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Spectrofluorimetric determination of hydrogen peroxide scavenging activity

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Abstract

Homovanillic acid (HVA) is widely used for the detection and imaging of oxidative enzymes—peroxidase, glucose oxidase and xanthine oxidase, but antioxidant activity has not been determined so far with the use of HVA. We have developed a simple, sensitive and in-field spectrofluorimetric method for the determination of hydrogen peroxide (H_2O_2) scavenging activity. The assay is based on the oxidation of HVA to its fluorescent biphenyl dimer in the presence of H_2O_2 and peroxidase. The presence of substances with H_2O_2 scavenging activity prevents the oxidation of HVA by removing H_2O_2. The decrease in fluorescence intensity is proportional to the antioxidative (H_2O_2 scavenging) activity. The method was evaluated using Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), BHA (3-*t*-butyl-4-hydroxyanisole) and ferulic, vanillic, caffeic, chlorogenic, protocatechuic and oxalic acids. Additionally, tea and herb infusions known for their antioxidant properties were evaluated.

Keywords: Spectrofluorimetry; Homovanillic acid; Antioxidant; H_2O_2 scavenging ability; Phenolic compounds; Herbs

1. Introduction

Reactive oxygen species such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) are inevitably produced as by-products of normal aerobic metabolism and are increased under stress conditions. Living organisms have developed complex antioxidant systems consisting of multiple defense enzymes such as superoxide dismutase, glutathione peroxidase, ascorbate peroxidase and catalase as well as non-enzymatic antioxidants, such as albumin, glutathione, ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), β-carotene (Provitamin A), uric acid, bilirubin, flavonoids and many phenolic compounds that were widely investigated recently [1–5].

Antioxidants are defined as any substances that, when present at low concentrations compared with those of an oxidizable substrate, significantly delay or prevent oxidation of that substrate [1]. These small-molecule antioxidants are present extra- and intra-cellularly. Antioxidants work by preventing the formation of new free radical species, by converting existing free radicals into less harmful molecules and by preventing chain reactions. The mechanisms by which these antioxidants act at the molecular and cellular level include their role in gene expression and regulation, apoptosis and signal transduction.

Owing to the number of different antioxidants having various scavenging activities against superoxide

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radical anion, H_2O_2, hydroxyl radical or peroxyl radical, many methods for measuring these properties have been developed. H_2O_2 scavenging activity is one of the methods for the estimation of reactive oxygen—scavenging ability in biological material. It cannot be evaluated as the total antioxidant activity due to the fact that antioxidants can act directly, for example by scavenging reactive oxygen species ($O_2^{\bullet -}$, H_2O_2, $^{\bullet}OH$) or by inhibiting their generation, or indirectly by regulating endogenous antioxidant defenses. H_2O_2 is easily and sensitively measured by using peroxidase-based assay systems. The most common systems use horseradish peroxidase (HRPO), and follows the oxidation of fluorogenic or chemiluminogenic substrates by H_2O_2. The most extensively studied substrates are *p*-hydroxyphenylacetic acid (*p*-HPA), homovanillic acid (HVA), *p*-hydroxyphenylpropionic acid (*p*-HPPA), tyramine [6] and scopoletin [7,8]. Unlike the more sensitive fluorogenic substrates, HVA does not inhibit polyamine oxidase and the fluorescent dimer is more stable than scopoletin [9]. Furthermore, HVA is mentioned as the most extensively employed reagent for fluorimetric detection of hydrogen peroxide [10]. Although HVA fluorescence intensity is the same as HPA but lower than that of HPPA; it is still high enough for this assay [11]. The system for H_2O_2 scavenging activity is based on the incubation of a putative scavenger with H_2O_2 and analyzing the reaction mixture for the loss of H_2O_2 [1,5].

The objective of this study is the evaluation of the spectrofluorimetric determination of H_2O_2 scavenging activity as a measure of antioxidant properties using HVA (4-hydroxy-3-methoxyphenylacetic acid) as a fluorimetric substrate. The method using HVA is only the part of battery measurements necessary to assess total antioxidant activity.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were reagent grade.

