DIPLOMA THESIS

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Evaluation of new garden thyme (*Thymus vulgaris* L.) clones and varieties

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1. Introduction

Medicinal plants have always been essential in our daily life. WHO has confirmed that herbal medicines serve the health needs of about 80 percent of the world's population. Among all other varied species of *Thymus*, *Thymus vulgaris* is used more than other species in therapeutic dosage forms. In traditional medicine, common thyme is cultivated in rural areas and it is used to treat diseases, insect bites, muscle pain and other inflammation related ailments. While in modern technology the presence of essential oil is extremely useful, it consists of an active compound, thymol, which shows anti-inflammatory, anti-fungal and antioxidant features (Hosseinzadeh et al. , 2015).

In the life we live now, people starts to look after their health benefits increasingly. Aside from the basic nutrition we take in everyday, consumers are demanding for food, which contains more useful substances, such as herbs. As herbal plants can be consumed in many types, in the form of essential oil, tea, or pills. The essential oil of *Thymus vulgaris* is widely known for its active compounds. Mostly used in cosmetics or perfumery, as it has a special and characteristic aroma. Fresh thyme has the highest antioxidant activity among other herbs. It's mineral and vitamins are also essential for our health. While thyme essential oil has some healing effect, it is combined with other herbs it could treat disease from bronchitis to skin disorder. Consuming thyme tea daily could help with arthritis (Dauqan, and Abdullah, 2017).

In the plant family of Lamiaceae, there are over 300 thyme species that range from small perennials to evergreen woody shrubs. Common or garden thyme (*Thymus vulgaris* L.) is a herb with a distinct odour and taste. The flowers, leaves, and oil are commonly used to flavor foods and are also used as medicine. Garden thyme has been used in worldwide for herbs, spices and much more (Stahl-Biskup and Venskutonis, 2012). Thyme contains chemicals that help in bacterial and fungal infections. It also support humans to relieve coughing and have antioxidant effects. Thyme's benefits include fighting acne, lowering blood pressure, boosting immunity, disinfecting, repelling pests, helping to treat yeast infections, and can be applied in aromatherapy, too.

The most valued compound of thyme raw material is essential oil, widely used in pharmacy, medicine, perfumery-cosmetic and food industries. Among all the type of thymes, mainly focusing on the French, English and German thyme. English thyme has a flat, pointy, green, fragrant foliage as a low-growing plant, while the French has small, narrow gray-green leave with upright stems. It is also called as the summer thyme as it offers a slightly subtler flavour than English thyme. German thyme foliage has lots of flavor with its smaller, slightly rounded leave. Also called as the winter thyme since it is cold and winter hardy. As some new garden thyme hybrids has higher yields in dry matter and essential oil (Rey et al., 2005).

The main aim of the following thesis is to evaluate the essential oil diversity of garden thyme (*Thymus vulgaris* L.) clones and varieties. Perennial stands of *Thymus vulgaris* varieties ('French Summer', 'Standard Winter', 'Pannon

Timol') and selected clones (TV 115, TV 121, TV135, TV143) were established in 2019 at the experimental station in Budapest, among similar field conditions, with the purpose to compare the overall performance, yields and active compounds of taxa included. After field measurements and sampling in spring and autumn periods of 2022, laboratory analysis including essential oil isolation and determination of compounds using GC (gas chromatography) were applied.

2. Objectives

The aims of our studies were as follows:

1. Characterizing morphology and essential oil properties of different *Thymus* taxa cut in spring and autumn periods of 2022.
2. Provide essential information on drug yields of taxa included.
3. Determination of quality parameters concerning varieties available for growers.

3. Literature review

3.1. Introduction of *Thymus vulgaris*

3.1.1. Botanical description

The Lamiaceae family is known for its flowering plants with aromatic scent, flavour and fragrance, widely used for culinary herbs or medicinal plants. Most of the plant family members are perennial or annual herbs while some species are woody shrubs. Leaves are usually hairy, simple, and oppositely arranged, mostly fragrant and contain essential oils with a strong scent if crushed (**Figure 1)**. Flowers are arranged in clusters with two-lipped, gaping mouth, tubular corolla with five-lobed calyces that looks like bell, mostly blooming during end of spring, early summer. Dry nutlet is a typical fruit of Lamiaceae (Xu et al.,2017). Among all 220 genera, thymus is among the most important ones (Stahl-Biskup. and Sáez, 2002).

Thyme is a small perennial shrub, which can grow to 20 cm tall, not more than 50 cm. The stems turn woody with years densely branching. Shoots are soft with four lateral edges, length of 10-25 cm. Thyme leaves are small, usually 2.5 to 5.0 mm long, hairy, opposite in lanceolate shape. Both surfaces are covered by glandular hairs which consist of essential oil, it will evaporate when the glandular hairs are damaged, every species having different scent (Talhouk et al, 2015). Larger hermaphrodite flowers exist on separate plants, while female flowers are usually smaller with white-pinkish color. May-June is the usual flowering period (Stahl-Biskup and Sáez., 2002). Four little spherical deep brown nutlets cover in sepals, it is the fruit. It needs full sun to grow to its best structure adapting well with the hot and dry summer (Morales, 2002). Thyme doesn’t tolerate excessive wet, under this conditions it will get rot diseases. Thyme prefers lightweight, well- drained soils with a pH of 5.0 to 8.0. It grows well with rough soils that may be unsuitable for other alternative plants (Reddy et al, 2014).

Figure 1. Morphological characteristics of *Thymus vulgaris* L.

3.1.2 Occurrence and ecological demands

Common thyme is native to dry dwarf shrublands, growing near the seashore of the Mediterranean Sea on maritime limestone hillsides. Thymus vulgaris develops at a yearly average temperature of 7-25 °C, grown under 400-2800 mm precipitation per year. Sunshine and warmth are essential without excessive water amount. Rot disease occurs when there's excessive wet. Thyme prefers lightweight, well- drained soils with a pH of 5.0 to 8.0. Loose or medium hard soils with good texture enriched with nutrients soil is demanded. Thyme is a drought tolerant and needs a well-drained, preferably near neutral or slight alkaline soil. It will grow well in quite poor, even stony, soil. Wet fields may cause slow development of plants and low level of essential oil. A warm and sunny position is needed for thyme. The more sunlight it gets, the stronger its flavor is. Different ecological environments and harvesting time result in variability of thyme (Alizadeh et al., 2011).

3.1.3 Drugs and active compounds of thyme

Thymi herba also known as the dried leaves and flowering tops of *Thymus vulgaris* L. or of *Thymus zygis* L. *Thymi aetheroleum* is the hydrodistilled essential oil from thyme. Both are official drugs listed in the European Pharmacopoeia (Ph. Eur. 7.0, 2010). To process the drugs, the dried aerial parts of *Thymus vulgaris* L. or *Thymus zygis* L. are used. Listed in the pharmacopoeal specifications, *Thymi herba* needs to contain minimum 12 ml/kg of essential oil (Pluhár et al, 2016). *Thymi herba* has been widely used for centuries, but appropriate quality control aiming to ensure its consistency, safety, and efficacy has space to improve. Quality control of herbal medicines is typically carried out by the measurement of the chemical markers shown in the medicines (Chang et al, 2020).

Essential oil is a complex fragrant liquid that has a compact percentage in aromatic plants. Exhibiting significant contribution in the cosmetic, food and pharmaceutical industries. To extract the essential oils effectively, different methods are applied. The main components of thyme oil include p-cymene (8.41%), γ-terpinene (30.90%) and thymol (47.59%) (**Figure 2**), responsible for the spicy aroma in the plant leaves in thyme essential oil (Sharafzadeh, 2011). The oil is accumulated in glandular peltated trichomes on both sides of the leaves (Stahl-Biskup and Sáez, 2002, Stahl-Biskup and Venskutonis, 2004) (**Figure 3**). The chemical composition of thyme oil is mostly produced from flowering plants (Stahl-Biskup & Sáez, 2002). During the life cycle of a plant, the flowering period has the highest level of oil production. Thyme oil contains a complex mixture of at least 70 varied molecules, including thymol, p-cymene, γ-terpinene, carvacrol, (E)-β-caryophellene, linalool and much more. Thymol turns out to be the most prevalent compound in thyme oil, with the effects including antimicrobial, anti-tumoral, anti-fungal, anti-parasitic, anti-oxidative and anti-inflammatory. P-cymene is also present in thyme oil with relatively high concentrations with antiviral, antioxidant properties. γ-Terpinene and carvacrol is related to thymol by its structure, has potent antimicrobial and anti-fungal activities (Vassiliou et al, 2023).

