

Comparison of Antioxidative Activity Data for Aqueous Extracts of Lemon Balm (*Melissa officinalis* L.), Oregano (*Origanum vulgare* L.), Thyme (*Thymus vulgaris* L.), and Agrimony (*Agrimonia eupatoria* L.) obtained by Conventional Methods and the DNA-Based Biosensor

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Abstract

HEILEROVÁ L., BUČKOVÁ M., TARAPČÍK P., ŠILHÁR S., LABUDA J. (2003): **Comparison of antioxidative activity data for aqueous extracts of lemon balm (*Melissa officinalis* L.), oregano (*Origanum vulgare* L.), thyme (*Thymus vulgaris* L.), and agrimony (*Agrimonia eupatoria* L.) obtained by conventional methods and the DNA-based biosensor.** Czech J. Food Sci., **21**: 78–84.

The antioxidative properties of aqueous plant extracts were evaluated using common methods such as the Rancimat and 2,2'-diphenyl-1-picrylhydrazyl (DPPH) free radical method. Moreover, a voltammetric procedure based on the protective effect of antioxidants against the oxidative DNA damage was employed using a disposable DNA biosensor fabricated as a screen-printed electrode chemically modified by calf thymus double stranded (ds) DNA. The total polyphenols were also determined spectrophotometrically with the Folin-Ciocalteu agent. The extracts of oregano and lemon balm exhibited significantly higher activity than those of thyme and agrimony. The results were treated statistically and their operational character is discussed.

Keywords: antioxidants; plant extracts; Rancimat method; DPPH radical; DNA biosensor

The antioxidative properties of phytochemicals may play an unsubstitutable role in the chemoprevention of human diseases (LIPPMAN *et al.* 1994; HALLIWELL 1997). Plant materials contain numerous types of antioxidants with varied activities (SHAHIDI 2000; MARIÁSSYOVÁ & ŠILHÁR 2000). Among herbs, lemon balm, oregano and thyme (*Lamiceae*) as well as agrimony (*Rosaceae*) are known for their antioxidative potency (ÖZCAN & AKGÜL 1995; TAKÁCSOVÁ & PRÍBELA 1995; LACROIX *et al.* 1997; MARINOVA & YANISHLIEVA 1997; CERVATO *et al.* 2000). Apart from

the plant vegetation stage, the solvent used for the extraction of the active agents is also an important factor (MARINOVA & YANISHLIEVA 1997; CERVATO *et al.* 2000). Aqueous extracts are of interest, particularly in gastronomy and medicine. Despite the wide investigations, there are still many unsolved questions in this topic, for instance, whether and when all phenolic phytochemicals function as antioxidants, what are their synergic effects, what are their levels the individual parts of a plant during vegetation, what are the optimum conditions for

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their extraction from plants and their application in food, what is the antioxidative capacity of the additives (including plant extracts) actually used in the food production, and others. To answer these questions, new analytical data and detection techniques/devices are needed.

With respect to the definition of antioxidants (HALIWELL 1999), the antioxidative activity is rather a complex parameter based on (bio)chemical reactivity. Antioxidants differ in the target substances they protect. Therefore, analytical data on such an activity (of both pure substances and extracts) should have an operational character given by the detection principles of the experimental techniques used; their evaluation is also of a great interest. Methods based on the inhibition of pure lipid oxidation (Rancimat method) and on the scavenging effect of hydrogen atom donors on the stable free radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH) (BRAND-WILLIAMS *et al.* 1995) are typically used. As many natural and synthetic antioxidants exhibit an effective protection against the oxidative DNA damage (cell death) induced by H₂O₂ (DUTHIE *et al.* 1997; JOHNSON & LOO 2000), bleomycin (NG *et al.* 2000), tert-butylhydroperoxide (SESTILI *et al.* 1998; VALLS-BELLÉS *et al.* 2002), NO-releasing compound (OHSHIMA *et al.* 1998) and γ -irradiation (CAI *et al.* 2001), methods utilising the DNA cleavage inhibition are also widely applied.

