# EFFECT OF HARVESTING AND PROCESSING OF STINGING NETTLE ON THE ANTIOXIDANT CAPACITY OF ITS INFUSIONS

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*The antioxidant properties of different matrices can be determined using various methods. In this work, two spectrophotometric methods based on the scavenging activity of the radicals DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) were used together with a spectrophotometric method based on the ferric reduction power of phenolic compounds. The results obtained using all three methods were compared and discussed for infusions prepared from different parts of stinging nettle (Urtica dioica L.). The relation with the time of harvest and location of grow were also examined. Finally, the antioxidant capacity of all the studied nettle infusions were correlated with the total phenolic content determined using Folin–Ciocalteu reagent.*

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**Introduction**

Nettle (*Urtica dioica* L.), also called Stinging Nettle, is commonly widespread through Europe and North America, occurring also in Northern Africa and some parts of Asia. This plant is well known for its toothed, hairy leaves, and for its sting, when stalk, root or leaves are usually the harvested parts [1].

Nettle has been used in traditional medicine as diuretic and laxative agent, to relieve pain and to treat arthritis, asthma, bronchitis, diarrhea, and some urinary disorders [2-6]. Along with this medicinal application, stinging nettle has been used for preparation of different meals as a leafy vegetable. The biological activity of stinging nettle can be particularly attributed to the presence of phenolic compounds. Phenolic compounds (also known as polyphenols) are the secondary metabolites synthesized in plants. The main classes of phenolic compounds are phenolic acids (derivatives of cinnamic and benzoic acids) and flavonoids (flavonols, flavanones, flavones and isoflavones, anthocyanidins, and flavanols) [7,8]. Whereas the flavonoids are usually present in plants as glycosides, polyphenols are widely found in fruits, vegetables, nuts, seeds, and also, in different kinds of herbs [8-10].

Increasing interest in these compounds is due to their antioxidant properties [11]. Many recent studies are focused on the determination of total antioxidant capacity of above mentioned samples that are analyzed using biochemical assays. A majority of such studies utilized antioxidant assays based on the measure of free radical scavenging ability. These methods can be divided in two groups – those based on a hydrogen atom transfer reaction (ORAC, TRAP) and procedures based on an electron transfer reaction (DPPH, FRAP, ABTS) [12,13]. The methods involving the radicals of 2,2-diphenyl-1-picrylhydrazyl (DPPH$^•$) or 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{+•}$) are being most often used [11]. Furthermore, ferric ion reducing antioxidant power (FRAP), where the colourless Fe$^{III}$-tripyridyltriazine complex is reduced to a blue solution with a Fe$^{II}$ complex is frequently applied [14]. The antioxidant capacity is usually expressed as an equivalent concentration of a standard Trolox solution (Trolox Equivalent Antioxidant Capacity, TEAC) [14,15], which enables to compare the results obtained by using different methods. Total phenolic content is commonly determined using the Folin–Ciocalteu method, being expressed in a concentration equivalent to gallic acid (Gallic Acid Equivalent, GAE) [11-14].

The aim of this study was to determine the antioxidant capacity of nettle water infusions using three spectroscopic methods, ABTS, DPPH, and FRAP. The preparation of infusions was optimized and the antioxidant capacity correlated with the total phenolic content being determined with Folin–Ciocalteu method. The differences between the individual nettle infusions were studied with respect to the effect of harvest time, location, and different part(s) of stinging nettle on the antioxidant capacity.

**Experimental**

Reagents and Materials

2,2-diphenyl-picrylhydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonoc acid) (ABTS), Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), Folin–Ciocalteu reagent and 4,6-tripyridyl-s-triazine (TPTZ) were obtained from Sigma-Aldrich. Iron trichloride, hydrochloric acid (35%), potassium persulfate, acetic acid (99.8%), sodium carbonate, and sodium acetate trihydrate, all being analytical grade, were purchased from Lachema (Neratovice, the Czech Republic). Water was purified using Ultra CLEAR UV apparatus (SG, Hamburg, Germany).