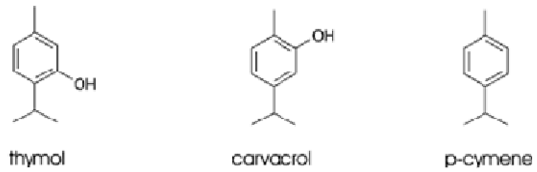

Figure 2. Main compounds of thyme oil: thymol, carvacrol and p-cymene

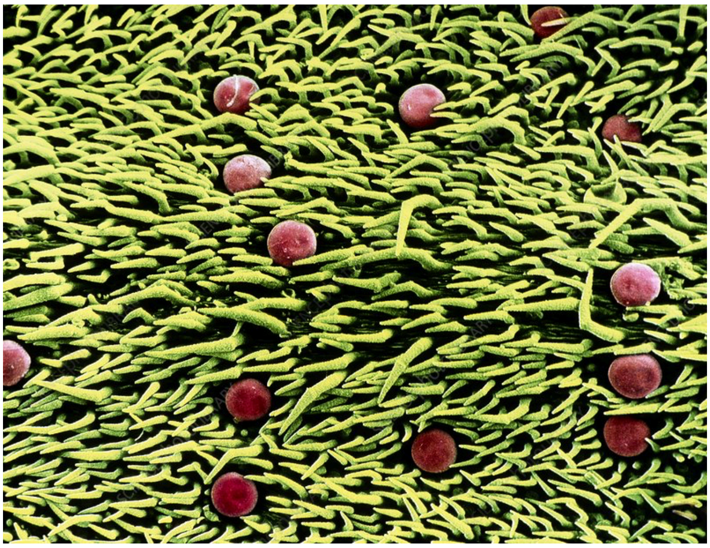

Figure 3. Thyme oil producing leaf glandular hairs

3.1.4 Essential oil variability of thyme

Aside from the essential oil content, the suitable portion of effective compounds detected by GC/MS as relative percentages should also be concerned in the therapeutic aspects of the drugs collected. In the case of the *Thymus* species, utilization of the drugs and industrial raw materials is generally based on monoterpene phenol compounds (thymol, carvacrol) and derivatives (thymol methyl ether, carvacrol methyl ether), as well as biosynthetic intermediates (*p*-cymene, *γ*-terpinene) that are detectable in typical thyme essential oils, where high thymol percentages are expected.

The results of a recent study show that the chemical composition of the essential oils obtained from the herb of two common thyme ecotypes represented by *T. vulgaris* L. cv. English Winter and cv. Summer Thyme de Provence cultivars were different. The oils were investigated by gas chromatography-mass spectrometry (GC-MS) which led to the identification of 80 and 73 constituents. In conclusion, 'English Winter' had the higher content of thymol, while 'Summer Thyme de Provence' was richer in p-cymene (Wesolowska and Jadczak., 2019). Based on the experiment carried out in Central Otago, New Zealand it was presented that in order to obtain more yields and phenol content of the essential oil, thyme is best to harvest during the flowering finished in December. The data showed high (16%) divergence in the level of phenolic components between the summer and winter harvest (McGimpsey et al,1994). In Caxias do Sul (Rio Grande do Sul State, Brazil), essential oils from the leaves of *Thymus vulgaris* L, higher yield with richer oxygenated compounds was determined, when it was harvested during spring (Atti-Santos et al,2004).

3.1.5 Traditional use and pharmacological properties

Thymus vulgaris known back to ancient Egyptians, Greeks, and Romans. Greeks used in baths, and Romans applied it to purify their rooms. In Unani medicine, aside from the root parts, all other parts are counted analgesic, anti-inflammatory, expectorant, digestive, carminative, emmenagogue, anthelmintic, lithotriptic, diuretic, aphrodisiac, and to improve digestion and appetite. Thyme oil has mostly been used for its anti-inflammatory purpose as a therapeutic purpose since ancient times. As a nervine tonic, it is used in the treatment of epilepsy, and also applied in dyspnea and asthma, diarrhea, dyspepsia with flatulence, gonorrhea, leucorrhea, and visceral catarrh. It is also effective against irritant to skin and mucous membranes. Thyme oil is used as a disinfectant and antiseptic, probably due to its phenolic content also for scenting soaps, making perfumes, and as a flavoring agent for food. Aerial parts contain volatile oil, flavonoids, triterpenes, tannins, and anthocyan glycosides. It contains thymol, carvacrol, p-cymene, α-pinene, terpineol, γ-terpinene, geraniol, linalool, and traces of cineole. Thymus vulgaris and its extracts includes high antioxidant activity and rosmarinic acid is identified as the predominant phenolic compound. Both thymol and carvacrol have also been responsible for the antioxidant activity, while others attributed the antioxidative action to rosmarinic acid (Akbar and Akbar, 2020., Stahl-Biskup and Sáez., 2002).

The antimicrobial activity of thyme oil was evaluated on 7 common food-related bacteria and fungus by using the disk diffusion method. The results show that Thymus vulgaris essential oil is tested to possess a strong antimicrobial property. In the future it will represent a new source of natural antiseptics in the pharmaceutical and food industry (Borugă et al, 2014).

A recent study showed that high number of flavonoids exhibited antioxidant and antibacterial activity, which were found in the extract of *Thymus vulgaris*. In general, thyme is an easily available source of natural antioxidants and antibiotics in food production and drugs (Prasanth et al, 2014). Composition of the essential oil was determined by GC (Gas chromatography)/ MS (mass spectrometry), while the antioxidant activity of the pure compounds were tested by DPPH, ABTS, and FRAP methods. DPPH assay according to Scherer and Godoy (2009). Classifying antioxidants as weak, when AAI < 0.5; moderate, when 0.5 < AAI < 1.0; strong, when 1.0 < AAI < 2.0; and extraordinarily strong, when AAI > 2.0. ABTS assay according to Re et al. (1999) with modifications. The results were expressed as IR50 (concentration capable of reducing 50% of free radicals) and were calculated using a calibration curve. FRAP (Ferric Reducing Antioxidant Power) assay was executed according to Benzie and Strain (1996). The results are expressed same as the ABTS assay (Lemos et al, 2017). Study shows GC/MS analysis revealed thymol as a major component of *T. vulgaris* with its contribution to the oil, 48.9%. The essential oils (EOs) exhibited a significant antimicrobial activity against all tested strains, in addition to their well-known traditional use in food and cosmetics, the exciting potential of tested *Thymus* essential oils for application in oral diseases and anticancer treatments, encourage further investigation (Nikolić et al., 2014).

3.2. Thyme cultivation

3.2.1 Propagation methods

Sowing seeds, preparing stem cuttings are the most widespread ways to grow thyme. To meet the demand of *Thymus vulgaris* plantations, the traditional way by seeds and vegetative propagation by cuttings are applied (El-Banna, 2017). To have the same exact plants as the parent plant, cuttings from late spring or late summer is needed. By sowing seeds, the plant might not turn out as exact as the parent plant. Best planted in late spring or early summer. Layering can also be used for propagation in small areas (Talhouk et al, 2015).

The sowing time is ideal around March, sowed in seed trays with nutrient rich soil mixture followed by irrigation, weed control and transplanting to bigger area during May. Cuttings take place in late spring for propagation, when one cut the woody stems at the basal part below the node, usually where the root formations are most viable. Remove the lower leaves and put the cut end into a container of moist soil mix or perlite. Place the pot in a warm, shady area with slightly damp. Vegetative cuttings propagation method could be challenging, a lot of care is needed, and the survival rate is limited. Layering of thyme plants, aerial stems are used for propagation, attached to the parent plant during rooting and later detached as an independent plant. An economic vegetative propagation method since no special tools are needed but the low rate of propagation and slow process might be a disadvantage.

3.2.2 Plant care

Environmental factors such as temperature, moisture level, soil condition and nutrients affect the growth and active substances in medicinal plants. Note to avoid areas with extreme hot or cold weathers. Warm and sunny area with well-drained soil is recommended to grow thyme. Thyme may not survive in the case of exposed to cold and damp soil.

One of the most important environmental factors is nutrient supply. A greenhouse experiment was carried out to evaluate the effect of nitrogen, phosphorous and potassium fertilizers on qualitative and quantitative characteristics of garden thyme. Each fertilizer treatments showed significant differences. The highest green and drug herb weights was achieved on 100 mg N, P_2O_5, K_2O/kg soil of pot (equal to 200 kg/ha). It is indicated that nitrogen and phosphorous are necessary for producing high yields. By increasing the nutrient content, it increases the fresh leave and dry weight of thyme. The results showed that potassium is useful in excessive amounts. Potassium affected the activity of several important enzymes such as ATPase and Rubisco.

Geographical environment, composition of essential oil where the plant is grown, physical and chemical characteristic of the soil, seed source, plant age and which part of the plant is used (Sharafzadeh, 2011) . It showed that phenolic essential oil compounds (thymol, carvacrol) and polyphenols (e.g., flavonoids) prefer warmer and drier climatic zones while other, non-phenolic substances usually accumulate in higher quantities in cooler and more humid areas. Different chemotypes prefer different conditions, it proves that chemotypes are not fully dependent on the environment and support a direct relation of essential oil and flavonoid patters with the genetic features of these plants. During different growth period but with same conditions, it shows that higher drug yield is obtained from lowland condition instead of mountainous. It was found that environmental conditions also affect the harvesting periods as well (Golmakani, and Rezaei., 2008). The plant can be damaged by few pests and diseases, such as the leaves can be damaged by aphids, rosemary beetles and sage leafhoppers.

3.2.3 Harvesting

As a woody perennial plant, it can be harvested 2 or 3 times annually for several years. Studies showed that harvesting time affects the yield and quality of thyme significantly. Delayed harvest cause in increased plant height and mass but decreased quality of herb (lesser quantity of essential oil and thymol). Harvesting by time ensured fairly good yield of herbs and high quality. The weather conditions prevailing during the vegetation period had a substantial effect on the yield and quality of herb. In a study of (Beata et al, 2015) they proved that thyme is cultivated in rows of 50 cm apart with inter-row spacing of 15, 30 or 45 cm. Plants are harvested in three stages, the beginning of blooming, full blooming and fruit set. In order to study the effects of plants space and time of harvesting, plant height, plant diameter, yields of dry and fresh herbage, content (%) and yield of oil, thymol and carvacrol were measured. Planting space had an effect on plant diameter and a very effect on other measured parameters except oil content.