HVA (Sigma) solution was prepared by dissolving compound in distilled water to give a final concentration of 1.25 mM HVA, according to [12,13].

HRPO (Merck) solutions were prepared by dissolving the enzyme in distilled water to give a final concentration of 1 or 10 U.

Hydrogen peroxide was diluted daily in distilled water. The concentrations of peroxidase and hydrogen peroxide were experimentally selected to give the highest fluorescence intensities.

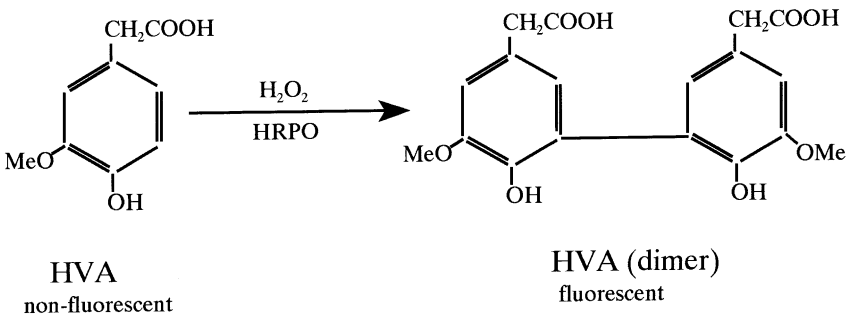
Sodium phosphate buffer was 25 mM at pH 7.5 and Tris–HCl buffer was 25 mM at pH 8.5.

2.2. Apparatus

Fluorescence spectra and intensities were measured at 20 °C with a "FluoroMax-2" spectrofluorimeter using 1 cm × 1 cm quartz cells.

2.3. Procedure

The assay is based on the oxidation of HVA to its fluorescent biphenyl dimer (Scheme 1) in the presence of hydrogen peroxide and peroxidase [14,15].



Scheme 1.

The presence of substances with H_2O_2 scavenging activity prevents the oxidation of HVA by removing H_2O_2 and causes a decrease in fluorescence that is proportional to the antioxidative activity. The conditions for the fluorimetric method were selected. Two buffers, phosphate buffer pH 7.5 and Tris–HCl buffer pH 8.5, two HRPO concentrations (1 and 10 U) in the presence of increasing concentration of hydrogen peroxide were tested. The highest fluorescence intensity was obtained for 1 mM hydrogen peroxide. The influence of peroxidase level and type of buffer was not significant. Due to the optimal conditions of the peroxidase–H_2O_2 reaction phosphate buffer and 1 U of peroxidase were selected for further studies (Figs. 1 and 2). The final testing procedure was as follows: to 0.5 ml of 25 mM phosphate buffer, pH 7.5, 0.2 ml of 1 mM H_2O_2 and 0.1 ml of sample solution were added. The mixture was vortex mixed and incubated for 5 min at 20 °C. After incubation, 0.1 ml of 1.25 mM HVA and 0.1 ml of peroxidase (1 U) was added, mixed and incubated for 5 min at 20 °C. The fluorescence intensity was measured at an excitation of 315 nm and an emission of 425 nm. The following compounds were tested: Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), BHA (3-*t*-butyl-4-hydroxyanisole), vanillic acid, ferulic acid, oxalic acid (Sigma), caffeic acid, chlorogenic acid (Fluka) and protocatechuic acid (Winlab). Additionally, the H_2O_2 scavenging activity of selected herb and tea infusions was tested: lemon balm (*Melissa officinalis*), peppermint (*Mentha piperita*), hibiscus (*Hibiscus sabdariffa*), St. John's wort (*Hypericum perforatum*), rooibos (*Aspalanthus linearis*), lime-tree flowers (*Inflorescentia tiliae*), aniseed (*Pimpinella anisum*), nettle (*Urtica dioica*), green tea and black tea (Yunnan). Tea and herb infusions were prepared as water extracts of powdered material: 10, 5 or 1 g of dry material was mixed with 1000 ml of boiling deionized water and incubated at 95 °C for 10 min. Next the herb and tea infusions were filtered and used for the determination of H_2O_2 scavenging activity. Herb teas were purchased from Herbapol, Lublin SA, Poland.

