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PHENOLICS CONTENT, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF SOME EXTRACTS OBTAINED FROM ROMANIAN SUMMER SAVORY AND LEBANON WILD THYME

Corina PREDESCU, Camelia PAPUC, Georgeta ŞTEFAN, Carmen PETCU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independenţei Street, 050097, District 5, Bucharest, Romania

Corresponding author email: durduncorina@yahoo.com

Abstract

The aim of the present study was to compare the antioxidant and antibacterial properties of summer savory (Satureja hortensis L.) from Muscel County flora (Romania) and wild thyme (Thymus serpyllum) from Lebanon. The aerial parts of plants were harvested in august, dried quickly and alcoholic extracts were prepared. Total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging activity, ferric reducing antioxidant power (FRAP) value, and antimicrobial properties were determined using the extracts. TPC showed that Lebanon thyme had higher concentration compared with Romanian thyme (13.78 ± 0.95 mg GAE/g DW and 12.14 ± 0.97 mg GAE/g DW, respectively). DPPH• was calculated as IC_{50} and the sample results were compared with gallic acid. FRAP results showed similar values 42.71 ± 4.24 µM Fe^{+2}/g DW (Romanian thyme) and 39.55 ± 4.21 µM Fe^{+2}/g DW (Lebanon thyme). The antibacterial activity of summer savory was found to have maximum effect against Staphylococcus aureus ATCC 9144. Bioactive compounds, measured as total phenolic content, were in higher concentration in both extracts which also relates to their antioxidant and antibacterial activities.

Key words*: Satureja hortensis, Thymus sepyllum, phenolics, flavonoids, antioxidant and antimicrobial activity.*

INTRODUCTION

In the past few years, the food industry producers have tried very hard to change direction toward a clean label. The replacement of synthetic preservatives with phenolic structures, such as butylated hydroxyanisole (BHA), with extracts obtained from aromatic plants rich in natural phenolics, has attracted the attention of the researchers, due to speculation about the possible toxic effects of synthetic antioxidants (Papuc et al., 2010).

Aromatic plants have been studied extensively because they are a rich source of natural antioxidants and antimicrobial substances which can be extracted relatively easily using different solvents. Summer savory and wild thyme, could achieve these demands due to their active compounds found in their extracts (Gedikoğlu et al., 2019). Recent trends for natural food additives made plant extracts with antioxidant and antimicrobial properties an important step to obtain a clean label and proved beneficial food products for the consumers (El-Guendouz et al., 2019; Gonelimali et al., 2018). It was reported that the antioxidant and antimicrobial effects of aromatic plants are closely related to the presence of phenolic compounds (Kulisic et al., 2005). *Satureja hortensis* L. (summer savory, thyme), member of *Lamiaceae* family, is a variety of an annual herbaceous crop species, flowering shrubs, found in many parts of the world, native from the western Mediterranean to southern Europe. Growing up to 30 cm tall, by 40 cm wide, it is a bushy evergreen subshrub with small, aromatic, grey-green leaves and clusters of purple or pink flowers in summer (Fierascu et al., 2018). Summer savory is known especially as aromatic herb and have an intense culinary use. In folk medicine, these herbs are used against headaches, toothaches, colds, asthma, and rheumatism (Gedikoğlu et al., 2019).

Thymus serpyllum is a perennial shrub, known as Breckland thyme, wild thyme, or creeping thyme; however, its specific name "*serpyllum*" is derived from the Greek word meaning "*to creep*", because of wild thyme's trailing habit. It is a species of flowering plant in the mint family *Lamiaceae*, native to regions of Europe, Asia and North Africa. It is a low, subshrub

growing to 2 cm tall with creeping stems up to 10 cm long. The oval evergreen leaves are 3–8 mm long. The strongly scented flowers are either lilac, pink-purple, magenta, or a rare white, all 4-6 mm long and produced in clusters. The hardy plant tolerates some pedestrian traffic and produces odours ranging from heavily herbal to lightly lemon, depending on the variety. It has high tolerance for low water and poor nutrient soils. The increase of pathogenic microorganism's multidrug resistant has led to extensive phytochemical and pharmacological studies of *T. serpyllum* as an important source of medicinal substances with antioxidant and antimicrobial properties (Jarić et al., 2015).

