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Antioxidant Activity and Phenolic Profile of Extracts of Basil

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Running title: **Antioxidants in Basil**

Abstract
Nowadays, there is a growing interest in natural extracts, especially with plant origin because of their strong antioxidant potential and absence of toxicity. The presence of polyphenols in medicinal and aromatic plants increases significantly their antioxidant and other biological activities. So the aim of current study was to investigate the antioxidant activity and phenolic profile of different extracts (water, ethanol: water (70:30, v/v) and ethanol: water (96:4, v/v) of commercially available basil. Provided HPLC analysis of phenolic acids revealed that the rosmarinic acid is the major compound in the investigated extracts (3.76 -13.59 mg/g extract), followed by *p*-coumaric (0.84-3.13 mg/g extract) and chlorogenic (0.63-1.67 mg/g extract) acids. Antioxidant activity of the obtained extracts was determined by four spectrophotometric methods differ in mechanism and reaction conditions, namely DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2`azinobis(3)-ethylbenozthiazoline-6-sulfonic acid), FRAP (ferric reducing antioxidant power) and CUPRAC (cupric reducing antioxidant capacity). The highest antioxidant activity, defined by all tested methods, represented as mM TE/g dry weight (DW) was determined in the water extracts (162.16 mM TE/g DW, 188.36 mM TE/g DW, 141.69 mM TE/g DW, 238.83 mM TE/g DW, respectively) as for mM TE/g extract – in the 96% ethanol extract (492.22 mM TE/g extract, 514.16 mM TE/g extract, 643.63 mM TE/g extract, 1051.78 mM TE/g extract).

Practical applications
The defined high quantities of phenolic acids in the water and water-ethanol extracts of commercially available basil made them valuable sources of these biologically active substances. The observed strong antioxidant activity of investigated extracts is a base for their possibly application as antioxidants in different food systems.

Key words: antioxidants, HPLC, basil, DPPH, ABTS, FRAP, CUPRAC

Introduction

Antioxidants are compounds that have an important application against oxidative damages in food systems and free radical induced oxidative stress-associated diseases in humans, such as diabetes, cardiovascular diseases, various cancers and aging processes (Patil et al., 2011; Shafique et al., 2011). In the recent years, investigations of the natural antioxidants intensified because of the proved toxic, mutagenic and carcinogenic effects of commercial synthetic ones (Dabija et al., 2011; Kaurinovic et al., 2011). Good sources of antioxidants are herbs and spices with an antioxidant capacity superior to that of many fruits and cereals (Dabija et al., 2011). Concentrated in just a few grams of material, they may represent the simplest way to increase the antioxidant capacity of a daily diet with health benefits (Suhaj, 2006). Usually, the antioxidant potential of the plant extracts was related to their polyphenolic compounds with strong redox properties (absorbed and neutralized free radicals, quenched singlet and triplet oxygen or decomposed peroxides) (Benedec et al., 2012).

Ocimum basilicum L. (sweet basil) belonging to the *Lamiaceace* family is a "bridge" between medicine, food and tradition. Basil contains plenty of phytochemicals with significant nutritional, as well as antioxidant capabilities and health benefits (Yayasinghe et al., 2003; Dabija et al., 2011). Sweet basil is cultivated for production of essential oils, dry leaves either as a spice or as an ornamental plant. It is used in culinary for flavoring and seasoning of various dishes (salads, sausages, fish dishes), especially in the Mediterranean and Asian cuisine (Dabija et al., 2011). Leaves and flowering parts of *O. basilicum* are traditionally used in medicine against headache, cough, diarrhea, constipation, stomach ulcer, as antispasmodic, carminative, digestive, galactogogue and tonic agents (Zheljazkov et al., 2007; Adiguzell et al., 2005).

Among the factors leading to differences in the composition and antioxidant activity of plant extracts, may be mentioned: the genetic factors, the degree of maturity of plants, cultivation techniques, post-harvest handling, storage conditions and solvents and conditions applied for extractions (Dabija et al., 2011).Therefore the purpose of current study is comparative analysis of antioxidant activity and phenolic compounds in different extracts (water, 70% and 96% ethanol (v/v) extracts) of commercially available basil for seasoning.