Time of harvest had significant effect on yield of fresh herbage, content of oil and content of thymol. Its effect on other parameters was incredibly significant except dry herbage and oil yield. The maximum yield of dry and fresh herbage, yield and content of oil and thymol yield were obtained in 15 cm space and beginning of blooming stage. Maximum thymol content was detected in the beginning of blooming and 45 cm space. However, 15 cm spacing and harvesting in the beginning of blooming was the best treatment in respect of yield of dry matter, oil and thymol per unit area. Spacing was interacted with harvesting time in its effect on carvacrol content and yield only. Spacing affected plant growth (by means of plant diameter), plant height, thymol and carvacrol content, and yields of fresh and dry herbage, oil, thymol and carvacrol per unit area. Highest plant growth was achieved by inter-row spacing of 45 cm, but the highest plant height was achieved by the lowest spacing (Moualla and Naser, 2015).

3.3 Primary processing and storage of thyme

3.3.1 Drying

Herbs to drug, pure active agent or medicine is the main aim of medicinal plants production and processing. Primary processing of thyme includes procedures of cleaning, drying, sorting or essential oil distillation, etc. (Poós and Varju., 2017). Process steps after drying are as follows: removing the leaves from the stem, get rid of contaminants to obtain a consistent high quality product. It is not recommended to dry the thyme above 40°C in order to prevent cut back loss of flavour through volatilisations of essential oil, and to preserve the appropriate colour. Fresh herbs after cleaning from dirt and other foreign materials proceed with drying should turn out with an intensive decent color, odour and flavour. The essential oil content and composition change during the drying process, lower drying temperature can preserve larger amount of essential oil (Pluhár et al, 2016).

At *Thymus vulgaris* the effect of different drying techniques in a natural way were investigated on the quantity and quality parameters of the final product analyzed by GC–MS and sensory profile methods. Measured essential oil amounts were between 0.69 ml/100 g and 1.84 ml/100 g calculated on the dry weight basis. The different primary processing methods also influenced significantly the essential oil composition. Based on the data of the sensory analysis, the preference of the spice was mainly determined by the freshness, ratio of the purple color, ratio of 1,8-cineol and thymol; even if the essential oil amount of the spice did not meet the requirements of the Pharmacopoeia. According to the results, sensory analysis data in most of the cases corresponds to the GC–MS measurements and gives a much more complex characterization of a garden thyme spice (Sárosi et al, 2013).

By drying the plants, it may cause some losses in the biological characteristic of the plants also chemical compounds. Studies show that two methods are applied to analyse the effect of drying on the aroma constituents on thyme and sage. The volatile constituents of herbs (fresh, freeze-dried and oven-dried at 30 °C and 60 °C) were isolated by dynamic headspace. Simultaneous distillation-extraction methods followed by capillary gas chromatography and coupled gas chromatography-mass spectrometry were applied. A total of 68 compounds are found in thyme and 44 in sage. Also, more than 100 components are screened relevantly. A substantial reduction in the amount extracted is only found in the case after drying at 60 °C. The results are caused by the loss of non-oxygenated monoterpenes. For thyme, the character change was more complex. Some aroma assessment criteria (coefficient of efficiency Ce) are proposed on the basis of the results obtained(Venskutonis, P.R., 1997). The air drying of thyme was investigated at a temperature range of 40–60 °C and at an air velocity of 2.0 m/s. The drying time varied from 120 to 495 min. Increased air temperature significantly reduced the drying time of the thyme (Doymaz., 2011).

3.3.2 Essential oil isolation

The common methods to obtain thyme essential oil from the herbs are hydro-distillation (HD), steam distillation or steam and water distillation (Stahl-Biskup and Sáez, 2002). Recently published studies have succeeded in utilizing a microwave oven for the extraction of active components from medicinal plants/herbs (Lucchesi et al., 2004, Stashenko et al., 2004a, Stashenko et al., 2004b, Wang et al., 2006). Lucchesi et al. (2004) reported on a solvent-less microwave method for the extraction of essential oils from three aromatic herbs (basil, garden mint, and thyme). In 30 minutes, the amount of essential oils obtained with this method was comparable, either from qualitative and quantitative, to those obtained after 4.5 hour of HD. In a trial to take advantage of microwave heating with the conventional HD, microwave-assisted Hydro distillation (MAHD) was developed and used for the extraction of essential oils from *Xylopia aromatica* (Lamarck) and *Lippia alba* (Mill.) (Stashenko et al., 2004a, Stashenko et al., 2004b). However, to the authors' knowledge no work has been published on the MAHD of thyme species. It shows that MAHD has substantial advantage over conventional HD, similar extraction yield obtained in a shorter time when using MAHD. Results show a high extraction efficiency with shorter time caused by the microwave causing a quick rupture of the glandular walls.

3.3.3 Storage

After primary processing (drying) of thyme herb, the drug is stored for either longer or shorter period, such as thyme its best to store at 10 °C, with increasing temperature the change of essential content changes drastically. An International Standard (ISO 6754:1996) indicates the minimal quality requirements for dried thyme. The quality involves several parameters of the finished product. The volatile oil content of the dried herb is a crucial issue contributing to the flavour intensity. A minimum of 0.5% essential oil equivalent to 5 ml/ kg dried herb is required in the whole thyme leaves.

To preserve the expected quality of thyme oil, a very cool and dry space is recommended. It is important to not expose the thyme oil to heat or metals. Air-tight glass bottle is a preferred container to store the thyme oil in the dark. Once the thyme oil is exposed to the air, putting it in the refrigerator its essential since it can prolong its shelf life and applicability. Deterioration begins if the liquid is far darker or a lot of viscous than traditional (Prasanth Reddy et al, 2014).

4. Materials and Methods

4. 1. Experimental materials

The Thymus field studied was planted on 4th June, 2019, by the plant density of 5 plants/m^2, where the planting sheme of 50 cm x 40 cm was applied. Each population involves altogether 40 plants. Three year-old perennial stands of *Thymus vulgaris* varieties (’French Summer’: FS, ’Standard Winter’: SW, ’Pannon Timol’: PT) and selected clones (TV 115, TV 121, TV135, TV143) were studied in 2022 at our experimental station, among similar field conditions in order to compare the overall performance, yields and active compounds of taxa included (**Table 1, 2**). Sampling of flowering shoots in 3 replications per varieties was applied.

Table 1. Names and study details of thyme taxa involved in the experiment

Taxa	Chemotype	Fragrance	Pharmacological effect	Propagation
’Pannon Timol’	Thymol	strong, characteristic for thyme, phenol-like	expectorant, spasmolytic, antimicrobial	seedling growing (seeds form own parent stand)
’Standard Winter’				seedling growing, (seeds from Jelitto, Germany)
’French Summer’				seedling growing, (seeds from Jelitto, Germany)
TV 115	Geraniol	Citrus fragrance	repellent	halfwood cuttings
TV 121	Linalool	lavender fragrance	antiinflammatory, calming, anxiolytic	halfwood cuttings
TV 135	Carvacrol	oregano odour	antimicrobial	halfwood cuttings
TV 143	α-Terpineol	lilac odour		halfwood cuttings

4. 2. Experimental conditions

The study area is located in Budapest (46° 55' 01" N, 17° 43' 19"E), Hungary, in the Medicinal Plant Unit of the Experimental Station of the Hungarian University of Agriculture and Life Sciences. Environmental conditions and soil characteristics are presented in **Table 3** and **Table 4**.

Table 2. Main compounds in the essential oil of chemotypes studied

Chemotype	Main essential oil compounds	Codes	Origin
'T'	thymol	TV 135	France
'G'	geraniol + geranyl acetate	TV 115	France
'L'	linalool	TV 121	France
"A"	α-terpineol + limonene+α-terpinyl acetate	TV 143	France

Table 3. Environmental conditions of the experimental area of Thymus taxa studied (Medicinal Plant Unit of the Experimental station, MATE University, Budapest)

Study conditions	Characteristics
Region	Budapest, Pest county, Hungary
Geographic location	Danube-Tisza Mid Region, NW of the Great Hungarian Plain
Exposure of growing area	47° 39′88″ N, 19° 14°92″ E
Soil type	alluvial sandy soil
Average annual temperature	11°C
Average annual rainfall, mm	500-600

Table 4. Soil characteristic of the experimental area of Thymus taxa studied (Medicinal Plant Unit of the Experimental station, MATE University, Budapest)

Soil parameters												
pH	K_A	$CaCO_3$ m/m%	Humus m/m%	NO_3^- +NO_2^--N mg/kg	P_2O_5 mg/kg	K_2O mg/kg	Mg mg/kg	Na mg/kg	Zn mg/kg	Cu mg/kg	Mn mg/kg	SO_4^{2-} mg/kg
6.49	<30	<1	1.2	1.2	291.0	36.7	53.0	n.d.	1.7	3.5	37.8	n.d.

4.3 Experimental methods

4.3.1 Sampling procedures

Sampling was due in May (in full flowering) and September (in vegetative phase) in 2022. Fresh samples were collected from randomly selected plants in three replications, then dried naturally. Fresh and dry yields as well as crumbled, stem free drug mass (*Thymi herba*, including only flower and leaf parts) were determined and subjected to laboratory analysis. Essential oils are isolated by hydrodistillation in the lab of the Department, while the oil composition was determined by GC/MS.

Figure 4. Thyme populations and clones at autumn and spring harvesting (Medicinal Plant Unit of the Experimental station, MATE University, Budapest, 2022)

Each Thyme varieties with different clones were collected for three samples each from different plant bush, during spring and autumn period. Before naturally dried, the herb was mandatory weighed to obtain the fresh mass with the stem. After having collected, the herb was naturally dried in the shade for a few days. When the herb was fully dried, each sample was measured for its dry weight with stem. Next removing soil particles, leaves and flowers from the stem manually was also needed. Flowers and leaves are collected to proceed to the next step. Dried flowers and leaves were scaled to observe the weight before processing it to obtain the essential oil.