Sample Preparation

The stinging nettle samples (*Urtica dioica* L.) as herbal teas and wild grow herbs were used for experiments (Table I). Three tea bag samples and other three loose tea samples were purchased in local supermarkets and pharmacies and stored in a dry and dark place. Eight samples of wild growing nettle were harvested during the 2011 and 2012 at the different localities in the Czech Republic (Table I). For

Table I    Studied samples of stinging nettle (*Urtica dioica*). Wild growing dried nettle harvested in different time and location (DN), nettle teas as tea bags (TB) and loose teas (LT)

| Symbol | Place and date of harvesting | Symbol | Form, distributor |
|--------|------------------------------|--------|-------------------|
| DN1 | Seč, May, 2011 | TB1 | Tea bag, Apotheke |
| DN2 | Seč, August, 2011 | TB2 | Tea bag, Jemča |
| DN3 | Seč, April, 2012 | TB3 | Tea bag, Megafyt |
| DN4 | Nymburk, April, 2012 | LT1 | Loose tea, Waldemar |
| DN5 | Třemošnice, April, 2012 | LT2 | Loose tea, Mikeš |
| DN6 | Pardubice, April, 2012 | LT3 | Loose tea, Oxalis |
| DN7 | Letohrad, April, 2012 | | |
| DN8 | Srnín, April, 2011 | | |

the proper analysis, the stalks, leaves, and flowers were used individually and in a mixture; the extracts being prepared by pouring of 200 ml boiling water over 1.5 g portion of nettle sample. After leaching (for 15 min), the extracts were cooled

down to a laboratory temperature, filtered with 0.45 µm PTFE syringe filters (Labicom, the Czech Republic) and used for analysis.


Spectrophotometric Measurements

All the experiments were performed on a UV/VIS spectrophotometer (model UV-2450; Shimadzu, Kyoto, Japan) connected to a computer for instrument control and data acquisition.

*Total Phenolic Content (TPC)*: The working solution was prepared by mixing deionised water and Folin–Ciocalteu reagent (9:1, v/v). Then, Nettle infusion (100 µl) was added to 1 ml deionised water and 1 ml working solution of Folin–Ciocalteu reagent. After 5 min, another 1 ml of sodium carbonate (7.5%, w/w) was added [16]. Absorbance of the resultant mixture was measured at a wave length of 750 nm after 35 min.

*Radical Scavenging Activity of DPPH*: The substance was dissolved in methanol to be 100 mmol $l^{-1}$ in concentration [17]. Afterwards, plant infusion (30 µl) was added to 3 ml of this solution. Absorbance decrease was monitored at 515 nm after 30 minutes.

*Scavenging Assay of ABTS*: The preparation of the ABTS radical cation was adopted from the literature [18] and slightly modified. The radical cation was produced by letting to react a solution of 3.6 mmol $l^{-1}$ ABTS in deionised water with 0.064 mol $l^{-1}$ potassium persulfate. The reaction mixture was kept in dark at laboratory temperature for 12-16 h before use, and then diluted with water to reach the absorbance of *ca.* 0.8. Finally, plant infusion (30 µl) was added to the ABTS working solution (3 ml) and the decrease in absorbance monitored at 734 nm after 30 min.

*FRAP Method:* The working solution was prepared by mixing 0.3 M acetate buffer (pH 3.6), with 10 mmol $l^{-1}$ TPTZ (2,4,6- tripyridyl-s-triazine) and 20 mmol $l^{-1}$ ferric chloride at the buffer-to-TPTZ-to-$FeCl_3$ ratio of 10:1:1 (v/v) [19]. Plant infusion (30 µl) was added to the FRAP reagent (3 ml) and the increase in absorbance of the resultant solution monitored at 593 nm after 10 min.

The antioxidant capacity of selected herbal infusions was determined as the change of absorbance of the solution measured after completing the reaction of the sample with the agent. Using both ABTS and DPPH methods, a relative decrease in absorbance was calculated: *decrease A* (%) = $(A_0 - A)/A_0 \cdot 100$, where $A$ is the absorbance at the end of reaction time, $t = 30$ min, and $A_0$ the absorbance of the blank sample. Regarding the FRAP method, the increase in the absorbance was observed in contrast to the ABTS and DPPH methods. The increase in the absorbance was then calculated by means of the following equation: $\Delta A = A - A_0$, where $A$ is the absorbance after 10 min (when the reaction started), $A_0$ is the absorbance of the blank. The change in absorbance (i.e., relative decrease or

increase) was recalculated to a concentration of the Trolox standard (TEAC), which was in relation with 1 g nettle expressed in the calibration equations with parameters gathered in Table II.