DNA-based biosensors could be of particular interest as simple and effective analytical devices for the characterisation of the antioxidant effects. Electrochemical sensors with a layer of the calf thymus double stranded (ds) DNA immobilised on the surface of voltammetric indication (mercury or solid) electrode are known for the detection of the DNA damage (LABUDA *et al.* 1999; PALEČEK & FOJTA 2001; PALEČEK & JELEN 2002). Recently, we have successfully tested the DNA-modified screen printed electrode for the estimation of the antioxidative activity of yeast polysaccharides (BUČKOVÁ *et al.* 2002) and phenolic acids (LABUDA *et al.* 2002). The detection scheme uses the [Co(phen)₃]³⁺ complex ion as a dsDNA redox marker for the quantification of DNA that survives the incubation of the biosensor in a mixture of the cleavage agent and the antioxidant. The aim of this work was to evaluate the antioxidative activity of samples of a complex matrix such as the plant extracts using the conventional (Rancimat and free radical) methods as well as the DNA biosensor, and to compare the data obtained. The content of polyphenols in the

extracts as determined photometrically using the Folin-Ciocalteu agent (SINGLETON *et al.* 1999) is also considered.

MATERIALS AND METHODS

Materials. Plants were collected as follows: Lemon balm, variety Citra (leaves) from the plantation in Mojmírovce, collected on 21 June 2000 and dried in a dry box at 50°C; Oregano (leaves and flowers) and Agrimony (leaves) from a meadow in Griňava, collected on 16 July 2000, and thyme (leaves and flowers) from a meadow in Modra, collected on 12 October 2000, all dried and stored at room temperature for 6 to 9 months. About 30 g of the dried herbs was powdered and 2 g was extracted by one-step extraction with 100 ml of water at 70°C for 1 h in whirling thermostate (MLW-Baureime, Germany). After cooling, the extracts were filtered through filter paper (Filtrak No. 390, Spezialpapierfabrik Niederschlag, Germany) and the volume was made up to 100 ml with water. The samples were stored frozen at -4°C and analysed within 2 weeks. One sample of each extract was examined for the antioxidant capacity using the individual methods in triplicates.

Rapeseed oil was the product of Palma (Bratislava, Slovakia) and it was stored at +4°C. The fatty acid contents were as follows: C 12:0 – 0.1%, C 14:0 – 0.1%, C 16:0 – 4.8%, C 18:0 – 2.0%, C 18:1 – 62.0%, C 18:2 – 20.0%, C 18:3 – 9.0%, free fatty acids 0.046%, and the peroxide number 0.53 mmol O/kg. The 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH) was from Aldrich; for the measurements, its methanolic stock solution of 0.0251 g/100 ml was diluted ten times with methanol. The Folin-Ciocalteu agent was from Merck. Tannic acid from Aldrich was used as 0.03% aqueous solution. Calf thymus dsDNA was obtained from Merck (1.24013.0100) and it was used as received. Its stock solution (5 mg/ml) was prepared in 0.01 mol/l Tris-HCl and 1 × 10⁻³ mol/l EDTA solution of pH 8.0 and stored at -4°C. The [Co(phen)₃]³⁺ (ClO₄)₃ complex was synthesised in our laboratory according to DOLLIMORE and GILLARD (1973) and checked by chemical analysis. Deionised and double distilled water was used throughout.

Preparation of the DNA biosensor. A working carbon electrode (SPE with 25 mm² geometric surface area) of the three-electrode screen-printed assembly (including also silver/silver chloride reference electrode and carbon counter-electrode,

FACH, Prešov, Slovakia) was chemically modified in laboratory by covering it with 5 µl of the DNA stock solution and leaving it to dry overnight.

Rancimat method. A Metrohm 743 Rancimat instrument (Herisau, Switzerland) and rapeseed oil as the lipid substrate were used to evaluate the lipid-oxidation inhibition activity of the extracts. 100 µl of the plant extract was pipetted into the measurement probe and 3 g of the oil was added. In the control measurement, 100 µl of water was pipetted instead of the plant extract. The experiment was carried out at 120°C with air passing through the sample mixture at 20 l/h. The inhibition activity was expressed as a difference between the induction period obtained for the sample and the control.