Materials and Methods

Materials

Chemicals and reagents

All chemicals and solvents used in the study were of analytical grade. DPPH (1, 1-Diphenyl-2-Picryl Hydrazyl), ABTS (2,2`azinobis (3)-ethylbenzthiazoline-6-sulfonic acid), $CuCl_2.2H_2O$, Neocuproine, ammonium acetate, Trolox, acetic acid, acetonitrile were obtained from Sigma-Aldrich (St.louis, Mo, USA). Potassium persulfate, HCl, $FeCl_3.6H_2O$ were purchased from Merck-Milipore (Germany).

Plant material

The sample of basil is produced from "Bioset" Ltd. Plovdiv and selled as dried and cutted spice.

Extraction procedure

Each plant sample (1.0 g) was extracted three times with 10 cm^3 of the relevant extraction solvent (96% ethanol, 70% ethanol (v/v) and distilled water) under reflux-heat at 70 ºC for 20 min. The residue of plant material was removed through filter paper filtration and the combined extracts were evaporated to dryiness under vacuum. The dried extracts were stored in refregirator at 4 ºC in dark and used for the next analyses. Before analysis the dried extracts were dissolved in the relevent solvent with appropriate dilutions.

HPLC analysis

Qualitative and quantitative determination of phenolic acids was performed by using Elite LaChrome (Hitachi) HPLC system equipped with DAD and ELITE LaCHrome (Hitachi) software. Separation of the phenolic acids was performed by Supelco Discovery HS C_{18} column (5 µm, 25 cm × 4.6 mm), operated at 30 ºC under gradient conditions

with mobile phase consist of 2% (v/v) acetic acid (solvent A) and acetonitrile (solvent B). The gradient program used is described in Table 1.

Table 1. Gradient program used for HPLC analyses.

Time, min	**0**	**1**	**40**	**45**	**46**	**50**
Solvent A, % (2 % acetic acid)	95	95	50	0	95	95
Solvent B, % (acetonitrile)	5	5	50	100	5	5
Flow rate, mL/min	0.8	0.8	0.8	0.8	0.8	0.8

Chlorogenic, caffeic, ferulic, p-coumaric, rosmarinic and cinammic acids (Sigma) were used for creation of standard calibration curves with linearity range of 10-100 μg/cm^3. The detection of componds was carried out at 280 and 320 nm and the flow rate was 0. 8 ml/min.

Analysis of antioxidant activity

Antioxidant activity was determined by DPPH, ABTS, FRAP and CUPRAC methods modifired and described previously by Ivanov et al., 2014.

In brief, each analyzed extract (0.15 cm^3) was mixed with 2.85 cm^3 freshly prepared 0.1 mM solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) in methanol. The reaction was at 37 °C in darkness and the absorbance at 517 nm were recorded after 15 min against methanol.

ABTS radical was generated by mixing aliquot parts of 7.0 mM 2, 2`azinobis (3)-ethylbenzthiazoline-6-sulfonic acid (ABTS) in distilled H_2O and 2.45 mM potassium persulfate in distilled H_2O. The reaction was performed for 16 h at ambient temperature in darkness and the generated ABTS radical is stable for several days. Before analyses, 2.0 cm^3 of generated $ABTS^{\cdot+}$ solution was dilutedwith methanol at proportions 1:30 (v/v), so the obtained final absorbance of the working solution was about 1.0 ÷ 1.1 at 734 nm. For the assay, 2.85 cm^3 of this $ABTS^{\cdot+}$ solution was mixed with 0.15 mL of obtained extracts. After 15 min at 37 °C in darkness the absorbance was measured at 734 nm against methanol.

