third minor constituent was monoterpenoid fenchone (7.8%). In the present study the quantity of fenchone varies between 4.7% and 6.4%. However, the content of thujones reported by Szołyga et al. (2014) is much bigger (almost 80%). The same main components of *T. occidentialis* extract were found in studies published by Bozsik et al. (2015), Tsiri et al. (2009) and Buben et al. (1992). In these studies trans-ferruginol was not identified, unlike our study where trans-ferruginol was present. Presented by Buben et al. (1992), a chemotype of *T. occidentalis* collected in Kostelec in the Czech Republic in 1991 contained *cis*-thujone in the greatest amount, then sabinen, fenchone and *trans*-thujone. This chemotype is almost identical to the obtained extract.

## CONCLUSIONS

In this study, thanks to carefully conducted analysis, in addition to mono and sesquiterpenes, a large amount of diterpenes and other compounds with high molecular weight were also detected. It turned out that *trans*-ferruginol is one of the main components of the analyzed extract. This diterpene content might reach almost 25% of the whole extract. Furthermore, the research showed that the best season to receive terpenoids from *T. occidentalis* leaves industrially by extraction is spring, due to the best, almost 10% efficiency and the highest concentration of monoterpenes and monoterpenoids, which are mainly responsible for the antimicrobial and antioxidant activities of the volatile mixture. These results might be important for companies engaged in industrial production of essential oils and also for people who use aromatherapy where an essential oil of the best quality is needed.

## AUTHORS' CONTRIBUTIONS

All contributions of the authors are equal and they have read and confirmed the final version of the manuscript for publication. The authors have declared that there is no conflict of interest.

## ACKNOWLEDGEMENTS

This work was financially supported by the Polish Ministry of Science and Higher Education under the grant 531-T010-D594-21.

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