4.3.2 Isolation and analysis of the essential oil

Phytochemical analysis from dried, crumbled plant material: EO content (mL/100 g DW), EO composition (%) is held. Complex data analysis was necessary to receive the full data in order to compare the difference. Clevenger type distillation apparatus was applied to isolate the essential oil (EO) from dried samples according to the Ph. Eur. (European Pharmacopoeia) (**Figure 5**). The EO content is measured and given in mL/100 g calculated on the dry weight (DW) basis. Then the EO samples were kept in glass vials in refrigerator prior to GC analysis. Gas chromatography (GC) coupled with mass spectrometry (MS) was used to determine the relative percentage (%) composition of terpenoid compounds within the EOs, will be carried out using an Agilent Technologies 6890N GC System instrument equipped with an Agilent Technologies MS 5975 detector. Temperature program has been optimized formerly at our department. The compounds will be identified by mass spectral libraries and by linear retention indices (LRI).

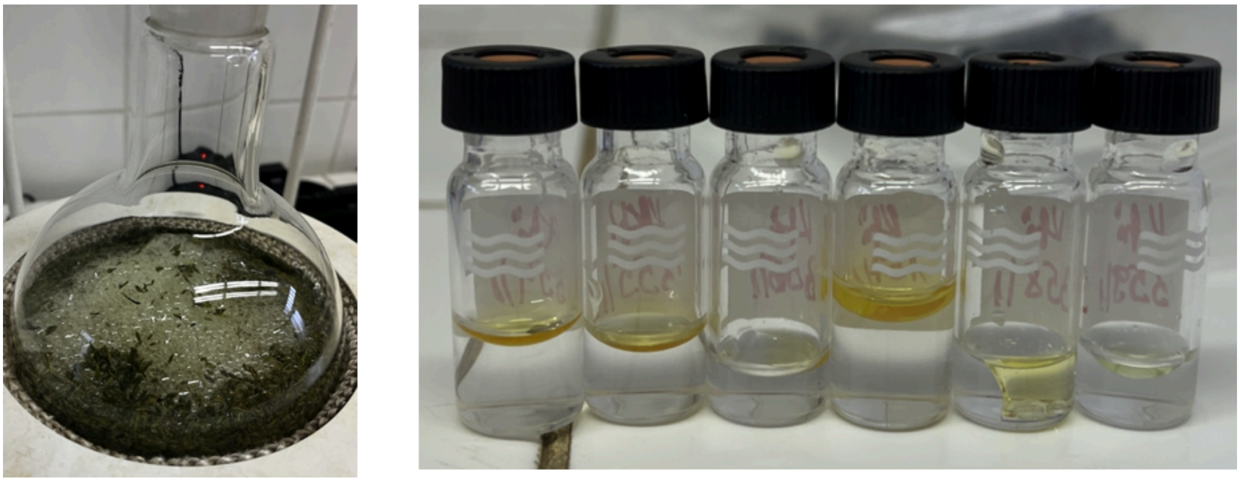

Figure 5. Clevenger distillation and Thyme essential oil samples

5. Results and discussion

5.1 Effect of harvest period on fresh and dry masses

According to the result achieved during the experiment, **Figure 6** shows that the clone coded TV135 had the highest amount for fresh weight, especially during spring harvest, when it weighted up to 43 gram/plant, followed by TV 143 (36.75 g/plant), TV 121 (35.25 g/plant) and Pannon Timol (PT: 34.33 g/plant), respectively.

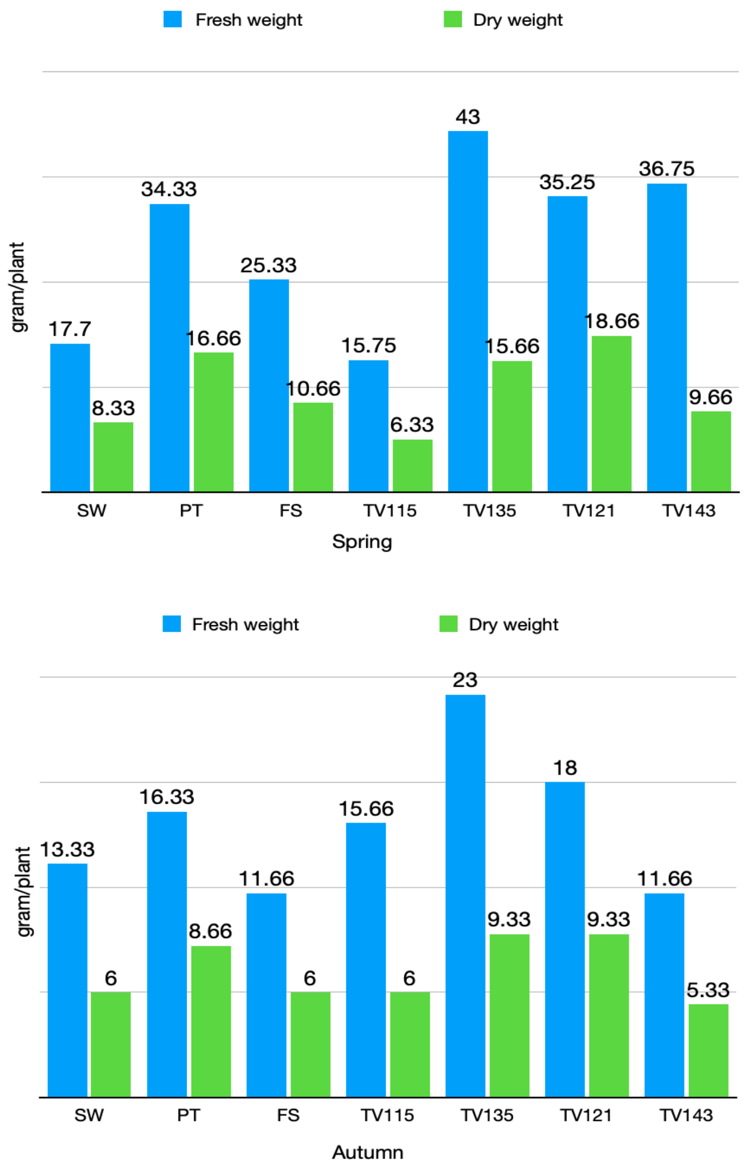

Figure 6. Average fresh mass values (g/plant) of garden thyme shoots and dry drug yields of *Thymi herba* during the periods of spring and autumn harvest in different taxa involved (Budapest, 2022)

The remaining taxa (TV 115, SW: Standard Winter and FS: French Summer) represented low fresh weight (15.75-25.33 g/plant). In the case of the dried drug mass, TV 121 was outstanding in spring and autumn as well, while in the period of autumn harvest, TV 135 showed the same remarkable average value (9.33 g/plant). If compared the two cutting periods, we found that spring harvest resulted generally in higher fesh and dry mass values. As in dry weight, TV121 shows up with the highest amount with 18.66 gram/plant. We can conclude that genetic features, regeneration ability of different varieties and the harvest season have all significant effect on the drug yields.

5.2. Effect of harvest period on essential oil content

The essential oil content of different thyme samples varied between low 1.19 mL/100 g DW) and quite high (3.32 mL/100 g DW) values. **Figure 7** shows that almost all the studied taxa in both cutting periods meet the pharmacopoeial requirement for *Thymi herba*, concerning min. 1.2 ml/ 100 g essential oil content. The seven taxa studied could be divided into three groups (with high, changeable and rather low values), according to the accumulation levels. Only three populations (PT, TV121 and TV135) of Thymus vulgaris produced fairly high amounts. From the remaining taxa, TV143 had a big difference between spring and autumn harvest, while this change of SW, FS and TV115 was not considerable .

The drug obtained by PT contained the highest amount of essential oil during both spring and autumn harvest, followed by TV143. Spring harvest mostly resulted in to have higher essential oil levels with an amount of 3.32 ml/100g detected in PT, followed by TV121 (2.68ml/100g).In the period of the autumn harvest, the highest essential oil quantities were found at PT (2.79 ml/100g) and TV121 (2.58 ml/100g). Our results shown that FS (1.19 ml/100g, 1.35 ml/100g) and TV115 (1.55 ml/100g, 1.37 ml/100g) have generally lower amount than the average, during spring and autumn harvest, either. Significant difference was found at TV143 between the essential oil content measured in spring harvest (2.78ml/100g) and autumn harvest (1.67ml/100g). In the case of SW, FS and TV135, the plants produced more essential oil in the period of autumn harvest, if compared to PT, TV115, TV121 and TV 143, where the EO levels were higher in spring. In the case of the essential oil, both the origin/variety and the harvest season have significant impact on essential oil production.