The FRAP reagent was freshly prepared before analyzes by mixing 10 parts 0.3 M acetate buffer (pH 3.6), 1 part 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ, Fluka) in 40 mM HCl (Merck) and 1 part 20 mM $FeCl_3.6H_2O$ (Merck) in distilled H_2O. The reaction was started by mixing 3.0 cm^3 FRAP reagent with 0.1 cm^3 of investigated extract. Blank sample, prepared with ethanol instead of extract was developed as well. The reaction time was 10 min at 37 °C in darkness and the absorbance at 593 nm of sample against blank was recorded.

For CUPRAC method reaction was started by mixing 1.0 cm^3 10 mM $CuCl_2.2H_2O$ (Sigma) in distilled H_2O, 1.0 cm^3 7.5 mM Neocuproine (Sigma) in methanol, 1.0 cm^3 0.1 M ammonium acetate buffer (pH 7.0), 0.1 cm^3 of investigated extract and 1.0 cm^3 distilled H_2O. Blank sample, with ethanol instead of extract was developed as well. The reaction was carried out for 20 min at 50 °C in darkness and the sample absorption at 450 nm was recorded against the blank.

The antioxidant activity defined by all of the tested methods was expressed as mM Trolox equivalents (TE) per g dry weight (DW) and g extract by using calibration curve, build in range of 0.05-0.5 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) dissolved in methanol.

Statistical analysis

Three independent extracts with the relevent solvent (96% ethanol, 70% ethanol (v/v) and distilled water) were prepared from analyzed sample of basil and each extract was analyzed for phenolic content and antioxidant activities in triple replication. The presented values are means (n = 3) with standard deviations (± SD). Figures were made by Microsoft Office Excel® 2010.

Results

HPLC analysis of phenol profile of basil extracts

HPLC analysis of phenolic acids of basil extracts revealed the presence of chlorogenic, caffeic, *p*-coumaric, rosmarinic and cinammic acid (Table 2).

The obtained data indicated that the rosmarinic acid was the major phenolic compound (between 1561.18 ± 8.14 and 2073.08 ± 9.23 μg/g DW) in all of the analyzed extracts, as the highest content was defined in the 70% ethanol extract. The highest quantities of the other identified phenolic acids were determined in the water extract, as ferulic acid was identified only in this extract of basil.

The quantities of identified phenolic acids were presented as mg per g extract (mg/g extract), as well (Table 3).

The highest amount of *p*-coumaric and cinammic acids was defined in the water extract, as rosmarinic acid was in the gratest quantity in 96% ethanol extract of basil. Water and 96% ethanol extracts contained similar quantities of chlorogenic and caffeic acids.

Analysis of antioxidant activities of basil extracts

Antioxidant activities of the three investigated extract were defined by DPPH, ABTS, FRAP and CUPRAC methods. The results were expressed as mM TE/ g DW and mM TE/ g extract (Figure 1-4). All of the analyzed extracts exhibited antioxidant potential, as the highest antioxidant activity signified as mM TE/g DW was defined in the water basil extract. On the other hand, the 96% ethanol extract was with the highest antioxidant potential, represented as mM TE/ g extract.

Discussion

Phenolic compounds are the most abundat plant secondury metabolites, that have been shown to possess multiple pharmacological activities (Kaurinovic et al., 2011). HPLC analyses of the phenolic profile of the obtained basil extracts revealed that the rosmarinic acid is the major phenolic compound (Table 2 and Table 3). That is of great importane because of its valuable biological activities, such as antiviral, antibacterial, anti-inflammatory, strong antioxidant, anti-allergic and anticancer activities (Makri and Kintzios, 2004; Osakabe et al., 2004; Chun et al., 2005; Yoshida et al., 2005). In plants, this molecule is supposed to act as a constitutively accumulated defense compound against pathogens and herbivores due to its tannin-like properties (Szabo et al. 1999).

Kruma et al., (2008) also found that rosmarinic acid is the main compound in the methanol extract of commercial (17.94 mg/100 g DW) and cultivated basil (12.18 mg/100 g DW). These quantities are similar with the defined content of rosmarinic acid in the 96 % and 70 % ethanol extracts in our study.