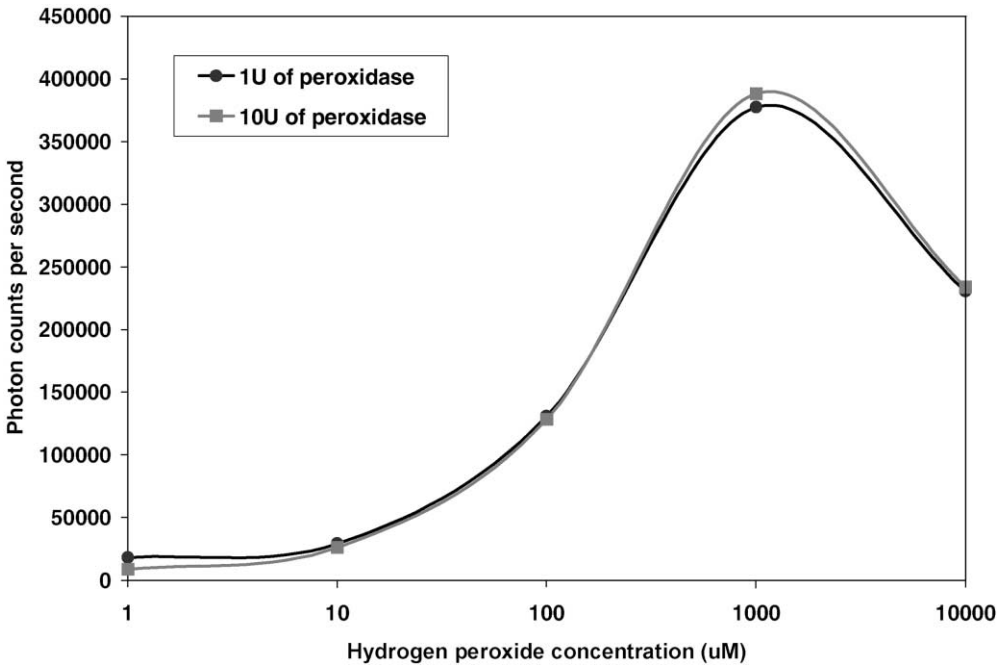


Fig. 1. The effect of the concentration of hydrogen peroxide and peroxidase activity (1 and 10 U) on the fluorescence intensity in 25 mM phosphate buffer pH 7.5.