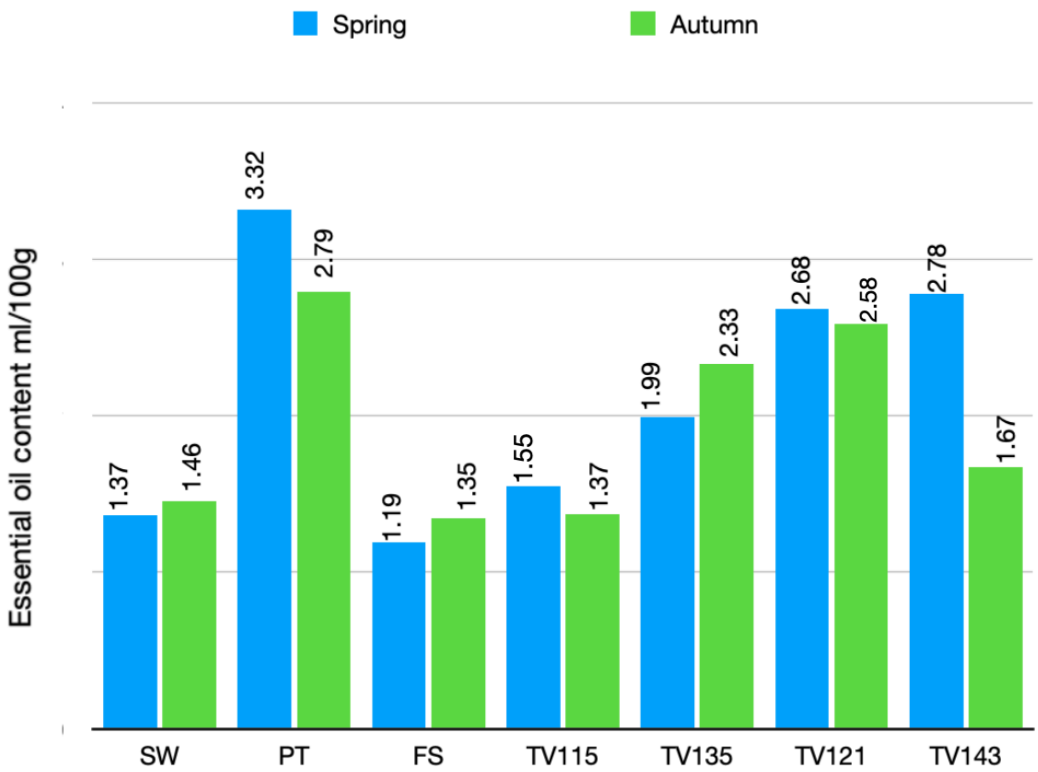

Figure 7. Average essential oil content values (ml/100 g) of *Thymi herba* during the periods of spring and autumn harvest in different thyme taxa involved (Budapest, 2022)

5.3. Effect of harvest period on essential oil composition

High levels of essential oil diversity of *Thymus vulgaris* were proven, based on samples collected from the MATE experimental field. More than 15 distinct chief compounds were identified in the essential oils of *Thymus vulgaris* samples of different varieties (**Table 5, Figure 8**). The main compound of the commercial essential oil is mostly thymol with the highest therapeutical relevance according to *Thymus* species. Breeding efforts of new chemotypes has been made to develop varieties with higher essential oil content as well as to maximize the thymol percentage.

In our study, thymol played an important role too, while other compounds (*p*-cymene, γ-terpinene, linalool, thymol methyl ether, neral, geraniol, borneol, geranial and carvacrol) were also significant in the chemotype pattern of some selected clones. The highest percentages of thymol, p-Cymene and γ-Terpinene were detected at SW, PT and FS. However, TV115 had geraniol, while linalool, carvacol and geranyl acetate also reached considerable levels in certain essential oil samples.

Table 5. Average percentage (%) composition of essential oils of *Thymi herba* obtained during the periods of spring and autumn harvest in different thyme taxa involved (Budapest, 2022)

			SW		PT		FS		TV115		TV121		TV135		TV143	
Compound	**RT**	**LRI**	**Spring**	**Autumn**	**Spring**	**Autumn**	**Spring**	**Autumn**	**Spring**	**Autumn**	**Spring**	**Autumn**	**Spring**	**Autumn**	**Spring**	**Autumn**
Camphene	**5.95**	**952**							0.17	1.49	0.66	1.01			0.34	0.77
Sabinene	**6.52**	**976**													1.73	1.69
1-octen-3-ol	**6.81**	**987**	0.78	1.25	0.80	1.15	0.73	1.16	0.75	1.29	0.77	0.64				
ß-Myrcene	**6.99**	**995**	0.58	1.32	1.13	1.67	0.66	1.05	0.06	0.48			0.72	1.61	1.36	1.33
α-Terpinene	**7.79**	**1018**	0.80	1.46	1.55	1.55	0.72	1.32	0.01	0.50			0.87	1.28		
p-Cymene	**8.09**	**1026**	8.66	15.37	10.37	20.22	11.05	15.60	0.23	5.62			14.61	22.40	0.70	1.28
Limonene	**8.19**	**1029**							0.04	0.86			1.55	4.73	1.59	1.76
1,8-Cineole	**8.38**	**1034**			1.00	1.19	0.54	0.69	0.30	1.14			1.61	1.54	0.46	0.65
γ-Terpinene	**9.20**	**1056**	6.48	13.12	9.67	12.07	4.99	13.46	0.09	5.04			3.59	9.00	0.71	0.70
cis-Sabinene hydrate	**9.73**	**1070**	1.47	1.40	1.12	1.26	1.30	1.51	0.19	2.08			3.25	12.60		
Linalool	**10.76**	**1097**	2.47	2.89	3.77	3.94	3.36	3.58	17.06	1.25			1.43	1.27		
trans-Sabinene hydrate	**10.76**	**1097**											0.78	1.18		
Octen-3-yl acetate	**11.19**	**1108**													0.66	1.50
Camphor	**12.68**	**1144**			0.58	1.16	0.35	0.80								
Borneol	**13.43**	**1162**	1.06	1.05	0.79	0.83	1.64	1.89	3.25	6.79	4.36	5.59	0.74	0.99	1.79	2.55
Terpinen-4-ol	**13.96**	**1175**											0.94	1.59		
α-Terpineol	**14.55**	**1189**													27.74	17.02
Nerol	**16.15**	**1227**							1.53	3.42	1.86	4.01				
Neral	**16.58**	**1238**							1.25	1.58	1.51	1.59				
Carvacrol methyl ether	**16.61**	**1238**	0.55	0.30			0.86	0.32								
Geraniol	**17.20**	**1252**							32.79	28.09	49.97	48.45	0.74	0.19		
Geranial	**17.86**	**1268**							1.87	2.24	1.90	2.38				
Bornyl acetate	**18.54**	**1284**													0.48	1.69
Thymol	**18.81**	**1290**	64.44	48.23	53.41	37.28	55.68	45.55	6.31	3.01	1.80	0.21	52.01	24.69		
Carvacrol	**19.20**	**1300**	4.50	3.75	4.05	3.63	4.27	3.45	2.90	10.18			4.68	3.15		
α-Terpinyl acetate	**21.00**	**1349**													56.06	61.29
ß-Bourbonene	**22.26**	**1383**							0.52	0.32						
Geranyl acetate	**22.43**	**1388**							12.03	7.42	18.03	14.08				
ß-Caryophyllene	**23.68**	**1420**	2.72	1.76	3.67	2.88	3.49	1.90	9.60	4.24	6.62	4.59	4.57	3.22	1.47	1.13
Geranyl propanoate	**25.24**	**1458**	0.49	0.42							2.61	1.80				
Germacrene D	**26.18**	**1482**							1.96	0.83					1.05	1.05
Neryl isobutanoate	**26.62**	**1492**									0.55	1.16				
Bicyclogermacrene	**26.81**	**1497**							0.35	0.10						
ß-Bisabolene	**27.23**	**1508**							0.57	0.07						
Geranyl butanoate	**29.33**	**1566**									1.59	1.58				
Caryophyllene oxide	**30.20**	**1590**	0.77	0.79	1.26	1.71	1.96	1.01	1.93	2.52	0.90	3.72	2.01	2.19		
tau-Cadinol	**32.26**	**1644**					0.50	0.05								
Total			95.67	93.11	93.16	90.52	92.09	93.30	95.73	90.50	93.11	90.81	94.09	91.64	96.13	94.41

Concerning genetic background, the availability of different chemotypes with different compounds in the essential oil is influenced by its chemotypes and harvest time. In our studies, thymol was the most frequent chemotype-determining monoterpene due to the wide distribution and occurrence of *Thymus vulgaris,* where this compound dominated the essential oils. Concerning all the samples, thymol and the relative compounds, p-cymene and *γ*-terpinene, are detected with the highest amount. From the experiment we can conclude, that in the essential oil of SW, PT, FS and TV135, thymol was detected in the highest amount among all the components. TV121 andTV115 had geraniol as their main component, while in TV143 α-Terpinyl acetate occupies more than half of the essential oil components. The results shows that higher essential oil content and thymol ratio were established at full bloom, during the spring harvest. (**Table 5, Figure 8**).

We have found the following components in the varieties and clones involved in our studies:

1. 'Standard Winter' (SW): 1-octen-3-ol, ß-Myrcene, α-Terpinene, p-Cymene, γ-Terpinene, cis-Sabinene hydrate, Linalool, Borneol, **Thymol**, Carvacrol, ß-Caryophyllene, Caryophyllene oxide, Carvacrol methyl ether, Geranyl propanoate.

2. Pannon Timol (PT): 1-octen-3-ol, ß-Myrcene, α-Terpinene, p-Cymene, γ-Terpinene, cis-Sabinene hydrate, Linalool, Borneol, **Thymol,** Carvacrol, ß-Caryophyllene, Caryophyllene oxide, 1,8-Cineole, Camphor.

3. **'French Summer' (FS):** 1-octen-3-ol, ß-Myrcene, α-Terpinene, p-Cymene, γ-Terpinene, cis-Sabinene hydrate, Linalool, Borneol, **Thymol,** Carvacrol, ß-Caryophyllene, Caryophyllene oxide, 1,8-Cineole, Camphor, Carvacrol methyl ether, tau-Cadinol.

4. **TV 115**: 1-octen-3-ol, ß-Myrcene, α-Terpinene, p-Cymene, γ-Terpinene, cis-Sabinene hydrate, Linalool, Borneol, Thymol, Carvacrol, ß-Caryophyllene, Caryophyllene oxide, 1,8-Cineole, Camphor, Carvacrol methyl ether, tau-Cadinol, Camphene, Limonene, Nerol, Neral, **Geraniol**, Geranial, ß-Bourbonene, Geranyl acetate, Germacrene D, Bicyclogermacrene, ß-Bisabolene.

