SHORT COMMUNICATION

Antioxidant activity evaluation by physiologically relevant assays based on haemoglobin peroxidase activity and cytochrome c-induced oxidation of liposomes

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ABSTRACT
Two new protocols for exploring antioxidant-related chemical composition and reactivity are described: one based on a chronometric variation of a haemoglobin ascorbate peroxidase assay and one based on cytochrome c-induced oxidation of lecithin liposomes. Detailed accounts are given on their design, application, critical correlations with established methods and mechanisms. These assays are proposed to be physiologically relevant and bring new information regarding a real sample, both qualitative and quantitative. The well-known assays used for evaluation of antioxidant (re)activity are revisited and compared with these new methods. Extracts of the Hedera helix L. are examined as test case, with focus on seasonal variation and on leaf, fruit and flower with respect to chromatographic, spectroscopic and reactivity properties. According to the set of assays performed, winter are the most antioxidant, followed by summer leaves, and then by flowers and fruits.

KEYWORDS Antioxidant (re)activity assays; haemoglobin ascorbate peroxidase assay; liposome peroxidation; principal component analysis; Hedera helix

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The Supplementary data contain Experimental sections, 16 additional figures, 2 tables and additional results and discussions http://dx.doi.org/10.1080/14786419.2015.1054824. 
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1. Introduction
It is accepted nowadays that the mechanism of action of antioxidants extends far beyond free radical scavenging or oxidants cleaning, with rather a multifaceted restoration of numerous redox signalling pathways (Jones 2006; Carocho & Ferreira 2013; López-Alarcón & Denicola 2013). Furthermore, there is consistent evidence that in certain situations, compounds that are widely considered as antioxidants may behave as prooxidants, depending on dosage, pH, chemical and enzymatic interaction (Procházková et al. 2011; Mot et al. 2014). In this context, while studying several natural extracts, we recently proposed three new directions for antioxidant capacity evaluation: one based on the interaction between a physiologically relevant antioxidant and haemoglobin; one involving semiquinone anion radicals generated by alkali treatment of a given extract; and another one based on the inhibition of induced peroxidation of liposomes (Mot et al. 2009; Mocan et al. 2014; Mot et al. 2015). In the present work, we mainly shed new light on the first and the third of these new assays, describe them in greater detail as well as emphasise the links between them and other well known in vitro antioxidant assays taking ivy leaf, fruit and flower extracts as a case study. *Hedera helix* L. (ivy) folium extracts are widely known, both in traditional and modern pharmacological usage, for their spasmolytic/bronchodilating activity, anti-inflammatory, antibacterial, antiviral, antimycotic, hepatoprotective, anti-coughing effects and others.

2. Results and discussion
2.1. Assays based on the inhibition of induced peroxidation of liposomes and on β-carotene bleaching
Inhibition of artificially induced peroxidation of lipids (linoleic acids, LDL and whole serum protein fraction) is a well-known method which gained popularity due to its similarity to the physiological phenomena, especially using LDL isolated from fresh blood samples and using either Cu(II) or azo initiators (Huang et al. 2005; Karadag et al. 2009; Niki 2010). However, this assay presents some easily observed drawbacks – such as inconsistency of LDL preparations due to the necessity to obtain blood samples from different individuals, the fact that Cu(II) alone fails to trigger the reaction thus requiring the presence of antioxidants (and due to the Fenton-like reaction of *in situ* generated Cu(I), the antioxidants may act as pro-oxidants), need of high dilution of LDL samples, and the fact that some antioxidants may chelate Cu(II) and stop its triggering effect. In order to correct some of these shortcomings but maintain physiological relevance, we propose to use lecithin-based liposomes instead of LDL, thus strongly increasing its reproducibility, and use cytochrome *c* as initiator, as liposome-cytochrome *c* interaction is well documented for decades (Goñi et al. 1985). Following the 236 nm absorbance, a sigmoidal curve was registered for all samples in 1000 min, all being less sharp than the control – as a manifestation of the antioxidant effect (Figure S1, Supplementary data). Two distinct parameters are proposed for effectively describing the antioxidant capacity of the samples: L1 (in min, inflection point, a measure of the length of the lag phase in essence) and L2 = L3 (in Abs min⁻¹, describing the steepness of the process, both describing the same fact, but differently calculated) as illustrated in Figure 1. L1 is expected to increase, and L2/L3 are expected to decrease, with the antioxidant capacity. L1, L2 and β-carotene bleaching results are plotted in Figure 1.

