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ESSENTIAL OIL AND EXTRACTS FROM THYMUS PRAECOX OPIZ SSP. POLYTRICHUS AS NATURAL ANTIOXIDANTS

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ABSTRACT

Spices and herbs have been used for centuries by many cultures to enhance the flavor and aroma of foods. Early cultures also recognized the value of using spices and herbs in preserving foods and for their medicinal value. In the present study the essential oil of wild growing Thymus praecox Opiz ssp. polytrichus was isolated by hydrodistillationprocess. Extracts of T. praecox ssp.polytrichuswere prepared by using solvents of varying polarity. Antioxidant potential and ability of essential oil and extractsto neutralize DPPH radicals was investigated. The essential oil showed significantly higher antioxidant activity compared with synthetic antioxidants BHA and BHT. This work provides the basis for the present rapidly increasing interest for the use of natural antioxidants as food antioxidants, functional food ingredients and/or as nutritional supplements.

Key words: *Thymus, hydrodistillation, essential oil, antioxidant activity*

INTRODUCTION

Essential oils are aromatic and volatile liquids, mixtures oforganic compounds extracted from plant materials and characterizedby a strong and generally pleasant flavor. They could be obtained from various plant materials (flowers, buds, seeds, leaves, twigs, bark, herbs, trees, fruits, roots and other plant parts). Essential oils and extracts isolated from herbs of the Lamiaceae family, like rosemary, sage, oregano and thyme are well-known for their antioxidant activity and possess the potential as natural agents for food preservations.

In recent years there is growing interest in finding natural antioxidants that could replace sinthetic antioxidants because of adverse toxicological reports on many synthetic compounds. Common antioxidants in the food, cosmetics and pharmaceutical industries such as butylatedhydroxytoluene (BHT), and butylatedhydroxyanisole (BHA) are synthetic and can produce carcinogeniceffects in living organisms [1].

Sources of natural antioxidants are primarily, plant phenolics that may occur in all parts of plants such as the fruits, vegetables, nuts, seeds, leaves, roots and barks [2]. The most important groups of natural antioxidants are the tocopherols, flavonoids, phenolic acids, terpenoids and carotenoids. Natural antioxidants play an important role in different systems:

1. in plants, they act as a protective agents against radiation and microbial infections,

2. in foods,they protect foods from lipid peroxidation and provide microbiological food safety, they delay or inhibit the formation of toxic products that result in oxidation of lipids, and thereby maintain the nutritional quality and prolong shelf life of foods, and

3. in biological systems, along with endogenous defenses (enzymes, vitamins, proteins, and others), dietary antioxidants may help prevent or slow the oxidative stress induced by free radicals, providing important health benefits for humans by maintaining our health and preventing disease [3].

The genus *Thymus* has about 215 species, and their essential oil and extract composition has been studied earlier especially on *Thymus vulgaris* L. and and*Thymus zygis* [4-8]. In the Flora of Serbia the genus *Thymus* is represented by 31 species with more than 60 varieties, most of which found in the grassland and dry sunny rocky limestone or serpentine habitats [9]. Due to the presence of essential oil which is rich in phenolic monoterpenes such as thymol and carvacrol *Thymus* species have antifungal, antibacterial and antioxidant effects. The thymol possesses useful antimicrobial and antioxidant properties. It is considered by many national authorities as Generally Recognized as Safe (GRAS) ingredient without teratogenic and mutagenic properties.

The most studied *Thymus* species *Thymus vulgaris*has been credited with a long list of medicinal uses: e.g. antiseptic, anthelmintic, carminative, expectorant, sedative, tonic, preservative, aromatic, stomachic, antispasmodic,antiplatelet effect etc [10-12]. Thyme has been used commonly as a culinary herb for adding flavour and as cough medicine, to treat dyspepsia and other gastrointestinal disturbances. According to Zarzuelo and Crespo (2002) wild growing *Thymus* species contains all the medicinal properties of the more commonly used *T. vulgaris*, though in a lesser degree. The two species of thyme most often referred to as wild thyme or mother of thyme, are *Thymus serpyllum* and *Thymus praecox* [13].