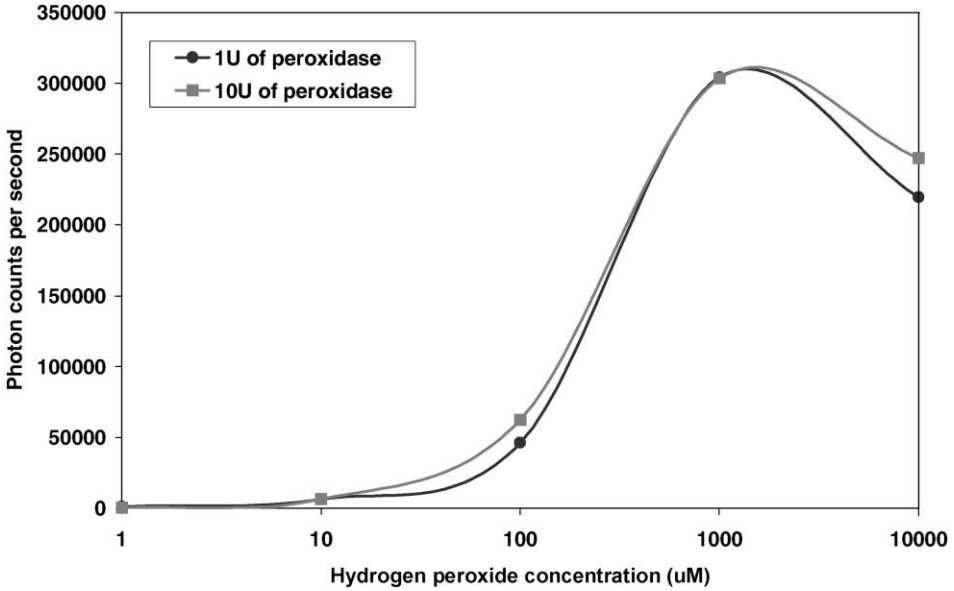


Fig. 2. The effect of the concentration of hydrogen peroxide and peroxidase activity (1 and 10 U) on the fluorescence intensity in 25 mM Tris–HCl buffer pH 8.5.

3. Results and discussion

The presence of many antioxidant components having different activities against various reactive oxygen species in biological samples causes the relative difficulty of measuring each antioxidant separately. Therefore, simple methods to estimate net or total antioxidant capacity of the samples are of considerable value [16]. Hydrogen peroxide together with superoxide radical anion can damage many cellular components and what is more important convert into more reactive oxygen species including hydroxyl radicals. The measurement of H_2O_2 scavenging activity can be one of the useful methods determining the ability of antioxidants to decrease the level of prooxidants such as H_2O_2. There are some spectrofluorimetric methods for the measurement of antioxidant activity, but only one for the measurement of H_2O_2 scavenging activity [1,5,8,17]. This last method is based on the oxidation of scopoletin to give a non-fluorescent product in the presence of HPRO and H_2O_2. If an antioxidant is incubated with H_2O_2 its ability to scavange H_2O_2 can be calculated from the fluorescence inhibition rate. We adopted the method for determining hydrogen peroxide level which utilizes HVA as a substrate as a method for measuring H_2O_2 scavenging activity. HVA appears to be an ideal spectrofluorometric substrate due to the fact that its solution is more stable and sensitive than that of scopoletin [9,14,15]. The conditions for the spectrofluorometric method were selected. The concentration of HVA was used according to [12,13]. The highest fluorescence intensity was obtained for 1 mM hydrogen peroxide and this concentration was chosen for the assay. The influence of peroxidase level and type of buffer was not significant, but due to the optimal conditions of the peroxidase–H_2O_2 reaction phosphate buffer pH 7.5 and 1 U of peroxidase were selected for further studies (Figs. 1 and 2). According to Li and Townshend [6] for the HRPO catalyzed reaction phosphate was used so that maximum fluorescence intensity could be obtained. Additionally, with increasing phosphate concentration the fluorescence intensity also increases [18]. In other assays, with the use of HVA as the substrate for the HPRO system phosphate buffer was used [12,13]. HVA is converted into a fluorescent dimer under the action of peroxidase and H_2O_2 and the reaction is complete within 1 min.

The fluorescence intensity of HVA was measured and was stable for at least several min (Fig. 3). The stability of fluorescence was checked for all tested