5. **TV121:** 1-octen-3-ol, Borneol, Thymol, ß-Caryophyllene, Caryophyllene oxide, Camphene, Nerol, Neral, **Geraniol,** Geranial, Geranyl acetate, Geranyl propanoate, Neryl isobutanoate, Geranyl butanoate.

6. **TV135:** ß-Myrcene, α-Terpinene, p-Cymene, γ-Terpinene, cis-Sabinene hydrate, Linalool, Borneol, **Thymol,** Carvacrol, ß-Caryophyllene, Caryophyllene oxide, 1,8-Cineole, Limonene, Geraniol, trans-Sabinene hydrate.

7**. TV143:** p-Cymene, γ-Terpinene, Borneol, ß-Caryophyllene, 1,8-Cineole, Camphene, Limonene, Sabinene, ß-Myrcene, Octen-3-yl acetate, *α-Terpineol*, Bornyl acetate, **α-Terpinyl acetate**, Germacrene D

Figure 8 and Table 6 show the average compositions of the essential oils obtained from individual samples belonging to the seven Thymus taxa. Altogether 37 compounds have been identified, representing 90.50–96.13% of the total volatile oils of 14 samples analyzed. Among monoterpenes, thymol played an important role, influencing the essential oil quality as the chief compound (up to 64.44%) of four varieties (Thymus vulgaris: SW, FS, PT, TV135). Other significant monoterpenes of the latter taxa were partly the precursors of thymol biosynthesis: p-cymene (Thymus vulgaris: SW, PT and TV135) and γ-terpinene (Thymus vulgaris: SW, PT, FS and TV135).

Ratio of **geraniol** (TV 115: 32.79 % > 28.09%) decreased, while that of the **α-Terpinyl acetate** (TV 143: 56.06 % < 61.09 %) increased from spring to autumn in the essential oil of the respective chemotypes by harvest time, while almost the same proportions of **geraniol** (49.97-48.45%) were detected in the essential oil in spring and in autumn cut shoots of TV 121 (**Figure 8, Table 6**).

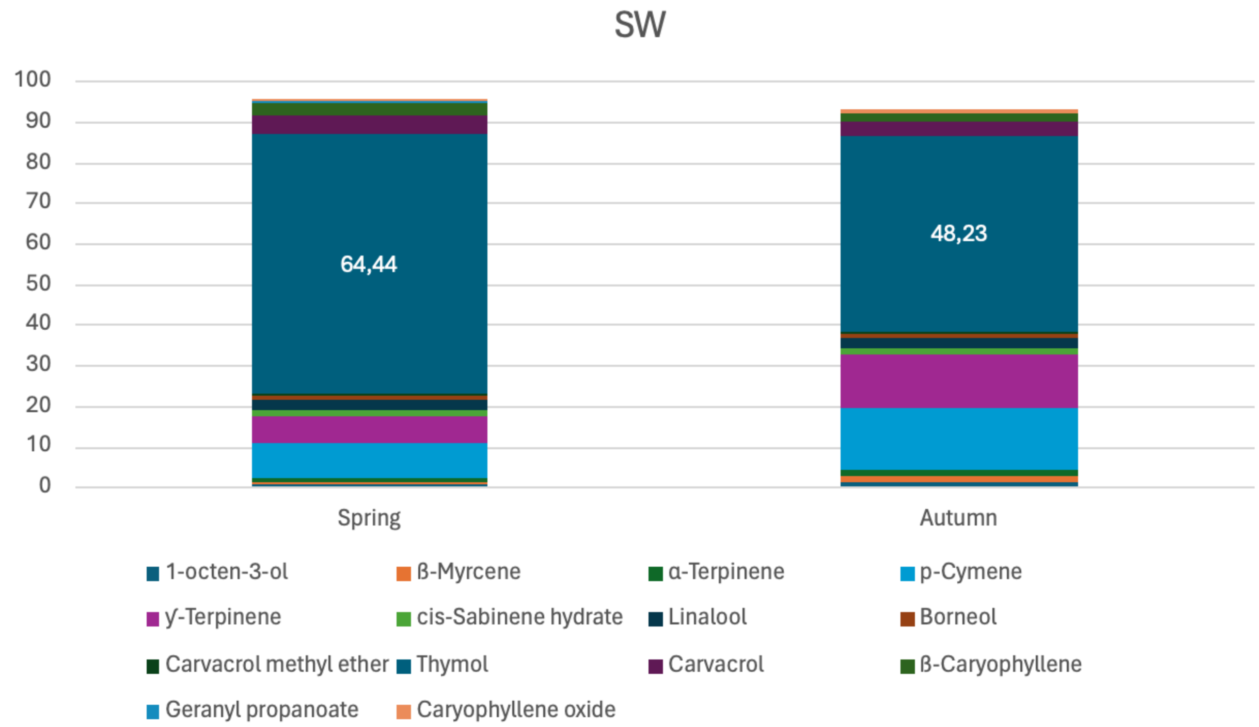

Figure 8/1. Essential oil composition of *Thymus vulgaris* L.'Standard Winter' (SW) in spring and autumn cut samples of 2022 (Budapest)

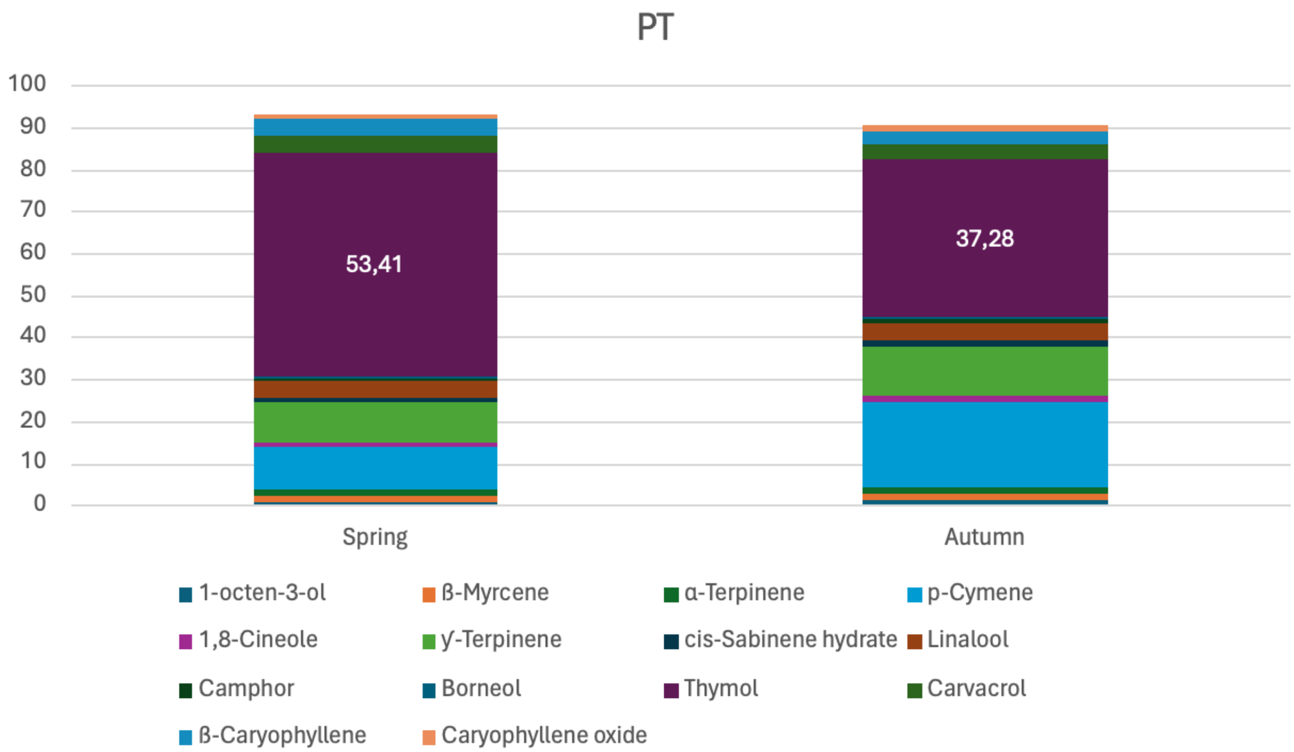

Figure 8/2. Essential oil composition of *Thymus vulgaris* L.'Pannon Timol' (PT) in spring and autumn cut samples of 2022 (Budapest)