In the β-carotene bleaching assay, the induced bleaching of the carotenoids by light, heat and peroxyl radicals (azo compound or oxidising lipids) is inhibited by the presence of the antioxidants (Karadag et al. 2009 and references cited therein). This method is more rarely used, due to its numerous reaction pathways. Trials to check the concentration dependence
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for rutin (the major flavonoid component of the extracts) revealed a non-linear relationship, hampering efforts to use a calibration curve for standardisation of the results (Figure S2, Supplementary data). The final results (Figure 1) are the degree of inhibition of a given sample at same concentration – but such values are useful just for comparisons within a given set of samples. For a more standardised method, an IC$_{50}$ value must be calculated. Very good correlations are observed between the β-carotene assay and TEAC, GAE, QF, EC$_{50}$; poor correlations are seen with ORAC, despite their similarity in mechanism (HAT). Correlations between L1–L3 and other parameters are discussed below. The known methods TEAC, GAE, ORAC and Folin–Ciocalteu are detailed and discussed in Supplementary data, Figures S3–S13.

2.2. Inhibition of haemoglobin ascorbate peroxidase activity (HAPX) assay

The HAPX assay was firstly discussed and developed for propolis extracts (Mot et al. 2009) and then successfully used for other natural extracts (Benedec et al. 2013; Tamokou et al. 2013). The first protocol proposed and used so far was based on inhibition of ascorbic acid consumption by the antioxidants present in the sample, by monitoring the slope at 290 nm (ascorbic acid) using the peroxidase activity of haemoglobin (HAPX1). This method may be physiologically relevant and useful for some of the extracts, but has some shortcomings. Thus, when following the absorbance in UV at 290 nm the degree of interference is very high and some of the oxidised compounds during the peroxidase cycle may absorb at this wavelength. For this reason, we kept the entire protocol (and the chemistry behind it) but now shift to monitor the haemoglobin involved in the assay rather than the substrate (assay now denoted HAPX2). The protocol has been detailed recently (Mot et al. 2015). This is of great importance since the interference from natural extracts at 405 or 575 is much less likely than at 290 nm, and haemoglobin has a very high extinction coefficient, enabling it to be monitored even at low concentrations. When met-haemoglobin (Hb-Fe(III)) is mixed with hydrogen peroxide in presence of ascorbic acid (and sample antioxidants), the haemoglobin is oxidised to HbFe(IV) but is quickly reduced by the ascorbic acid back to Fe(III); thus, as long as ascorbic acid (and tested antioxidants) is present in the mixture, haemoglobin mainly exists as met form (relatively stable absorbance at 405 nm). As soon as the ascorbic acid (or antioxidants) is completely oxidised, met is promptly transformed into ferryl (HbFe(IV)) by the remaining hydrogen peroxide, producing a sudden change in UV–vis spectra (Figure 2(A)). The higher the antioxidant level, the longer the time haemoglobin exists as met form.
Ascorbic acid is very important to be added in both control and samples, since it has a great affinity for haemoglobin and may act as a mediator between antioxidant molecules which have low affinity for haemoglobin. Another great advantage of this protocol (HAPX2) is that a calibration curve may be used and the results can thus be standardised for further comparisons. In this study, we used rutin as standard compound (the main polyphenolic component of the studied extracts) and a good calibration curve could be constructed (Figure S14, Supplementary data). Hence, the rutin equivalents of the samples were determined using this assay (HAPX2). The results obtained have a much higher correlation with other ET methods than the HAPX1, and greater discrimination between the antioxidant capacities of the samples (Figure 2(C)).

(Figure 2(B)).

2.3. Seasonal and plant parts variation of polyphenolic composition in H. helix extracts

H. helix extracts contain a variety of active compounds as reviewed. Though phenolic acids were identified by some other authors, there is only one paper which quantifies some flavonoids (Trute & Nahrstedt 1997). In this study, we identify and quantify using HPLC-DAD-MS 5 important phenolic components out of 18 standards available. A table containing the results can be found in Table S1 and Figure S15 (Supplementary data). It is visible that rutin and quercetin after hydrolysis levels follow the same pattern as main antioxidant assays (ORAC, TEAC, GAE, HAPX2, DPPH). Leaves from winter (December) seem to be richest in polyphenolic compounds, in good agreement with total phenolic content assay (GAE). Correlations between most of the previously mentioned parameters and their values suggest importance for this activity. A detailed comparison between the methods may be found in the Supplementary material.
3. Conclusions

Extracts of the *H. helix* L. are examined in terms of antioxidant-related chemical composition and reactivity, with focus on seasonal variation and on leaf, fruit and flower. Two new protocols for addressing such issues are proposed and described, with detailed account on design, application, mechanisms and correlation with established methods: one based on a chronometric variation of a haemoglobin ascorbate peroxidase assay and the other based on cytochrome c-induced oxidation of lecithin liposomes. *H. helix* leaf extract collected in December appears to be most antioxidant according to majority of assays.

Disclosure statement

No potential conflict of interest was reported by the authors.

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