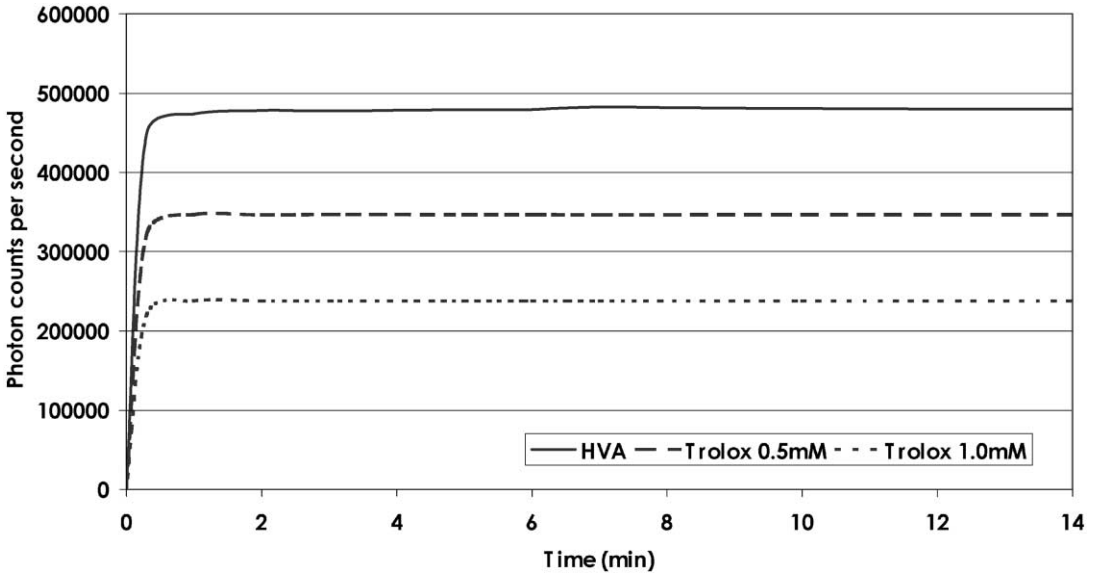


Fig. 3. The fluorescence stability of HVA and the reaction mixture containing standard antioxidant Trolox in two concentrations: 0.5 and 1.0 mM. The fluorescence intensity at 425 nm, plotted as a function of time, shows that the reaction is complete after 1 min.

compounds added to the reaction mixture and it was high. The fluorescence intensity for the standard antioxidant, Trolox, is presented (Fig. 3).

The H_2O_2 scavenging activity of tested compounds was measured in different concentration ranges. Trolox, a water-soluble analogue of Vitamin E, was used as a standard and the relationship between the percentage fluorescence inhibition and concentration was established. For comparison purposes the obtained results are presented as the percentage fluorescence inhibition using the following formula:

$$\text{inhibition ratio (\%)} = \frac{[(A - A_b) - (A_1 - A_b)]}{(A - A_b)} \times 100,$$

where A is the fluorescence of the HVA solution and A_1 the fluorescence of the reaction mixture containing the test sample. The fluorescence of the blank (A_b) was obtained without adding HVA to the reaction mixture and was subtracted from both A and A_1. It allows the influence of factors interfering with H_2O_2 and HVA–peroxidase system to be avoided.

Although H_2O_2 is a typical redox compound which has a high oxidation potential, most substances do not react rapidly with H_2O_2 and in order to achieve oxidation, activation of H_2O_2 is required. This is usually caused by the presence of transition metals [19,20]. Also, the presence of reducing sugars does not interfere with H_2O_2 in these assay conditions as aldoses are degraded only at low temperatures (0 °C), high concentration of H_2O_2 and under alkaline conditions [19]. Glucose is a good scavenger of hydroxyl radical but not H_2O_2 [21]. Schachl et al. [22] proved that simple sugars did not interfere with hydrogen peroxide in the H_2O_2 assay. Furthermore, glucose can be oxidized producing H_2O_2 only in the presence of glucose oxidase.

A linear relationship between the concentration of antioxidant and the percentage of HVA oxidation inhibition was obtained for Trolox (0.2–1.6 mM), BHA (0.02–0.3 mM), ferulic acid (0.005–0.2 mM), caffeic acid (0.05–2.5 mM), chlorogenic acid (0.025–2.5 mM), protocatechuic acid (0.05–6.0 mM), vanillic acid (2.0–20 mM), pyrogallol (0.2–1.7 mM) and oxalic acid (2.0–30 mM). The regression equations and correlation coefficients fitted by the least-squares method are presented in Table 1. The relative standard deviations for 10 replicate determinations of the following concentrations: 0.25, 0.5, 1.0, 1.5 mM Trolox were 1.1, 0.5, 0.6 and 0.4%, respectively; 0.06, 0.11, 0.17, 0.22, 0.277 mM BHA were 1.4, 0.7, 1.2, 1.0 and

Table 1
The regression equations and correlation coefficients fitted by the least-squares method for calibration of phenolic compounds and oxalic acid[a]