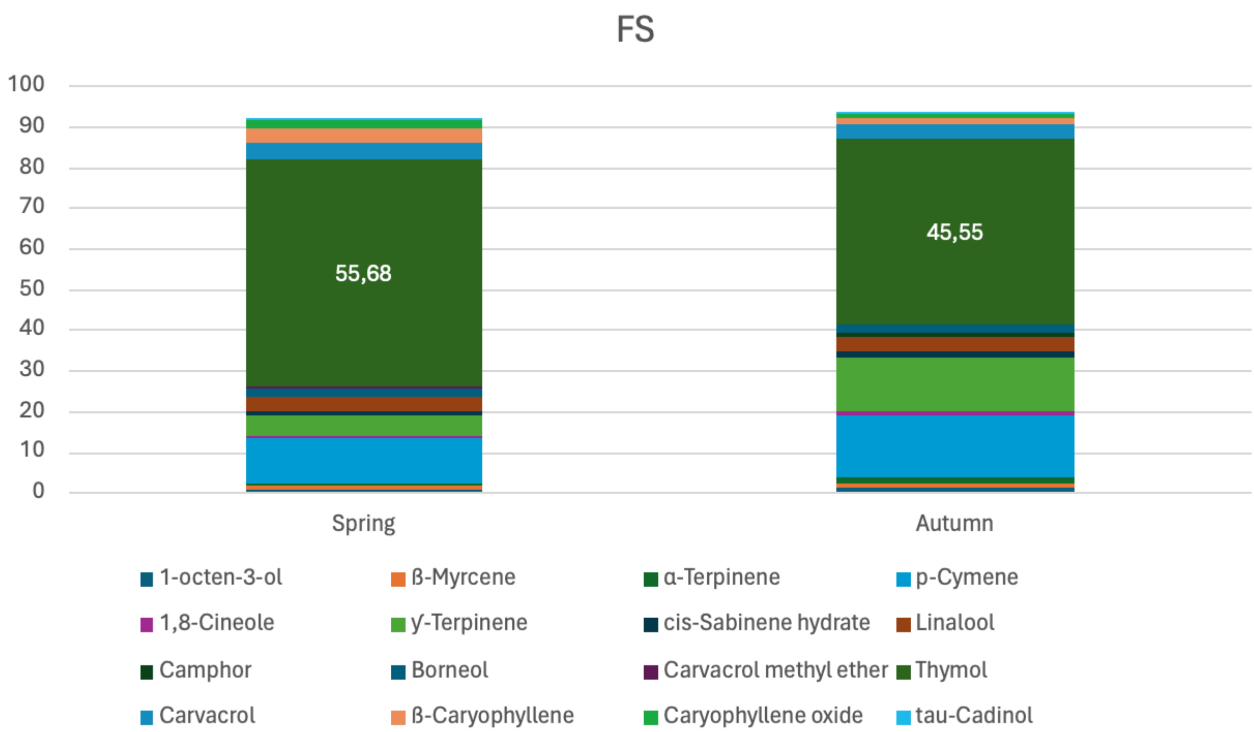

Figure 8/3. Essential oil composition of *Thymus vulgaris* L.'French Summer' (FS) in spring and autumn cut samples of 2022 (Budapest)

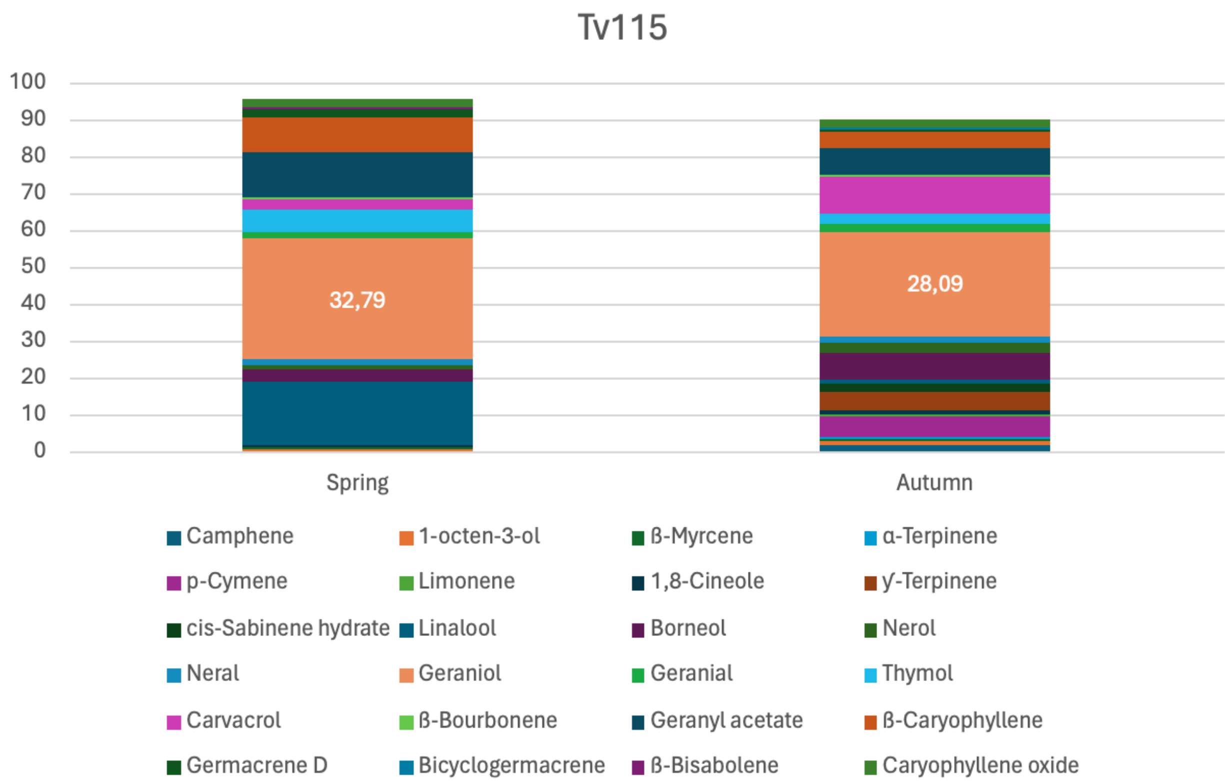

Figure 8/4. Essential oil composition of the TV 115 clone of *Thymus vulgaris* L. in spring and autumn cut samples of 2022 (Budapest)

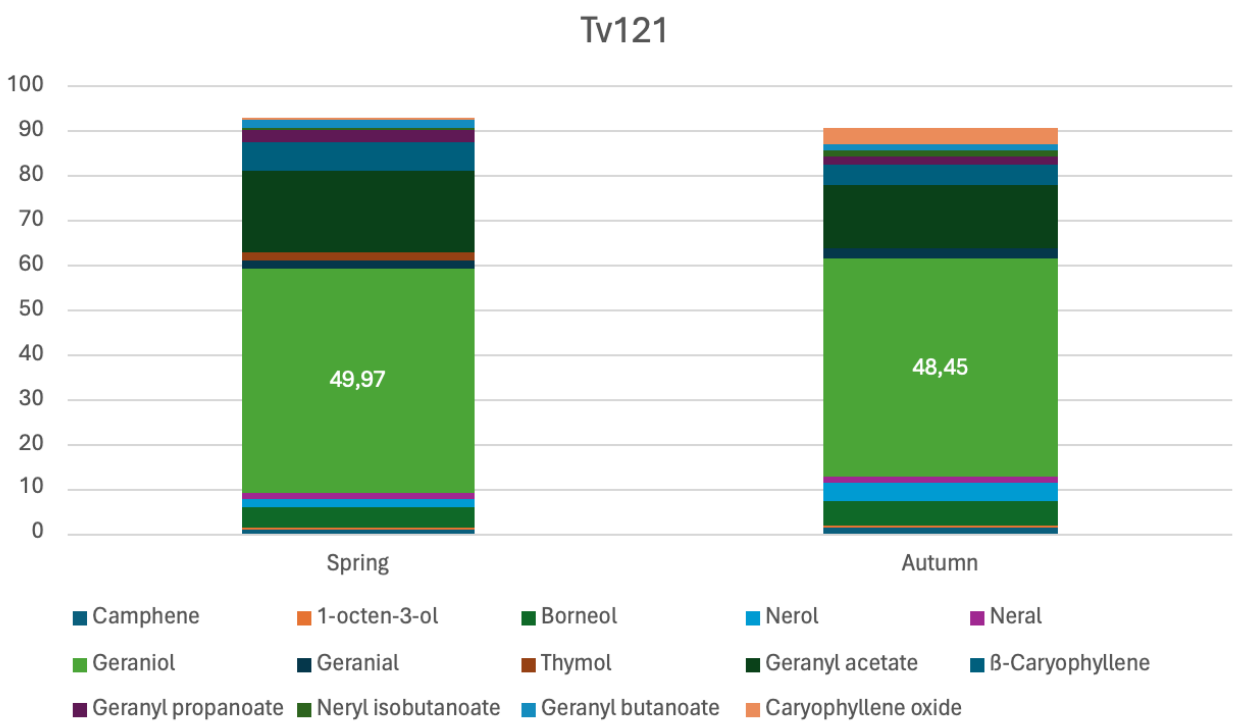

Figure 8/5. Essential oil composition of the TV 121 clone of *Thymus vulgaris* L. in spring and autumn cut samples of 2022 (Budapest)

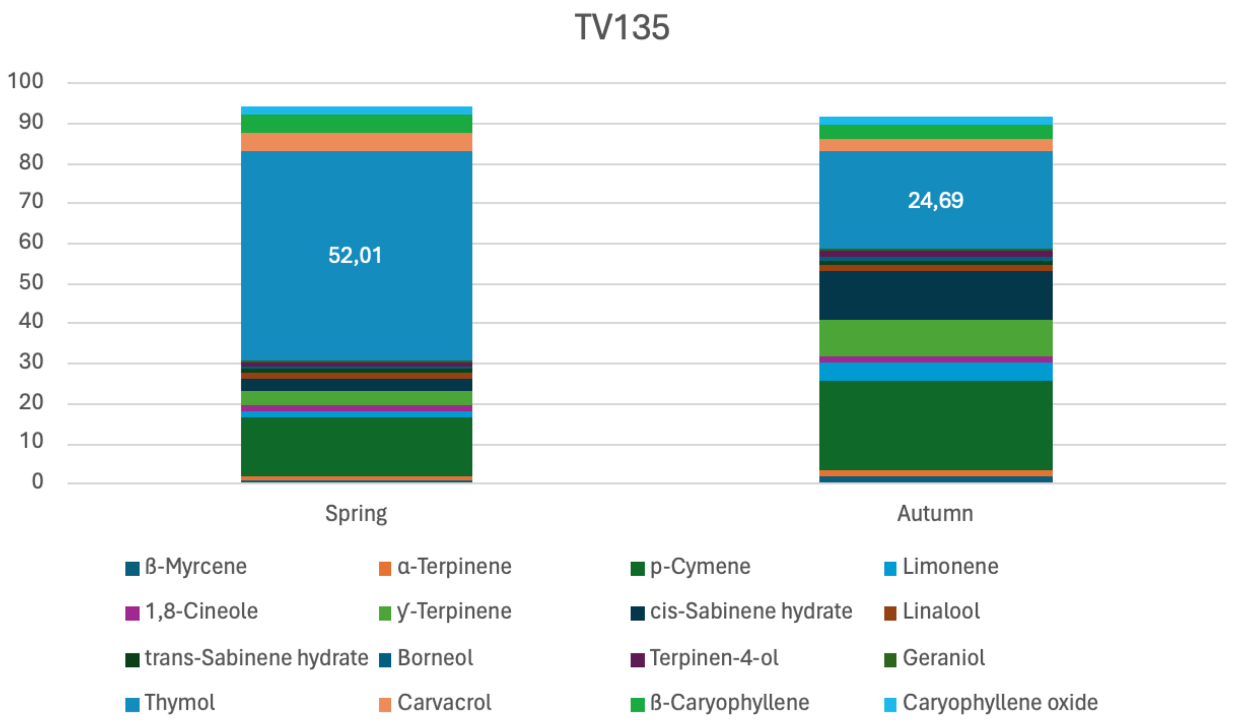

Figure 8/6. Essential oil composition of the TV 135 clone of *Thymus vulgaris* L. in spring and autumn cut samples of 2022 (Budapest)