Besides, the obvious influence of the solvent (96% ethanol, 70% ethanol, water) on the amount of extracted phenolic acid was observed. The highest quantity of rosmarinic acid was detemind in the 96 % ethanol extract (13.59 ± 0.05 mg/g extract), followed by 70% ethanol and water extracts. These results are in acordance with other studies that determined methanol and ethanol as better extragents for rosmarinic acid (Kruma et al., 2008; Park, 2011).

The other identified phenolic acids in the obtained basil extracts were in lower cincentrations, but also possessed valuable biological activities. Caffeic and cinammic acids have been reported to possess anticancer, anti-inflammatory, strong antioxidant, antimicrobial and antiviral activities (Gomes et al., 2003; Chung et al., 2004; Wang et al., 2009; Guzman, 2014). Ferulic acid is widely distributed in the plant kingdom and has a wide range of potential therapeutic effects useful in the treatments of cancer, diabetes, lung and cardiovascular diseases, as well as hepatic, neuro and photoprotective effects, antimicrobial and anti-inflammatory activities (Brenelli de Paiva al., 2013). Chlorogenic acid also has been shown to present multiple beneficial properties, including analgesic, anti-carcinogenic, anti-diabetic, anti-inflammatory, anti-microbial, anti-obesity, cardioprotective, hypotensive and neuroprotective effects (Plazas et al., 2014). Therefore the basil is good source of valuable phenolic acids with health benefits for humans.

The high quantity of phenolic acids in the obtained basil extracts could be considered as prerequisite for expected high antioxidant activities.

Thus the antioxidant potential of received basil extracts was defind by four most applyed *in vitro* spectrophotometric methods (DPPH, ABTS, FRAP and CUPRAC), differ in reaction mechanism and conditions.

All of the analysed extracts showed the ability to quench free radicals, such as DPPH and ABTS by hydrogen and/or single electron transfer (HAT and/or SET mechanism). This capability was most expressed by the compounds extracted with water (Figure 1 A, B) and 96% ethanol (Figure 2 A, B).

The reduction ability of the obtained extracts was defined by FRAP and CUPRAC methods. The highest potential to donate single electron, expressed as mM TE/g DW was determined in water extract (Figure 3A and 4A), as for mM TE/g extract in 96 % ethanol extract (Figure 3B and 4B).

The observed highest antioxidant potential (represented as mM TE/g extract) of 96% ethanol extract defined by all tested methods was due to the lowest amount of extract (131.5 mg/g DW) obtained from 1 g of dried weight of basil, compared to the water (413,0 mg/g DW) and 70% ethanol extarct (214.6 mg/g DW). Besides, the 96% ethanol extract was the extract with highest amount of rosmarinic acid - 13.59 ± 0.05 mg/g extract (Table 3). This phenolic acid is known as the main component in *Lamiaceae* plants with a strong antioxidant activity and thus, the observed antioxidant properties of the plants of this family could depend strongly on its amount (Peng et al. 2005). Redox properties of rosmarinic acid have an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides Among other factors, antioxidant activity of polyphenols is attributed to their hydroxyl groups. Ferulic acid has one hydroxyl group, caffeic acid contains two, while rosmarinic acid posseses four of them (ortho-dihydroxybenzene/catechol structure), (Furtado et al., 2008).

Therefore the observed high antioxidant activity of basil extracts could be associated with the presence of rosmarinic acid, as well as with synergism of others identified phenolic acids.

Patil et al., (2011) also defined that 96% ethanol extract (0.3 mg/ml) of Indian population of basil had highest antioxidant activity determind by DPPH method (45% of inhibition). Dabija et al., (2011) established that the compound extracted with 96%

ethanol possessed the highest ability to scavenge DPPH radical compared to those in the water, methanol and a mixture of methanol-acetone-water-formic acid extracts.

Conclusions

The defined high quantities of phenolic acids in the water and water-ethanol extracts of commercially available basil made them valuable sources of these biologically active substances. The observed strong antioxidant activity of investigated extracts is a base for their possibly application as antioxidants in different food systems.

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Table 2. HPLC analysis of phenolic acids of basil extracts, represented as µg/g DW.