Phenolic compound	Regression equation	Correlation coefficient (R^2)
Trolox	$y = 58.99x - 5.69$	0.9999
Ferulic acid	$y = 450.54x - 0.259$	0.9999
BHA	$y = 100.1x - 1.430$	0.9966
Pyrogallol	$y = 50.98x + 0.340$	0.9928
Caffeic acid	$y = 43.48x + 3.243$	0.9987
Chlorogenic acid	$y = 21.65x + 6.751$	0.9971
Protocatechuic acid	$y = 9.290x + 5.490$	0.9939
Oxalic acid	$y = 2.99x + 4.834$	0.9977
Vanillic acid	$y = 2.212x + 3.316$	0.9962

[a] y: fluorescence intensity (arbitrary units), x: constration (μM).

0.8%, respectively; 0.01, 0.05, 0.1, 0.15 mM ferulic acid were 1.0, 1.1, 0.9 and 0.5%, respectively; 0.055, 0.55, 1.1, 2.0 mM caffeic acid were 2.8, 2.3, 1.1 and 0.9%, respectively; 0.028, 0.28, 0.56, 1.41, 2.25 mM chlorogenic acid were 2.6, 2.0, 1.9, 1.6 and 1.1%, respectively; 0.065, 0.65, 1.3, 3.24, 5.2 mM protocatechuic acid were 2.7, 2.3, 2.0, 1.7 and 1.1%, respectively; 2.97, 5.95, 11.9, 17.85 mM vanillic acid were 1.9, 0.9, 1.3 and 0.8%, respectively; 0.39, 0.79, 0.99, 1.19, 1.59 mM pyrogallol were 1.8, 1.1, 1.3, 1.0 and 0.7%, respectively and 5.5, 11.1, 16.66, 22.21, 27.76 mM oxalic acid were 1.3, 1.5, 1.1, 0.9 and 0.6%, respectively.

The H_2O_2 scavenging activity of biological samples was evaluated. Three concentrations (0.1, 0.5 and 1.0%) of several herb and tea infusions known for their antioxidant properties were tested (Fig. 4). The highest activity was shown in the case of lemon balm followed by green tea, black tea and peppermint. Nettle and aniseed infusions exhibited the lowest activity. Rooibos tea sample possessing significant antioxidant activity against superoxide anion radical [23] showed only slightly over 50% activity than that obtained for lemon balm. Comparison was made with a calibration graph generated using Trolox. The unit of antioxidant activity (Trolox equivalent) was defined as the concentration (mmol l^{-1}) of Trolox having the equivalent antioxidant activity to 1 mM solution of phenolic compounds and oxalic acid or 1 mg ml^{-1} solution of herb and tea infusions (Tables 2 and 3). Ferulic acid showed the strongest H_2O_2 scavenging activity expressed as a molar Trolox equivalent which was about eight times