The calibration solutions were prepared by diluting of the Trolox standard in methanol, when the amount of Trolox added to the reaction mixture was in the range of 0.010-0.085 mmol Trolox for the ABTS method, 0.020-0.120 mmol for the DPPH method, and 0.010-0.040 mmol for the FRAP method, respectively. The total phenolic content was expressed as mg of gallic acid (Gallic Acid Equivalent, GAE) being related to 1 g of a dry nettle. The increase in absorbance was calculated using the same equation as that for the FRAP method, only the absorbance was measured after 35 min. The change in absorbance was recalculated to the respective GAE by means of calibration equations with parameters shown in Table II.

Table II    Parameters of linear regression of the data obtained by determining TEAC and TPC using the respective method (absorbance vs. concentration of Trolox and absorbance vs. concentration of gallic acid). $R^2$ – coefficient of determination, significance level – 95 %

| Method | Slope | $SD$ (slope) | Intercept | $SD$ (intercept) | $R^2$ | $p$-value (intercept) |
|--------|-------|--------------|-----------|------------------|-------|------------------------|
| DPPH | 738.8 | 5.3 | 0.398 | 0.397 | 0.9997 | 0.087 |
| ABTS | 1270 | 6.3 | 0.779 | 0.571 | 0.9996 | 0.548 |
| FRAP | 15.08 | 0.24 | −0.002 | 0.006 | 0.9961 | 0.899 |
| TPC | 30.45 | 0.36 | −0.012 | 0.007 | 0.9991 | 0.674 |

Statistical Evaluation of the Experimental Data

Statistical analysis was performed with the aid of a QC.Expert 2.5 (TriloByte, the Czech Republic) and Statistica 10 (StatSoft, the Czech Republic) software.

All the statistical tests were carried out at a significance level of 95 % ($\alpha$ = 0.05). The antioxidant capacity determination experiments made in five replicates for each sample ($n = 5$); the respective values being expressed as a mean and the standard deviation ($SD$). The calibration data for the Trolox Equivalent Antioxidant Capacity for all the methods used (ABTS, DPPH, FRAP) and for the total phenolic content were measured at eight concentration levels of the standard (Trolox or gallic acid); again, each in five replicates ($n = 5$). The calibration data were fitted using the least square linear regression method; the regression parameters (i.e. slopes and intercepts of regression lines) being presented with their standard deviations. The significance of intercept of regression lines was tested using Student's $t$-test and the linearity of calibration curves checked by

inspecting the plots of residuals. Finally, the statistical significance of the key-factors, such as the methods for the antioxidant capacity determination, the type of sample, and the sample treatment was tested by means of analysis of variance (ANOVA).

## Results and Discussion

Optimization of the Methods

Optimization of the antioxidant assays (ABTS, DPPH, FRAP) and of Folin–Ciocalteu method was performed using a nettle herbal tea Apotheke (TB1, Table I). The optimal volume of nettle infusion subjected to analysis was determined as the gradually increasing volume of the sample added to reaction mixtures. As optimal volumes of nettle infusions, 30 µl and 100 µl were chosen for measurement of antioxidant capacity and TPC, respectively.

These volumes were kept constant for all the nettle samples. Further, the effect of leaching time (5, 10, 15, and 20 min) on antioxidant capacity was evaluated, when the antioxidant capacity of nettle infusion increased with the leaching time up to 15 min. However, after 20 min of such a leaching, the decrease in antioxidant capacity of nettle infusion was observed and, therefore, the same period of 15 min for leaching time was used for all the nettle samples. This time is also recommended by the producers of nettle teas.