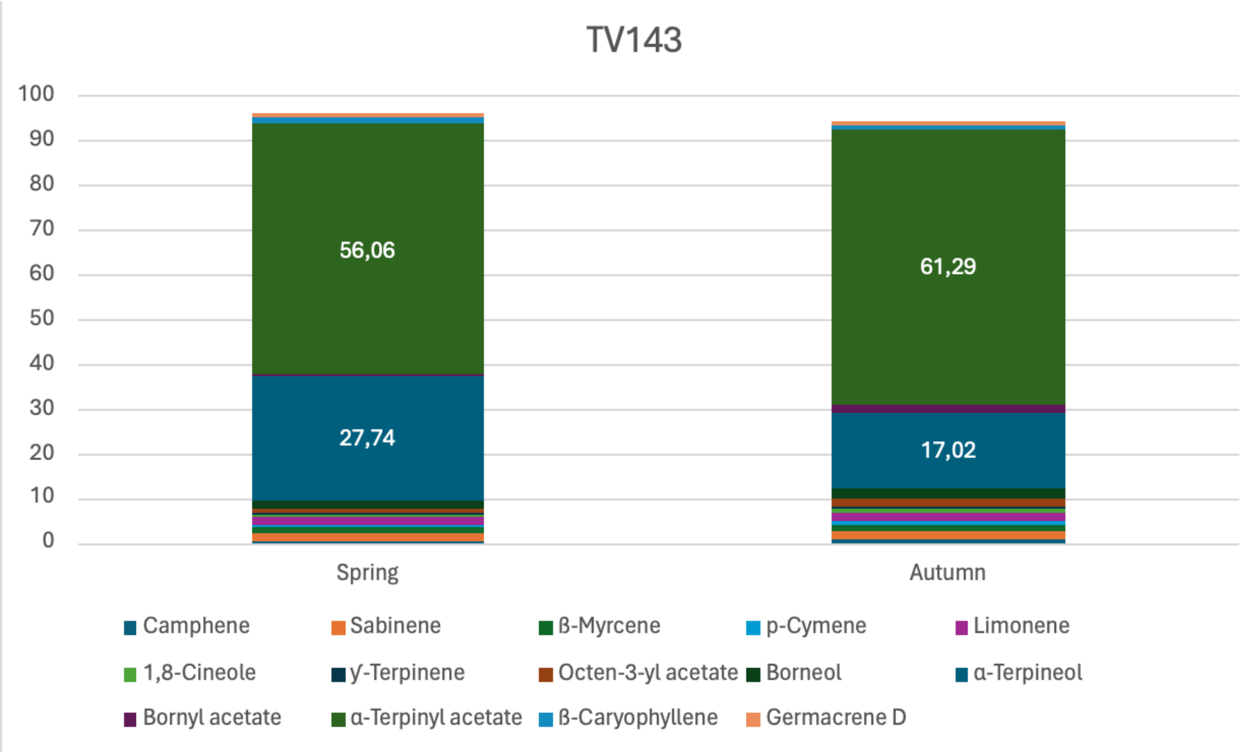

Figure 8/7. Essential oil composition of the TV 143 clone of *Thymus vulgaris* L. in spring and autumn cut samples of 2022 (Budapest)

Table 6. Summary of the main essential oil compound spectra of each thyme varieties involved in our studies detected in spring and autumn of 2022 (Budapest)

Variety/ clone	Harvest period	Thymol	p-Cymene	γ-Terpinene	Carvacrol	cis-Sabinene hydrate	Linalool	Geraniol	Geranyl acetate	α-Terpenyl acetate	α-Terpineol
		%									
Standard Winter	spring	64.44	8.66	6.48							
	autumn	48.23	15.37	13.12							
Pannon Timol	spring	53.41	10.37	9.67							
	autumn	37.28	20.22	12.07							
French Summer	spring	55.68	11.05	4.99							
	autumn	45.55	15.60	13.46							
TV 135	spring	52.01	14.61		4.68						
	autumn	24.69	22.40			12.60					
TV 115	spring						17.06	32.79	12.03		
	autumn				10.18			28.09	7.42		
TV121	spring							49.97	18.03		
	autumn							48.45	14.08		
TV 143	spring									56.06	27.74
	autumn									61.29	17.02

6. Summary

Medicinal plants have always been an essential in our daily life. WHO has confirmed that herbal medicines serve the health needs of about 80 percent of the world's population. Among all other varied species of *Thymus*, *Thymus vulgaris* is used more than other species in therapeutic dosage forms. In traditional medicine, common thyme is cultivated in rural areas. Cultivated in other part of the countries, it is used to treat diseases. The most valued compound of thyme raw material is essential oil, widely used in pharmaceutical, perfumery-cosmetic and food industries.

The main aim of the thesis was to evaluate the essential oil diversity of garden thyme (*Thymus vulgaris* L.) clones and varieties. Perennial stands of *Thymus vulgaris* varieties ('French Summer', 'Standard Winter', 'Pannon Timol') and selected clones (TV 115, TV 121, TV135, TV143) were established in 2019 at the experimental station in Budapest, among similar field conditions, with the purpose to compare the overall performance, yields and active compounds of taxa included, in two cutting periods (spring and autumn). After field measurements and sampling, laboratory analysis including essential oil isolation and determination of compounds using GC (gas chromatography) were applied. In this experiment, we aim to characterizing morphology and essential oil properties of different *Thymus* taxa, provide essential information on drug yields and determination of quality parameters concerning varieties available for growers.

Concerning dried drug mass values, TV 121 proven to be outstanding in spring and autumn as well, while in the period of autumn harvest, TV 135 showed the same remarkable average value (9.33 g/plant). If compared the two cutting periods, we found that spring harvest resulted generally in higher fesh and dry mass values. We can conclude that genetic features, regeneration ability of different varieties and the harvest season have all significant effect on the drug yields.

The essential oil content of different thyme samples varied between low 1.19 mL/100 g DW) and quite high (3.32 mL/100 g DW) values. Almost all the studied taxa in both cutting periods meet the pharmacopoeial requirement for *Thymi herba*, concerning min. 1.2 ml/ 100 g essential oil content. The seven taxa studied could be divided into three groups: with high, changing and rather low essential oil levels. Only three populations (PT, TV121 and TV135) produced fairly high amounts. The drug obtained by PT contained the highest amount of essential oil during both spring (3.32ml/100g) and autumn harvest (2.79ml/100g). TV143 showed significant seasonal variability, having big difference between spring (2.78ml/100g) and autumn (1.67ml/100g) harvest, while this change of SW, FS and TV115 was not considerable. In the case of the essential oil, both the origin/variety and the harvest season had significant impact on essential oil production.

High levels of essential oil diversity were found among *Thymus vulgaris* taxa involved, where the most abundant chemotype was that containing high level (%) of thymol in the essential oil. More than 15 distinct chief compounds were also identified in the essential oils of different varieties and clones (e.g. *p*-cymene, *γ*-terpinene, linalool, thymol methyl ether, neral, geraniol, borneol, geranial and carvacrol).

From the experiment we can conclude, that in the essential oil of SW, PT, FS and TV135, thymol was detected in the highest amount, while TV121 and TV115 belong to the geraniol and TV143 is α-Terpinyl acetate chemovarieties.

The results showed that higher essential oil content and **thymol** ratio can be determined at full bloom, during the spring harvest. Ratio of **geraniol** (TV 115: 32.79 % > 28.09%) decreased, while that of the **α-Terpinyl acetate** (TV 143: 56.06 % < 61.09 %) increased from spring to autumn in the essential oil of the respective chemotypes by harvest time, while almost the same proportions of **geraniol** (49.97-48.45%) were detected in the essential oil in spring and in autumn cut shoots of TV 121.

Aside from the essential oil content, the suitable portion of effective compounds detected by GC/MS as relative percentages should also be concerned in the therapeutic aspects of the drugs. In the case of the *Thymus* species, utilization of the drugs and industrial raw materials is generally based on monoterpene phenol compounds (thymol, carvacrol) and derivatives (thymol methyl ether, carvacrol methyl ether), as well as biosynthetic intermediates (*p*-cymene, *γ*-terpinene) that are detectable in typical thyme essential oils, where high thymol percentages are expected. In other applications, further chemotypes may also have importance in the future, what can be supported by our data obtained in the present study.

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