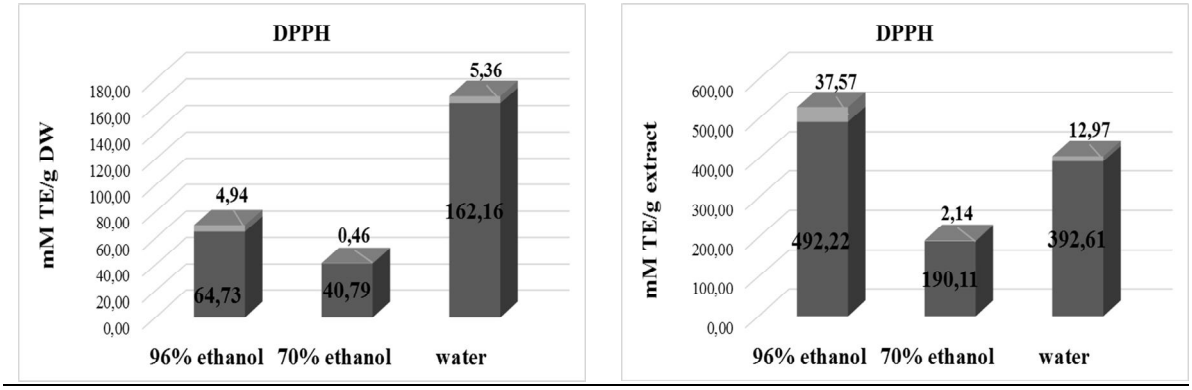
Extract / Phenolic acid*	96 % ethanol	70 % ethanol	water
Chlorogenic acid	219.60 ± 4.71	135.70 ± 4.18	675.70± 5.46
Caffeic acid	65.46 ± 3.12	39.84 ± 2.23	234.20 ± 2.89
Ferulic acid	nd	nd	753.76 ± 5.88
***p*-coumaric acid**	126.68 ± 4.24	182.15 ± 4.68	1297.26 ± 7.85
Rosmarinic acid	1786.74 ± 7.18	2073.08 ± 9.23	1561.18 ± 8.14
Cinammic acid	34.98 ± 2.06	11.57 ± 0.91	237.40 ± 2.52

*- µg/g DW; nd – not identified

Table 3. HPLC analysis of phenolic acids of basil extracts, represented as mg/g extrcat.

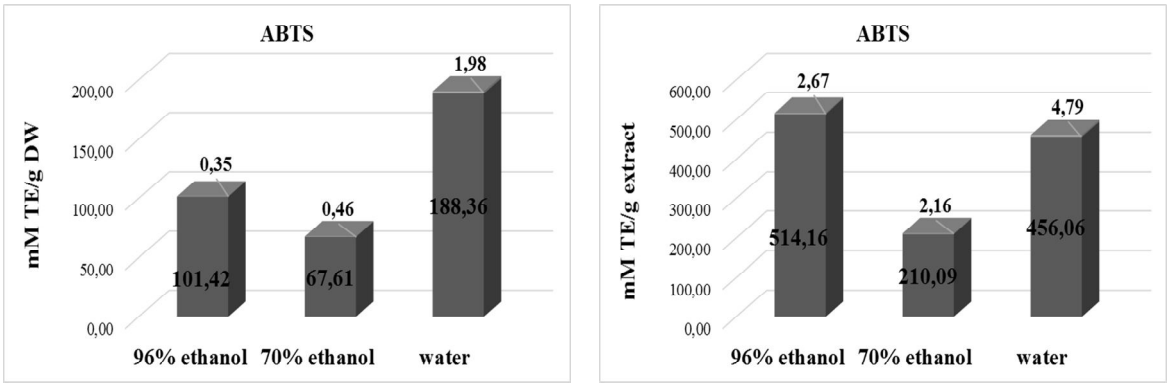
Extract / Phenolic acid*	96 % ethanol	70 % ethanol	water
Chlorogenic acid	1.67 ± 0.03	0.63 ± 0.02	1.63 ± 0.01
Caffeic acid	0.50 ± 0.02	0.18 ± 0.01	0.56 ± 0.01
Ferulic acid	nd	nd	1.82 ± 0.02
***p*-coumaric acid**	0.96 ± 0.03	0.84 ± 0.02	3.13 ± 0.02
Rosmarinic acid	13.59 ± 0.05	9.58 ± 0.04	3.76 ± 0.02
Cinammic acids	0.27 ± 0.01	0.06 ± 0.01	0.57 ± 0.01