Determination of the Antioxidant Capacity

Eight harvested nettle samples, three loose nettle teas and three tea bag nettle teas were subjected to the study (see Table I). The comparison of antioxidant capacity of all nettle infusions is shown in Fig.1. High values of the antioxidant capacity were obtained for the infusions prepared from the nettle harvested in spring (April and May) as the time of harvesting is a very important factor for total content of antioxidants. The young nettles that start to grow during a spring contain high amount of antioxidants and thus, the antioxidant capacity of their infusions is also high. As the plant is growing and getting older, the antioxidants are consumed for the natural protection against environmental stresses. This situation is clearly illustrated by the samples harvested at the same location but in different time (see Fig.1, DN1-DN3). Antioxidant capacity of the sample DN2 harvested during summer (in August) is the lowest in comparison with the samples DN1 and DN3 that were harvested during spring (in April). The year of harvesting does not strongly affect the antioxidant capacity. The antioxidant capacities of tea bags and loose nettle teas are in all cases lower than infusions from the nettles harvested

in spring (Fig. 1). This marked difference between the harvested samples and those being purchased can be explained by processing of commercial samples. The nettles picked in different places and different time can be mixed together in
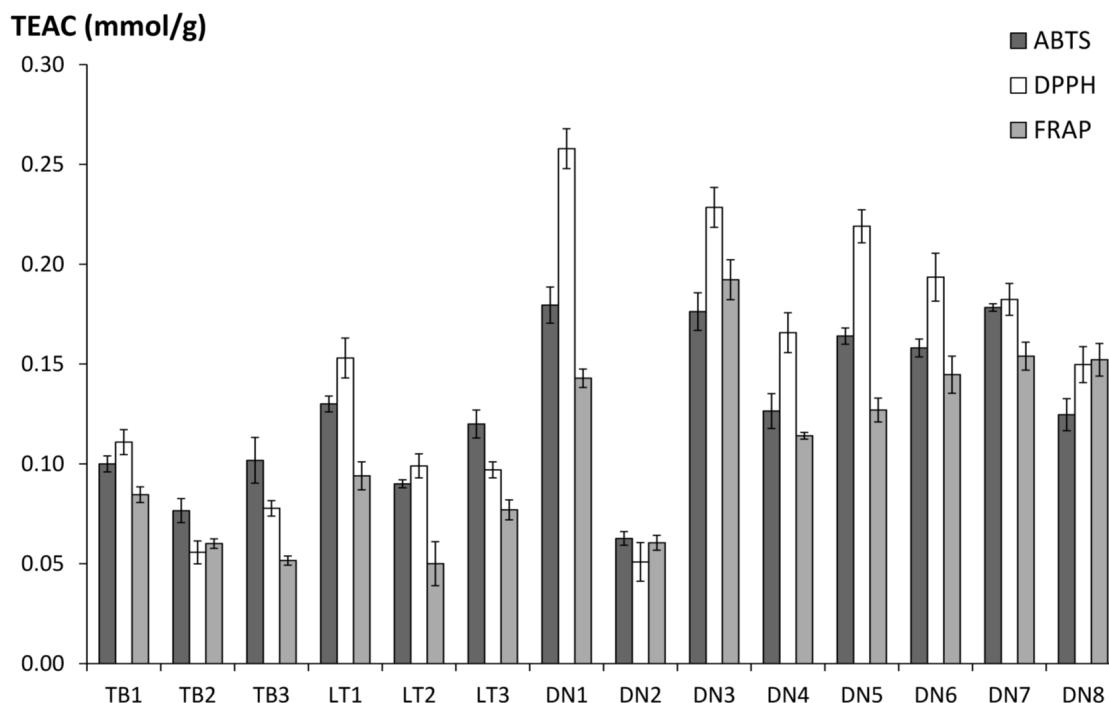


Fig. 1    Antioxidant capacity of nettle infusions measured using three spectrophotometric methods (DPPH, ABTS, FRAP). The samples are listed in Table I and the results expressed as TEAC $\pm$ SD, $n = 5$. For the respective conditions, see Experimental

purchased samples that affect principally the antioxidant capacity of samples. From this point of view, the infusions from the picked herbs are better to be used, because of the known origin of herbs and preferably higher content of antioxidants.

Concerning the methods used for the determination of antioxidant capacity, the highest values were obtained using the DPPH method in almost all cases (Fig. 1). The differences between the results obtained by all three methods can be attributed to the different reaction mechanisms of agents with antioxidants, which have been tested using analysis of variance. The difference between all three methods was statistically significant for the tea bags and harvested nettle (Fig. 2). For loose tea samples, the DPPH and ABTS methods had provided similar results. Except the FRAP method, where the tea bags and loose tea samples gave similar value of TEAC, the results obtained using the individual methods for different types of samples, showing again the statistical significance.