Based on *in vitro* tests, Uysal et al. (2015) reported that their chemical constituents, such as phenols and flavonoids, provide antimicrobial and antioxidant properties.

The ***objective*** of this research was to evaluate the antioxidant and antimicrobial activities of *Satureja hortensis* L. (summer savory), harvested from Muscel County, Romania and *Thymus serpyllum* (wild thyme) growing in Lebanon using a multiple-method approach in relation to their chemical composition, comparatively with synthetic antioxidants used in food industry.

MATERIALS AND METHODS

Reagents and chemicals. Spectrophotometric grade ethanol, 2,4,6 three(2-pyridyl)-S-triazine (TPTZ) reagent $AlCl_3$ anhydrous, sodium nitrite, sodium hydroxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium bicarbonate anhydrous, gallic acid, catechin and Folin - Ciocalteu reagent were supplied by Sigma Aldrich (Germany). Iron (III) chloride hydrate and ferrous sulfate ($FeSO_4 \cdot 7H_2O$) were purchased from Fisher Scientific (UK). Tryptic soy agar, tryptic soy broth, and Muller-Hinton agar were supplied by Merck (Germany). Antimicrobial susceptibility test disks were purchased from Oxoid (UK).

Hydroalcoholic extract preparation. Fresh plant parts (flower, leaves and stems) of both *Satureja hortensis* L. (summer savory) from Muscel County, Romania and *Thymus serpyllum* (wild thyme) growing in Lebanon were collected. Samples were washed, dried and powdered and stored at room temperature, in darkness. For extraction, ten grams of each sample were weighed into a beaker and 100 ml of 60% aqueous ethanol (1:10 ratio of w/v) was added. After 30 min the sample was placed in water bath for 3 hours at 60°C. Next, the homogenates were filtered using Whatman no. 1 filter paper. The two filtrates were placed in 250 ml round-bottom flasks.

Total phenolic content. To determine the total polyphenol content, 0.5 ml of the sample extract and 7 ml of distilled water were mixed with 0.5 ml of Folin - Ciocalteu's reagent. After 5 min, 2 ml of 20% Na_2CO_3 solution was added, and the mixture was incubated for 60 min in the dark. The reaction mixture absorbance was measured at 760 nm, and the reaction mixture without the extract was used as a blank. Gallic acid was used as standard, and a 5 points standard curve was prepared (0-10 mg/dl). The TPC of the plant extract was expressed as gallic acid equivalents/g dry weight plant (mg GAE/g DW) (Singleton and Rosi, 1965).

Total flavonoid content. The total flavonoid content was determined according to the method of Zhishen et al., 1999. 1 ml of extract was placed in a ten ml flask that contained 5 ml distillate water. Then, 0.3 ml of 5% $NaNO_2$ solution were added. After 5 minutes 0.6 ml of 10% $AlCl_3$ were added and after another 5 minutes, 2 ml of 1 M NaOH solution were added and the volume was brought to ten ml with distilled water. The mixture was left 15 minutes at room temperature and the absorbance was read at 510 nm. The results were expressed as mg catechin equivalent/g dry weight plant (mg CAT/g DW).

DPPH radical scavenging activity. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity was measured according to Blois (1958). Next, 50 µl plant extract (at different concentrations in methanol) was mixed with 5 ml of a 0.004% (w/v) DPPH▪ methanol solution. The reaction was allowed to stand at room temperature for 30 min, and absorbance was read against a blank at 517 nm. The inhibitions of the DPPH radical in percent were calculated as follows:

$$I\,(\%) = \left(\frac{A\ blank\ -\ A\ sample}{A\ blank}\right) \times\ 100$$

Where, A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts. Extract concentration providing 50% inhibition (IC_{50}) was calculated using the graph ‑ plotted inhibition percentage against the extract concentration. Gallic acid and butylated hydroxy anisole (BHA) were used as positive controls in concentration of 10 mg/100 ml. The IC_{50} value for each sample was determined graphically by plotting the percentage discoloration of DPPH• solution as a function of the sample concentration.