Essential oil and extract from *Thymus* species can be extracted using a variety of methods. Thyme essential oil can be obtained by steamdistillation and hydrodistillation, while thymeextracts can be obtained by extraction with organic solvents or with supercritical fluid extraction with supercritical carbon dioxide. This paper aims to show the comparison between different extraction procedures reflected in antioxidant activities of one practically unknown wild growing *Thymus* species from Serbia. To the best of our knowledge, there is no data available in the open literature on antioxidant activities of *T. praecox* ssp. *polytrichus.*

MATERIALS AND METHODS

Plant materials and chemicals

Thymus praecox Opiz ssp. *polytrichus* was collected in 2012, in the south of Serbia (the mountain Pasjača) and determinated as *T. balcanus* Borb. by Prof. Dr M. Veljić. According to the Flora Europaea *Thymus balcanus*is described as *Thymus praecox* Opiz subsp. *polytrichus*. The plant material was collected at the flowering stage during June, and dried in the shadow, at the place protected from direct sunlight. The voucher specimen (No. 16881) was deposited in Herbarium of the Institute of Botany and Botanical Garden“Jevremovac” of the Faculty of Biology, University of Belgrade, Serbia (BEOU).

2,2-Diphenyl-1-picrylhydrazyl free radical (DPPH) and dimethylsulphoxide (DMSO) were purchased fromFlukaChemie GmbH (Buchs, Switzerland). Synthetic antioxidants butylatedhydroxyanisole

(BHA) and butylatedhydroxytoluene (BHT) were purchased from Sigma Aldrich, Germany. n-N-hexane and 96% ethanol (Beta Hem, Serbia) were used as solvents for Soxhlet extraction

Isolation of essential oil and extract preparation

The air-dried aerial parts of *T. praecox* ssp.*polytrichus*were ground with a grinder to a particle size of around 0.5 mm. The plant material (50 g) mixed with 500 ml of distilled water and subjected to the hydrodistillationfor 3 h using Clevenger-type apparatus, according to the method for producing oil recommended by the European Pharmacopia[14]. The apparatus consists of a 1000 ml round-bottomed flask, a condenser assembly, Figure 1, closely fitting the flask, and a suitable heating device allowing good control. The obtained essential oil was dried with anhydrous sodium sulphate and stored at +4°C.

Extracts of air-dried and ground leaves and stemof *T. praecox* ssp.*polytrichus*wereprepared by using two solvents with different polarity and theextraction protocol is given below.

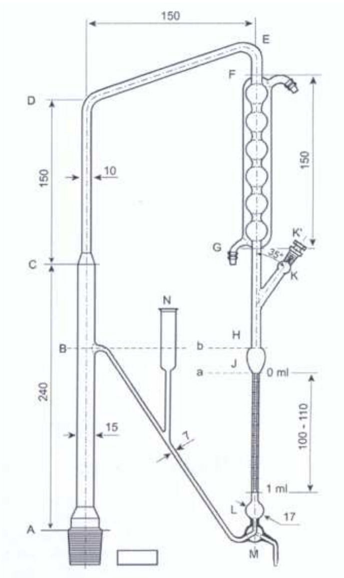

Figure 1.Clevenger apparatus according to the procedure prescribed by the Euro Ph. 6.0.