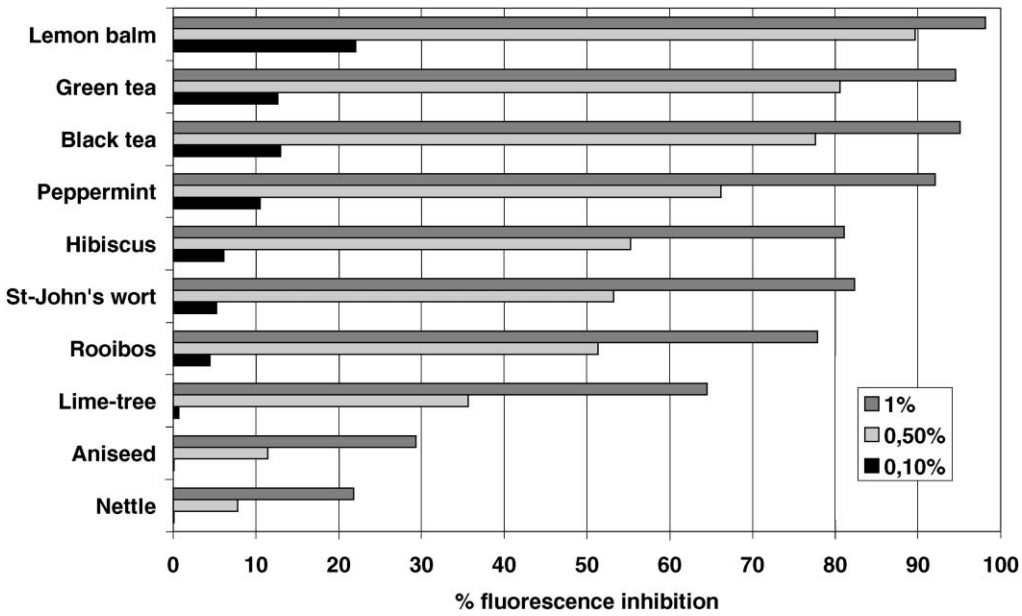


Fig. 4. The comparison of H_2O_2 scavenging activity of tea and herb infusions (0.1, 0.5 and 1.0%) expressed as a percentage of fluorescence inhibition of HVA.

Table 2
Antioxidant activity (Trolox equivalent) of phenolic compounds ($mmol\,l^{-1}$)

Phenolic compound	Trolox equivalent
Trolox	1.00
Ferulic acid	7.73
BHA	1.82
Pyrogallol	0.97
Caffeic acid	0.89
Chlorogenic acid	0.58
Protocatechuic acid	0.35
Oxalic acid	0.23
Vanillic acid	0.19

that obtained for Trolox while BHA exhibited less than twice the activity of Trolox. The majority of the herbs and tea infusions showed relatively little antioxidant action against H_2O_2. Only four of the examined infusions showed substantial antioxidant activity as follows: melissa 0.42 mM, black tea 0.25 mM, green tea 0.24 mM and peppermint 0.20 mM. The results obtained by Mantle et al. [24] also indicate that lemon balm had the strongest antioxidant activity of all the examined plant essential oils when tested against oxoferryl radical species (determined via chemiluminescence assay).

The spectrofluorometric method utilizing HVA acid as a substrate is useful for rapid estimation of H_2O_2 scavenging ability. However, using this method one should consider two matters. First, if the antioxidant is not a substrate for peroxidase and does not compete with hydrogen peroxide, thus, causing a change of fluorescence intensity independent of antioxidant activity. Due to the fact that ascorbate and thiol compounds are examples of substrates for most peroxidases, ascorbate was not estimated in this spectrofluorometric method, although they show antioxidative properties against superoxide radical, hydroxyl radical and hydrogen peroxide. Secondly, superoxide radical anion inhibits peroxidase and may affect fluorometric measurements in systems generating $O_2^{\bullet -}$ [1,5].

Table 3
Antioxidant activity (Trolox equivalent) of herbs and tea infusions ($mg\,ml^{-1}$)

Herbs and tea infusions	Trolox equivalent
Melissa	0.47 ± 0.05
Black tea	0.32 ± 0.02
Green tea	0.31 ± 0.03
Peppermint	0.27 ± 0.02
Hibiscus	0.20 ± 0.02
St. John's wort	0.18 ± 0.02
Rooibos	0.17 ± 0.01
Lime-tree flower	0.11 ± 0.01
Aniseed	>0.01
Nettle	>0.01

The described spectrofluorometric method is another assay in addition to the system using oxidation of fluorescent scopoletin that enables easy and relatively sensitive measurement of hydrogen peroxide scavenging activity. It has to be remembered that methods assaying total antioxidant activity (TAA) need to be interpreted on the basis of the type of reactive oxygen species (ROS) and the source of oxidative stress. Additionally, most of the in vitro analytical methods are based on the antioxidant activity measurement of the selected ROS and the assessment of total antioxidant activity requires the use of many different assays utilizing relevant ROS.

4. Conclusions

The present fluorimetric method may be useful for rapid quantifying the H_2O_2 scavenging activity and may be convenient for the evaluation of the antioxidant activity of phenolic compounds as well as natural antioxidants in biological material.

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