The ferric reducing antioxidant power (FRAP) FRAP assay is based on the ability of phenolics to reduce yellow ferric tripyridyl triazine complex (Fe(III)-TPTZ) to blue ferrous complex (Fe(II)-TPTZ) by the action of electron-donating antioxidants (Riahi et al., 2013). The FRAP reagent was freshly prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM TPTZ in 40 mM HCl), and $FeCl_3 \cdot 6H_2O$ (20 mM) in a ratio of 10:1:1 (v/v/v). To carry out the assay, 1.8 mL of FRAP reagent, 180 µl distilled water, and 20 µl of plant extract were mixed. After 15 min at 37°C, the absorbance was measured at 595 nm, using the FRAP solution as a blank. The antioxidant capacity of plant extracts was determined from a standard curve plotted using the $FeSO_4 \cdot 7H_2O$ linear regression. Results were expressed as µmols Fe^{2+}/g DW. BHA and ascorbic acid were used as controls in concentration of 10 mg/100 ml.

Antimicrobial activity. Two Gram-positive and three Gram-negative bacteria were used as test organisms: *Staphylococcus aureus* ATCC 9144, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076, and *Salmonella typhimurium* ATCC 14028.

Disk diffusion assay (DDA) (Oke et al., 2009). All the bacterial species were first inoculated into tryptic soy agar and incubated overnight at 37°C. After checking for purity, the bacteria were suspended in a 0.9% NaCl solution. A spectrophotometer was used to adjust the final cell concentration (1.5×10^8 cfu/ml) by reading the DO at 600 nm. Then, 100 µl of the bacterial suspensions was spread on Mueller-Hinton agar. The 6-mm-diameter, sterile, empty disks impregnated with 20 µl of extracts were placed on the inoculated agar. Antibiotic standard disks were used as a control. The inoculated plates were incubated at 37°C for 24 h. As positive controls, ciprofloxacin and Ampicillin (30 µg/disk) were used for bacterial strains. Antibacterial activity was determined by measuring the *zone of inhibition* in mm without including the diameter of the disk (Valgas et al., 2007).

RESULTS AND DISCUSSIONS

Total phenolic and flavonoid content. Plants from *Lamiaceae* family are known to be rich in compounds possessing strong antioxidant activity. Thyme and wild thyme, are found in many parts of the world, especially in the Mediterranean region, are also regarded as medicinal herbs and condiments. Because of the highest concentration in active compounds, as phenols and flavonoids, thyme has positive effect on health of the consumers, when it is used as tea or added in food. In fact, the food products that contain natural antioxidants have a double benefit: better conservation during the viability period and the excess of antioxidants get to the consumers.

Table 1. Total phenolic compounds and total flavonoid content in hydroalcoholic extracts of Romanian and Lebanon wild thyme

Analysed plant	Total phenolic content (TPC) mg GAE/g DW	Total flavonoid content (TFC) mg CE/g DW
Lebanon wild thyme	13.78 ± 0.95	8.54 ± 0.84
Summer savory	12.14 ± 0.97	7.49 ± 0.79

Values are the average of duplicates ± standard deviation.

The concentration of phenols in plants is dependent on climate and geographical position (Liu et al., 2018). *Satureja hortensis* L. (summer savory) from Muscel County, Romania and *Thymus serpyllum* (wild thyme) growing in Lebanon contain important concentrations of phenols and flavonoids. These findings are similar with other results regarding *Satureja hortensis* L. and *Thymus serpyllum* phenol and flavonoid contents (Ballester−Costa et al., 2017; Plánder et al., 2012; Kulisic et al., 2005). Lebanon wild thyme extract have shown that contains a high content of phenolic 13.78 ± 0.95 mg GAE/g DW and flavonoid 8.54 ± 0.84 mg CE/g DW

compounds when compare to Romanian thyme which had 12.14 ± 0.97 mg GAE/g DW and 7.49 ± 0.79 mg CE/g DW (Table 1). It was calculated that for both types of thyme, about 60% of the phenols were flavonoids. It was also calculated that the Lebanese wild thyme had 14% more polyphenols than the Romanian one. Also, the concentration of flavonoids was lower for the Romanian thyme compared to the Lebanese one with 14%. Statistically significant difference and positive correlation between the concentration of phenol and flavonoid contents of Romanian and Lebanese thyme ($p < 0.05$, $R^2 = 0.9999$).