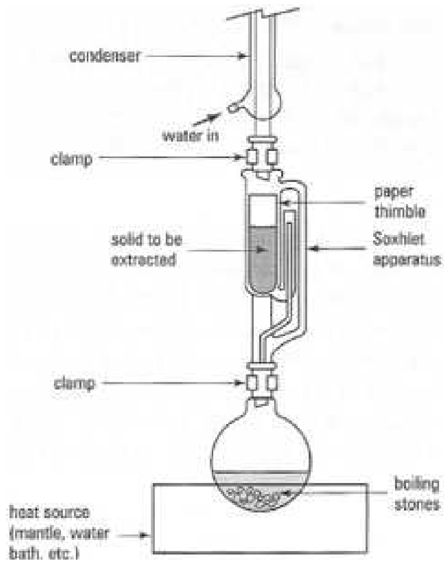

Figure 2. A Soxhlet extraction system

Powdered plant material (15 g) wasplaced in a thimblewhich is placed inside the Soxhlet extractor and extracted with 300 ml n-hexane for 2 h, at a temperature not exceedingthe boiling point of the solvent. The mixture was filtered and then evaporated under reducedpressure using a rotary vacuum evaporator, and then the weight of extract was measured. Similarly, ethanol extract was extracted by Soxhletextraction using 15 g of plant material and 96% ethanol as a solvent with 300 ml ethanol for 6 h. Procedure for obtaining n-hexane/ethanol extract was follow: after Soxhletextraction with n-hexane, the plant materialfrom thimblewas dried for 24 h and 15 g of plant material wassubjected to a Soxhlet extraction with 96% ethanol for7 h, then filtered and evaporated by rotary vacuum evaporator. Extractionwas carried out in duplicate and the results were averaged. These two solvents were chosen becauseof their opposite polarities allowing extracting different kinds ofcompounds (non polar to polar). The extraction yield (Y) was calculated from the formula:

$$Y\ (\%, w/w) = \frac{m_e}{m_{pm}} \cdot 100$$

where, m_e is the mass of the extract and m_{pm} is the mass of the plant material

Evaluation of antioxidant activity

T. praecox ssp. *polytrichus*extracts and essential oilwere tested for thescavenging effect on DPPH radical according to the method of Soler-Rivaset al [16], adapted for 96-well microplates Being rapid,simple and independent of sample polarity, the DPPH assay is veryconvenient for the quick screening of many samples for radicalscavenging activity [17]. As a positive control syntheticantioxidants BHA and BHT were used.Ten microlitres of the examinedextract solutions, in series of different concentrations (0.031-10 mg/mL) and examined essential oil solutionsin series of different concentrations (1.56-100 µL/mL), was added to 100 µl of 90 µmol/L DPPH solution inmethanol, and the mixture was diluted with 190 µLof methanol.In the control, the exact amount of extract/essential oil was substituted with solvent, and in the blank probe, only methanol (290 µL) and extract/essential oil (10 µL) were mixed. Absorption at 515 nmwas measured by the microplate reader (MultiskanSpectrum, Thermo Corporation) after 60 min of incubationat room temperature. The antioxidant activity of the extracts/ essential oil was expressed as concentration of extract/essential oil that inhibited DPPH radical formation by 50% (IC_{50}).

Statistical analysis

Percent of inhibition achieved by differentconcentration of extract/essential oil was calculated by the following equation: $I (\%)=(A_0 - A)/A_0x100$, where A_0 wasthe absorbance of the control reaction and A was the absorbance of theexamined samples, corrected for the value of the blank probe. Corresponding inhibition-concentration curves were drawn using Origin software,version 8.0, and IC_{50} values (concentration of extract that inhibited DPPH formation by 50%) weredetermined. For antioxidant assay,
all of the results were expressed as mean ± SD of three differenttrials).

RESULTS AND DISCUSSION

Yield of essential oil obtained by hydrodistillation was 0.63% w/w. The ethanolic extract has highest yield (10.8 % w/w) followed by n-hexane/ethanol (9.7 % w/w) and then n-hexane (3.6 % w/w). According to the presented results much higher extraction yields were obtained by the solvent extractions with ethanol than with n-hexane due to the lower selectivity of the ethanol compared to that of n-hexane. Literature data on chemical composition of essential oil of *T. praecox* ssp.*polytrichus*are scarce,while for the extracts no literature data. The essential oils from this *Thymus* species collected in the Tyrolean Alps, Macedonia and Bosnia were studied earlier [18-20].

Bischof-Deichnik et al. (2000), in an analysis of samples from 16 sites in the Austrian and Italian Alps, found 12 different essential oil types, with the thymolchemotype being the most frequent[18].Vidic et al. (2010) reported that yield of hydrodistilled oil of *T. praecox* ssp.*polytrichus* from Bosnia was 0.77%, while main components of distilled essential oil were linalool (13.9%) and nerolidol (10.4%)[19]. Kulevanova et al. (1998) reported as the main components trans-caryophyllene (22.42%), β-pinene (12.48%) and α-pinene (5.64%) in essential oil from Macedonian [20]. Variations in the yield and chemical composition of essential oil *T. praecox* ssp. *polytrichus* could be explained in terms of different origin of plant material, quality of herbs, harvest date, influence of different climatic and other factors on the biosynthesis of these secondary metabolites, and diffrent distillation operating conditions.