Next, the effect of nettle processing was investigated. In almost all cases the antioxidant capacity of infusions prepared from the freshly harvested nettle
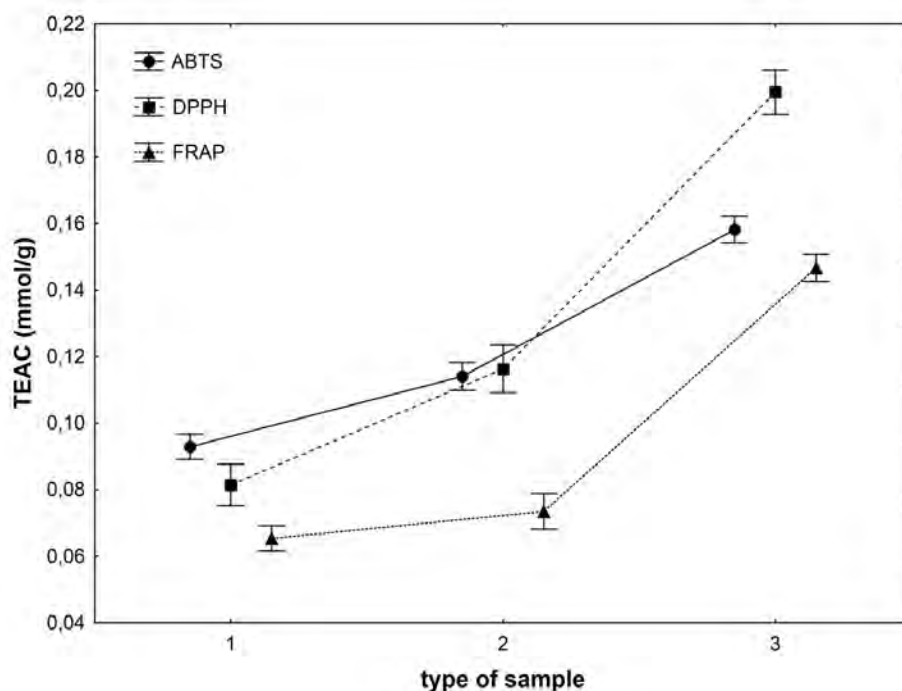
Fig. 2   The analysis of variance for two factors: (i) type of sample and (ii) the method used. Three types of methods (ABTS, DPPH, FRAP ) and three types of samples (1 – tea bags, 2 – loose tea samples, 3 – dried harvested samples) were tested for statistical significance; the results being expressed as a weighted average and the standard deviation ($\alpha = 0.05$)

samples had been higher than an infusion prepared from the same nettle but after air drying. Especially the DPPH assay provided a very high value of antioxidant capacity of infusions prepared from fresh nettle (Fig. 3). This outstanding difference between the TEAC values obtained by DPPH assay for infusions from fresh or dried nettle samples could be caused by a degradation of valuable antioxidants during the air drying, when assuming that these antioxidants react solely with the DPPH radical. The respective results were further analyzed using ANOVA, confirming the previous discussion.

The DPPH and ABTS methods have provided statistically different results for the individual treatments of the sample(s), whereas, in the case of FRAP method, the results for both treatments are almost the same.

Concerning the individual parts of the nettle, the leaves have had the most severe impact on the antioxidant capacity, followed by flowers and stalks (Fig. 4). The value of TEAC measured for infusions from a fresh whole nettle corresponds to average value of TEAC measured for infusions from the individual parts of fresh nettle.