*- mg/g extract; nd – not identified



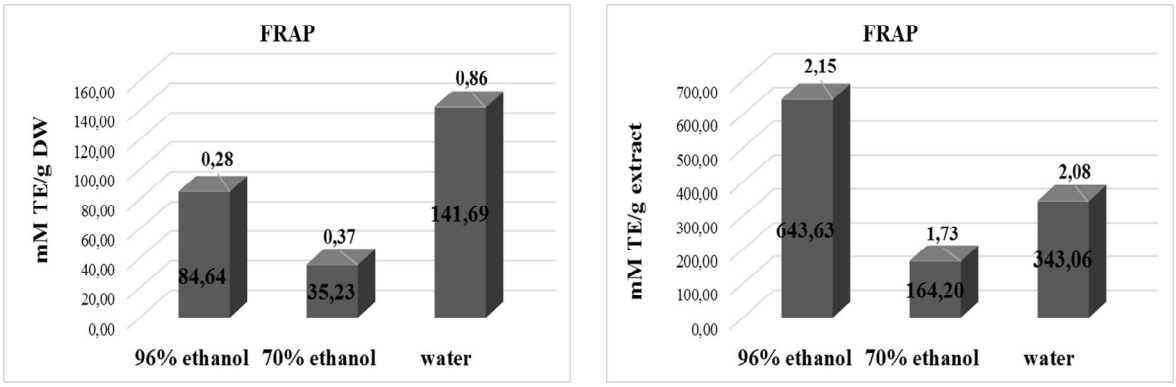
A **B**

Figure 1. Antioxidant activity of different basil extracts, defined by DPPH method, represented as: A- mM TE/g DW; B- mM TE/g extract.



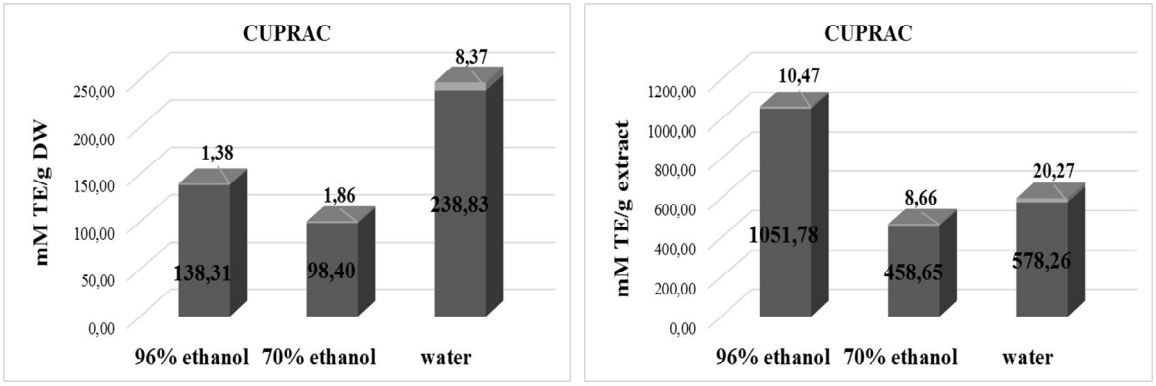
A **B**

Figure 2. Antioxidant activity of different basil extracts, defined by ABTS method, represented as: A- mM TE/g DW; B- mM TE/g extract.



A **B**

Figure 3. Antioxidant activity of different basil extracts, defined by FRAP method, represented as: A- mM TE/g DW; B- mM TE/g extract.



A B

Figure 4. Antioxidant activity of different basil extracts, defined by CUPRAC method, represented as: A- mM TE/g DW; B- mM TE/g extract.