Thevalues for IC_{50} of the extracts and essential oil are presented in Table 1.A lower IC_{50} indicated a higher antioxidant activity. Essential oil, ethanol and n-hexane/ethanol extract exhibited better antioxidant activity than n-hexane extract which mean that polar extracts exhibited stronger activity than non-polar extract in the case of *T. praecox* ssp. *polytrichus*. Because there are no data on antioxidant activity of *T. praecox* ssp. *polytrichus* essential oil and extract in the open literature, our results could be compared only with results for other *Thymus* species.

In the present study, DPPH radical scavenging capacity ofessential oil and extracts (ethanol extract and n-hexane/ethanol extract) of *T. praecox* ssp.*polytrichus*was found to be greater than thatreported for *T. vulgaris*, *T. fallax*, *T. serpyllum* and*T. linearis* essential oil (IC_{50}:5.84 μg/ml, 215 μg/ml, 34.8μg/ml, 42.9μg/mlrespectively) [21-23], and for hydroalcoholic extracts of *T. pubescens* (IC_{50}: 31.5 μg/mL), *T. kotschyanus* (IC_{50}: 47.22 μg/ml), and *T. daenensis* (IC_{50}: 48.68 μg/mL) [24].

Table 1. IC_{50} values

T. praecox ssp.*polytrichus* essential oil and extracts, BHA and BHT	IC_{50} (μg/ml)
essential oil	0.50 ± 0.01
n-hexane extract	146.5 ± 3.9
n-hexane/ethanol extract	3.64 ± 0.17
ethanol extract	2.32 ± 0.15
BHA	0.97±0.03
BHT	5.58± 0.64

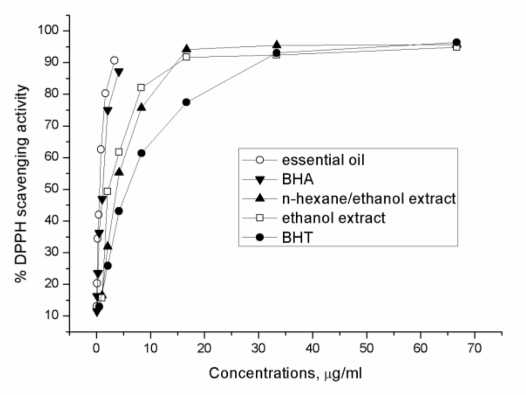

Figure 3.Free radical scavenging capacities of the essential oil and extracts measured in DPPH assay

Also *T. vulgaris*essential oil in some studies [21,23,25] had lowerantioxidant activity than synthetic antioxidantsBHA and BHT, while in this studythe essential oil showed significantly higher antioxidant activity compared with synthetic antioxidants BHA and BHT. The majority of natural antioxidantsare phenolic compounds or polyphenols and the antioxidant activity of many natural extracts and essential oils is due to such phenolic compounds. The antioxidant activity of phenolic compounds is mainly due to their redox properties, simultaneous hydrogen atom donation to free radicals, electron transfer and metal chelating [26]. Lee et al. (2005) showed that the main components dichloromethane extract of *T. vulgaris*, particularly eugenol, thymol and carvacrol, have higher antioxidant activity than synthetic antioxidant BHT and α-tocopherol [27].Other compounds isolated from thyme that showed strong antioxidant activity are caffeic and rosmarinic acid, biphenyl compounds of monoterpenoid origin, flavanones (narirutin, eriodictyol, eriocitrin, hesperidin), methyl rosmarinate [28-30].

CONCLUSION

Thymus species are not only favourite remedies in traditional medicine, but also favourite medicaments in science and conventional medicine.In foods they commonly used mainly for their flavour, aromas and preservation.This investigation showed that the essential oil and extracts of *T. praecox* ssp. *polytrichus* represent a promising alternative to the use of synthetic antioxidantsin the pharmaceutical and food industries. Such natural antioxidants are important in the pharmaceutical and food industries not only because of their usefulness as a preservation methods but also because of their beneficial effects on human health Usage of natural antioxidant prevents different diseases such as diabetes, cancer, atherosclerosis, cardiovascular diseases, inflammatory bowel diseases, skin aging, old age dementia and arthritis. They act as scavengers of reactive oxygen species and metal chelators that protect human cells and reduce oxidative damages.

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