Finally, the correlation of antioxidant capacity with total phenolic content expressed as the amount of gallic acid was performed for the samples studied (Fig. 5). From the dependence of TEAC vs. GEA, it is evident that the antioxidant capacity is quite well correlated with the total phenolic content in the nettle
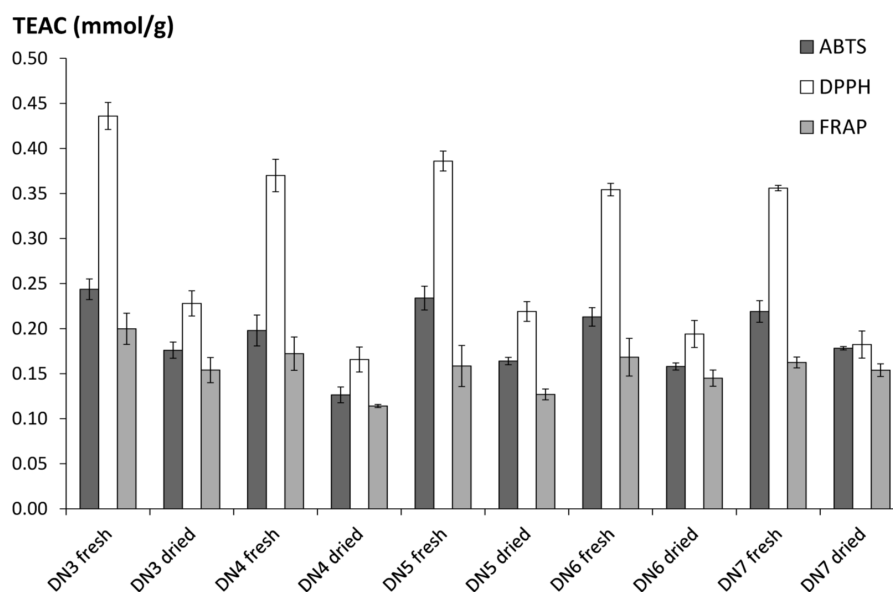
Fig. 3 Comparison of antioxidant capacity of infusions prepared from fresh and dried wild nettle samples analyzed using three different techniques (ABTS, DPPH, FRAP). The samples are listed in Table II, when the results are expressed as TEAC ± SD, $n = 5$. For the respective conditions, see Experimental
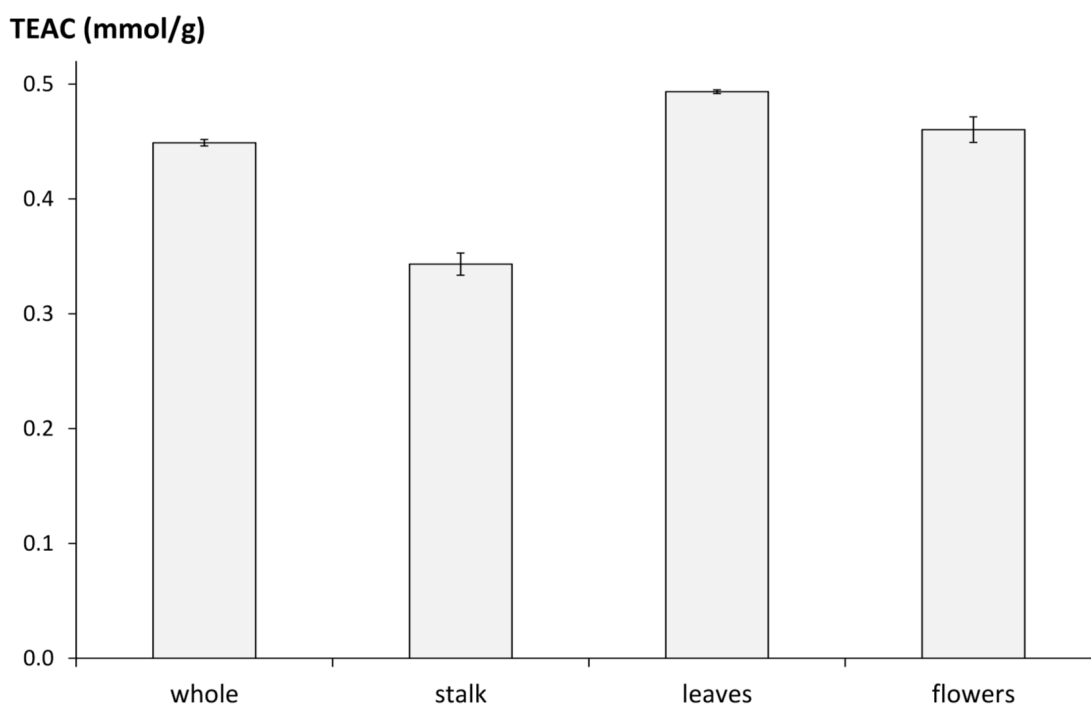