Scavenging DPPH radical. The procedure reported previously (BRAND-WILLIAMS *et al.* 1995) was adapted as follows: 3.9 ml of the DPPH solution in methanol (0.025 g/l) was pipetted into 1 cm cuvette and the absorbance value A_0 was read against blank at 515.6 nm using UV-visible spectrophotometer UV-1601 (Shimadzu, Japan). The original plant extract was diluted with water in the ratios of 0.5 ml/10 ml, 1 ml/10 ml, 2 ml/10 ml and 3 ml/10 ml, and 0.1 ml of the diluted extract was added into the cuvette containing the DPPH solution. After shaking, the decrease in absorbance was measured immediately at 5 s intervals during 300 s. The measurements were carried out in triplicates. The exact DPPH concentrations (g/l) in the reaction medium (the initial one, $c(\text{DPPH})_0$, and those at a given time, $c(\text{DPPH})_t$) were obtained using the calibration curve (coefficient of correlation 0.9999)

$$A = 0.0509 + 24.8264c (\text{DPPH})$$

From the time dependence of

$$\text{Residual DPPH (\%)} = [c(\text{DPPH})_t/c(\text{DPPH})_0] \times 100$$

the values under steady state conditions were obtained for the individual diluted extract samples. These values were plotted against the mass ratio

$$\frac{[\text{m}(\text{dry plant in 0.10 ml of the diluted extract})/\text{m}(\text{DPPH in 3.9 ml of its solution})]}{}$$

to obtain the efficient concentration (mass ratio) needed to decrease the initial DPPH concentration by 50% (the EC_{50} value) as well as the antiradical power (ARP equal to $1/\text{EC}_{50}$).

Use of the DNA-Based Biosensor. The procedure reported previously (BUČKOVÁ *et al.* 2002; LABUDA *et al.* 2002) was exploited. Briefly, the new DNA sensor (DNA/SPE) was pretreated by immersion into 10 mmol/l phosphate buffer, pH 7.0, for 15 min and rinsed with water. Then, the $[\text{Co}(\text{phen})_3]^{3+}$ marker was accumulated from 5 ml of its 5×10^{-7} mol/l solution in 0.010 mol/l phosphate buffer under stirring for 120 s at an open circuit. The differential pulse voltammogram (DPV) was recorded immediately from +0.4 to -0.5 V at the pulse amplitude of 100 mV and the scan rate of 25 mV/s using a computerised voltammetric analyser ECA pol, model 110 (Istran, Bratislava, Slovakia), fitted with the DNA/SPE assembly. With the software used, the current was measured with 2 mV scan step at this scan rate. The marker DPV peak current (I_0) at -0.130 V was evaluated against the base-line using the standard software and was corrected by the subtraction of the mean marker DPV peak current measured at the unmodified SPE ($n = 10$) under identical conditions. Subsequently, the DNA/SPE was regenerated by the removal of the accumulated $[\text{Co}(\text{phen})_3]^{3+}$ ions from the DNA layer treating the sensor in the solution of a high ionic strength (0.100 mol/l phosphate buffer pH 7.0) under stirring during 120 s. The negligible marker peak current was checked by the DPV record in blank. The peak current I_0 was obtained in triplicates.

The DNA damage and the antioxidative effects of the plant extracts were detected after 5 min incubation of the sensor in the cleavage mixture (2×10^{-4} mol/l FeSO_4 , 4×10^{-4} mol/l EDTA, 9×10^{-3} mol/l H_2O_2 , in 0.010 mol/l phosphate buffer solution with 10% methanol without or with the addition of the plant extract under the application of the electrode potential of -0.5 V in aerobic conditions at room temperature). The marker peak current I was obtained in triplicates using the marker accumulation/measurement/sensor regeneration scheme as described above and employing the same DNA/SPE for the given composition of the cleavage mixture. The average signals I_0 and I were calculated from the second and third measurements. To compensate for the differences in the properties of the individual strips of the DNA-biosensor, the normalised (relative) signal value I/I_0 was obtained which represents the survived portion of the original DNA.

Spectrophotometric determination of polyphenols. Polyphenols were determined spectrophotometrically as described elsewhere (SINGLETON *et*

al. 1999) using photometer Spekol 11 (Zeiss, Jena, Germany). Briefly, 1.0 ml of the diluted plant extract (1:1 with water), 1.0 ml of the Folin-Coicalteu agent and 10 ml of 20% Na_2CO_3 were pipetted into a 100 ml volumetric flask and the volume was made up to the mark with water. After allowing the solution to stand still at room temperature for 30 min, the absorbance at 700 nm was measured against blank (without extract). The linear calibration curve ($R = 0.9999$)

$$A = -0.00095 + 0.00089c \text{ (tannic acid)}$$

was used for the quantification of polyphenols.