DPPH radical scavenging activity. DPPH (2,2 - diphenyl - 1 - picrylhydrazyl) free radical scavenging activity was used to investigate the antioxidant activity of two thyme extract by comparation with gallic acid. The effect of antioxidants on DPPH radical scavenging is due to their ability to donate hydrogen. DPPH▪ is a stable free radical which accepts a hydrogen radical from an antioxidant molecule (AH) to become a stable diamagnetic molecule, in accordance with the equation below:

$$DPPH▪ + AH \rightarrow DPPH▪▪H + A▪$$

The concentration of antioxidant needed to decrease the initial DPPH concentration by 50% (IC_{50}) is a parameter widely used to measure the antioxidant activity (Brighente et al., 2007).

The free radical scavenging activity is higher when IC_{50} value is lower. The amount of extract needed to decrease the initial radical DPPH concentration by 50% is used for the free radical scavenging activity and is established as IC_{50}. Results of the DPPH radical scavenging activity test are shown in Table 2. IC_{50} of Lebanon thyme extract (85.25 ± 7.31 µg/ml) was significantly ($p < 0.05$) higher than that found for Romanian thyme extract (102.94 ± 8.14 µg/ml) radical scavenging activity when compared to BHA.

Some researchers reported that the phenolic compounds with hydroxyl groups attached to their structures present in aromatic plants are responsible for the important antioxidant effect (Shahidi et al., 1992). When comparing the sample results with the standard gallic acid (4 ± 0.35 µg/ml), the extracts in the current study had a much lower free radical scavenging activity. A significantly higher correlation was established between total flavonoid content and DPPH radical scavenging activity ($p<0.05$). The results indicated that both Romanian and Lebanon thyme extract have effective DPPH radical scavenging activities. It was observed that Lebanon wild thyme extract had the most effective DPPH radical scavenging activity.

Table 2. DPPH radical scavenging activity of hydroalcoholic extracts of Romanian and Lebanon wild thyme

Sample	IC_{50} value of DPPH (µg/ml)
Lebanon wild thyme extract	15.25 ± 1.31
Summer savory extract	17.94 ± 2.14
Gallic acid 10 µg/100 ml	4.51 ± 0.35
BHA 10 µg/100 ml	14.84 ± 1.57

Values are the average of triplicates ± standard deviation.

The extract of Lebanon thyme, with significant DPPH • scavenging activity, also had a higher quantity of total phenolics. This extract, which have a high antioxidant activity, also had a great quantity of flavonoids, as summarized in Table 1.

The ferric reducing antioxidant power (FRAP)

Prior et al. (2005) found out that FRAP mechanism is based on electron transfer rather than hydrogen atom transfer. The basis of FRAP assay is the ability of antioxidant compounds to reduce Fe^{3+} to Fe^{2+}.

The FRAP reaction is taking place in acidic medium (pH value equal to 3.6) in order to maintain iron solubility. The reaction mechanism is based on decreasing of ionization potential at low pH that drives hydrogen atom transfer and increases the redox potential. In the presence of 2,4,6-trypyridyl-s-triazine the reduction of Fe^{3+} to Fe^{2+} occurs. The reduction reaction is accompanied by the formation of a blue complex with Fe^{2+} with a maximum absorption at 593 nm. The reducing power appears to be related to the degree of hydroxylation and extent of conjugation in antioxidant compound (Huang et al., 2005). The results of evaluation of Ferric reducing antioxidant power (FRAP) are shown in Table 3.

Antioxidative activity of examined plant extracts measured with FRAP method bring more information regarding benefic effects of them. Results of the evaluations presented as

µmols Fe^{2+}/g DW, showed increasing activity with phenol extract's concentration. Highest FRAP values were found for ascorbic acid (54.14 ± 5.61 µmols Fe^{2+}/g DW) and in sample of Lebanon thyme extract (42.71 ± 4.24 µmols Fe^{2+}/g DW) and Romanian thyme extract (39.55 ± 4.21 µmols Fe^{2+}/g DW). Also, high activity showed BHA (34.12 ± 3.42 µmols Fe^{2+}/g DW). Similar results were observed by Gedikoğlu et al. (2019) and Birasuren et al. (2013). As the result of the FRAP analysis herbs extracts and standards were ranked as follows: BHA < Romanian thyme extract < Lebanon wild thyme extract < ascorbic acid.