Fig.4 Antioxidant capacity of nettle infusions prepared from different parts of fresh wild nettle sample DN3 harvested in Seč (in April, 2012). The DPPH method was used for analyzing the TEAC and the results expressed as TEAC ± SD, $n = 5$. For the respective conditions, see Experimental

infusions, when the ABTS method is used. The respective coefficient of determination is $R^2 = 0.9549$, whereas the DPPH and FRAP methods do not provide such a good correlation, but their values are still reasonably tight ($R^2 = 0.9101$ for DPPH and $R^2 = 0.8710$ for FRAP). Two separated groups of nettle infusions can be seen in Fig. 5, indicating also the difference between antioxidant properties of the harvested samples and commercial ones. Thus, the infusions from harvested samples contain a higher amount of polyphenolic compounds with antioxidant properties compared to those prepared from nettle teas and purchased in local stores.
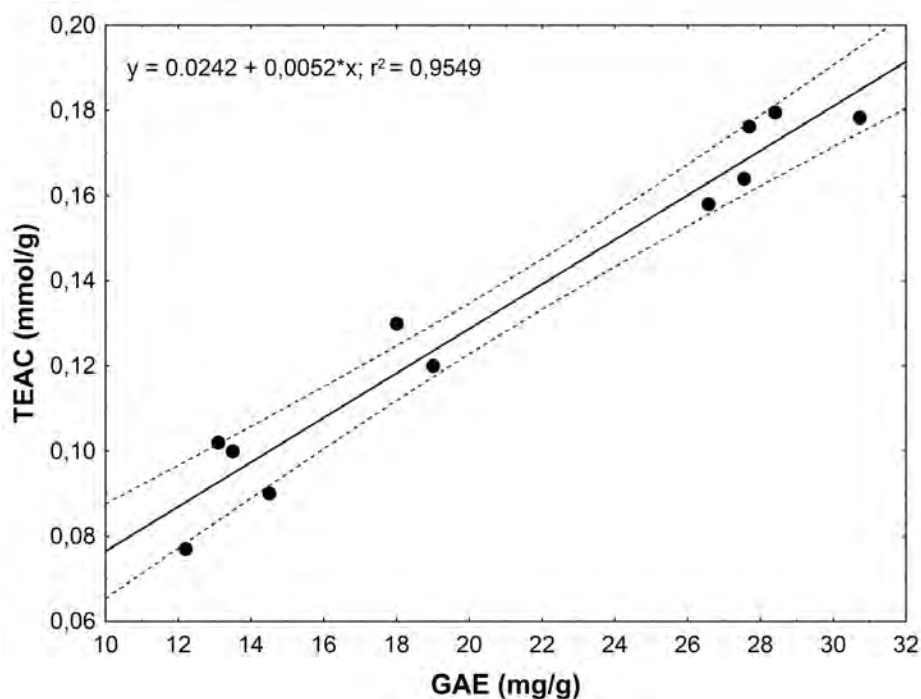


Fig. 5  Correlation between the antioxidant capacity of nettle infusions measured by ABTS method (TEAC) and the total phenolic content determined using Folin–Ciocalteu method (GAE)

## Conclusion

The antioxidant properties and total phenolic content of the nettle infusions have been determined using different spectrophotometric methods. The antioxidant capacities obtained using ABTS, DPPH and FRAP methods were compared and discussed for infusions prepared from different parts of nettle. Further, the total phenolic content was determined using Folin–Ciocalteu reagent and the results correlated with the antioxidant capacity of nettle infusions. The effect of time and location of harvesting was examined together with the relation on the type of sample purchased from various producers. From the study performed, it is evident

that the commercial samples has contained lower amount of phenolic compounds with antioxidant properties compared to the wild growing samples. This can be explained by mutual mixing of the nettle samples in purchased herbal teas harvested in different time. Finally, in the nettle infusions, the time of harvest has had the highest influence on the content of phenolic compounds with antioxidant properties.

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