Statistical treatment of the data. To determine any significant difference ($\alpha = 0.05$), the data were analysed using the standard *t*-test.

RESULTS AND DISCUSSION

The addition of plant extracts to rapeseed oil extended the induction period of its oxidation. In the control experiment, this induction period was (5.20 ± 0.02) h. The inhibition time periods obtained with the individual extracts from the corresponding induction period by subtracting that of the control experiment are presented in

Figure 1. For agrimony, however, the induction period was under the detection limit (5.26 h) and the inhibition time is not presented. The antiradical activity of the extracts against the DPPH radical expressed as the antiradical power is also shown in Figure 1 and it reveals the order of oregano > lemon balm > thyme.

For the investigation employing the DNA biosensor, the experimental conditions were first optimised to obtain the necessary sensitivity and selectivity. The composition of the DNA cleavage system and the period of incubation time were chosen so as to reach about 90% degradation of DNA in the control experiment, i.e. the DNA marker signal (I) of about 10% of that measured with the untreated biosensor (I_0). This is the consequence of the decrease of the ability of the original DNA to bind the marker due to extensive structural changes involving DNA strand breaks (LABUDA *et al.* 1999; BUČKOVÁ *et al.* 2002). The negative polarisation potential of -0.5 V applied to the sensor during its incubation in the cleavage system of $[\text{Fe}(\text{EDTA})]^-$ and H_2O_2 controls the iron(III)/iron(II) reduction/oxidation cycle that generates reactive oxygen species (FOJTA *et al.* 2000). No additional chemical reductant such as ascorbic acid (LABUDA

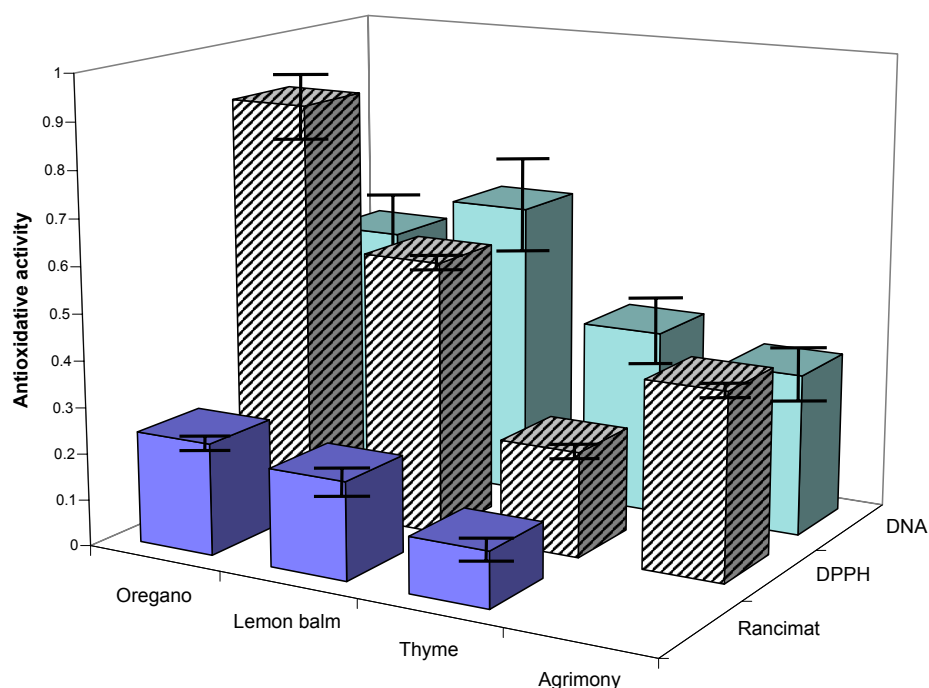


Figure 1. Antioxidative activity of the plant extracts expressed by the inhibition time period (in h) regarding the control at the oxidation of rapeseed oil using the Rancimat method; the antiradical power (ARP) in the DPPH method and the relative marker signal I/I_0 of the DNA biosensor in the system of cleavage agent and the plant extract (2 mg of the dry plant in the form of extract per 1 ml of the cleavage mixture). Error bars represent the standard deviation derived from the average value ($n = 3$)

et al. 1999; Bučková *et al.* 2002) is needed which is of a great interest in the evaluation of natural samples containing ascorbic acid.