Statistical analysis of relationships between ferric reducing antioxidant power of ethanol herbs extracts and total polyphenol content showed high correlations (R^2 = 0.9999, $p < 0.05$). Also, a significantly higher correlation was established between FRAP and DPPH radical scavenging activity ($p < 0.05$).

Table 3. Ferric reducing antioxidant power (FRAP) value of hydroalcoholic extracts of Romanian and Lebanon thyme

Sample	FRAP (µmols Fe^{2+}/g DW)
Lebanon wild thyme extract	42.71 ± 4.24
Summer savory extract	39.55 ± 4.21
Ascorbic acid (10 µg/100 ml)	54.14 ± 5.61
BHA (10 µg/100 ml)	34.12 ± 3.42

Values are the average of duplicates ± standard deviation.

Khosh-Khui et al. (2012) find out that water deficiency might increase antioxidants levels depending on plant genotypes and this can explain why Lebanon wild thyme extract showed higher antioxidant activity.

Antimicrobial activity. Two Gram-positive bacteria (*Staphylococcus aureus* ATCC 9144, *Staphylococcus epidermidis* ATCC 12228) and three Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076, and *Salmonella typhimurium* ATCC 14028) were used to test antimicrobial activity of Romanian thyme extract and Lebanon thyme extract. Table 4 present the antimicrobial activities of thyme extracts and BHA against various organisms. The two extracts possessed antimicrobial activity against all tested bacteria, but the highest activity was showed by Lebanon wild thyme extract. BHA showed the lowest antimicrobial activity. This result regarding the antimicrobial activity of synthetic antioxidant were similar with other researches (Gavarić et al., 2015).

Thyme extracts are known to possess some antimicrobial activities and are used in various food preparations as flavour enhancers (Nzeako et al., 2006). Even the antimicrobial pathways are not fully known and understood, it looks that its action is due to the compounds present in the thyme extract (phenols and flavonoids among the others) (Gavarić et al., 2015). The synergistic effects of different compounds present into extracts can contribute to the antimicrobial activity through different mechanisms. Different extract compounds can interfere with bacterial membrane and thereby increase the cell leakage or act indirectly antimicrobial by facilitating the influx of antimicrobial phenolic compounds (Burt, 2004). For this reason, it is recommended to use plant extracts than pure compounds (Burt, 2004).

Table 4. Antimicrobial activity of hydroalcoholic extracts of BHA and Romanian and Lebanon wild thyme

Sample / Microorganism	Lebanon wild thyme extract	Summer savory extract	BHA (10 µg/100 ml)
	Zone of inhibition in mm		
Staphylococcus aureus	27.5 ± 3.1	28.1 ± 2.1	13.6 ±0.9
Staphylococcus epidermidis	24.1 ± 1.5	23.3 ± 1.9	14.1±1.0
Escherichia coli	25.2 ± 1.4	25.6 ± 2.1	11.2 ±0.9
Salmonella enteritidis	18.4 ± 1.1	16.4 ± 1.5	14.2 ±1.2
Salmonella typhimurium	13.2±1.1	11.0 ± 1.1	6.±0.7

Values are the average of triplicates ± standard deviation.

CONCLUSIONS

Both hydroalcoholic extracts were found to contain a noticeable amount of phenolics and flavonoids. Phenolics and flavonoids, may be the compounds responsible their antioxidant and antimicrobial activities in these plants extracts. The thyme extracts contain compounds with antioxidant activity that act as hydrogen/electron donors. Romanian thyme extract and Lebanon wild thyme extracts showed strong DPPH radical scavenging activity and strong ferric reducing antioxidant power when compared with standard. The results of this study show that the extract possessed antimicrobial activity against the tested bacteria and can be used as an easily accessible source of natural antibiotic. In

addition, the extracts of these plants can be regarded as plant-derived antioxidant and antimicrobial mixture in different fields (foods, cosmetics, pharmaceuticals).

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