In Figure 1, a portion of the original dsDNA that survives the incubation of the biosensor in the system of the cleavage agent and the extract is expressed using the relative marker signal I/I_0 . The antioxidative activity of the individual plant extracts can be ordered as follows: oregano \cong lemon balm > thyme \cong agrimony.

The spectrophotometric determination of polyphenols using tannic acid for the calibration revealed the following values (mg/l of the extract): 1532 ± 23 , 647 ± 21 , 620 ± 20 , and 601 ± 27 for oregano, lemon balm, thyme, and agrimony, respectively.

It is a common analytical practise to compare the results obtained by different techniques or by different researchers/laboratories in order to check wheater reproducible, reliable and indeed meaningful data are obtained. Generally, the detection principle and the detection window of an analytical technique used define the results based on chemical reactivity (e.g. speciation of metals) (VAN DEN BERG & DONAT 1992). The antioxidative activity is also such a parameter. In other words, it should be defined operationally regarding the procedure used. For instance, changes in the order of the antioxidant activity of caffeic acid, rosmarinic acid, and related compounds in the dependence on the experimental conditions used in the lipid oxidation in the Rancimat method were reported (CHEN & Ho 1997).

The mean values of the antioxidant data obtained in this work by the given technique were compared using the *t*-test. As concerns the Rancimat and DPPH methods (as well as the spectrophotometric determination of polyphenols), the extract of oregano possessed the highest antioxidative activity followed by that of lemon balm. As concerns the results obtained with the DNA biosensor, the difference between the activity of oregano and lemon balm was not significant ($\alpha = 0.05$). A high activity of oregano in comparison to those of other herbs including thyme was reported previously (VEKIARI *et al.* 1993; ECONOMOU *et al.* 1991; JAGAIT 1996) while a higher activity in lemon balm than in oregano was also reported (MARINOVA & YANISHLIEVA 1997). Generally, the extracts of thyme and agrimony exhibited weaker antioxidative properties in all test. Their lipid oxidation inhibitory effect and the DPPH antiradical activity are

controversial and the difference in the signals of DNA protection is not significant ($\alpha = 0.05$). The spectrophotometric measurement of polyphenols showed no significant differences for lemon balm, thyme and agrimony.

In conclusion, this work has confirmed the operational character of the antioxidative activity data that were determined for plant extracts using the common model systems as well as the protection against oxidative DNA damage using the DNA-based biosensor. Different activity of the extracts under study is evidently given by their different chemical composition and different reactivity of their components towards detection systems used. Moreover, each of compounds involved may be present together with its precursor(s) and reaction product(s). Thus, the mode of action may be varied and could involve multiple mechanisms of actions, depending on the type and source of the material used (SHAHIDI 2000). For these reasons, the simple determination of polyphenols as active dietary antioxidants is not sufficient for the evaluation of the extracts.

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Súhrn

HEILEROVÁ L., BUČKOVÁ M., TARAPČÍK P., ŠILHÁR S., LABUDA J. (2003): **Porovnanie údajov antioxidačnej aktivity pre rastlinné extrakty medovky lekárskej (*Melissa officinalis* L.), pamajoránu obyčajného (*Origanum vulgare* L.), materinej dúšky (*Thymus vulgaris* L.) a repíka lekárskeho (*Agrimonia eupatoria* L.) získaných konvenčnými metódami a DNA biosenzorom.** Czech J. Food Sci., 21: 78–84.

Pomocou všeobecne používaných metód ako Rancimat a metóda voľného radikálu 2,2'-diphenyl-1-picrylhydrazylu (DPPH) sa hodnotili antioxidačné vlastnosti vodných rastlinných extraktov. Naviac sa použil voltampérometrický postup založený na ochrannom účinku antioxidantov voči oxidačnému poškodeniu DNA viazanej na DNA biosenzore realizovanom v podobe elektródy pripravenej sieťotlačou a chemicky modifikovanej dvojvláknovou (ds) DNA z teľacieho brzlíka. Stanovil sa tiež celkový obsah polyfenolov spektrofotometricky s činidlom podľa autorov Folin-Ciocalteu. Výsledky sú štatisticky spracované a diskutuje sa ich operačný charakter.

Kľúčové slová: antioxidanty; rastlinné extrakty; metóda Rancimat; DPPH radikál; DNA biosenzor

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