SUPPLEMENTARY MATERIAL

Antioxidant activity evaluation by physiologically relevant assays based on hemoglobin 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 hemoglobin 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. Principal component analysis (PCA) allow straightforward comparisons of these antioxidant assays based on mechanism and reinforce the need to use more than a single parameter in examining such reactivity. 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.

Keywords: antioxidant (re)activity assays; hemoglobin ascorbate peroxidase assay; liposome peroxidation; principal component analysis; *Hedera helix*.

Experimental section

1.1. Chemicals. AAPH (2,2'-azobis-2-methyl-propanimidamide dihydrochloride), DPPH (di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium), beta-carotene, rutin, kaempferol, tween, linoleic acid, methanol, ethanol, trolox, soy lecithin, chloroform, horse heart cytochrome *c*, ascorbic acid, sodium hydroxide are of high analytical purity and obtained from several companies (Sigma, Fluka, Merck). Standards: chlorogenic acid, p-coumaric acid, caffeic acid, rutin, apigenin, quercitrin, isoquercitrin, hyperoside, kaempferol, quercetol, myricetol and fisetin from Sigma (Germany); ferulic acid, sinapic acid, gentisic acid, patuletin and luteolin from Roth (Germany); and cichoric acid and caftaric acid from Dalton (USA). Methanol of HPLC analytical-grade and hydrochloric acid of analytical-grade were purchased from Merck (Germany). Methanolic stock solutions (100 mg mL^{-1}) of the above standards were prepared and stored at 4°C , and protected from daylight. They were appropriately diluted with double distilled water before being used as working solutions.

1.2. Extract preparation. Ivy (*Hedera helix* L.) was collected from the A. Borza Botanical Garden of Cluj-Napoca ($46^{\circ}45'36''\text{N}$ and $23^{\circ}35'13''\text{E}$) and was identified by Dr. M. Parvu, Babes-Bolyai University of Cluj-Napoca. A voucher specimen (CL 664210) is deposited at the Herbarium of Babes-Bolyai University, Cluj-Napoca, Romania. Small fragments (0.5-1 cm) of *H. helix* L. (ivy) were extracted with 70% ethanol (Merck, Bucuresti, Romania) in the Mycology Laboratory of Babes-Bolyai University, Cluj-Napoca, Romania by cold repercolation method (Mishra and Verma, 2009 and Gurumoorthi, 2012) at room temperature, for 3 days (Gurumoorthi, 2012). The ivy fresh material was harvested at different time intervals: leaves in 18th of June 2011 (P 1), 23th of September 2011 (P 2), 29th of December 2011 (P3); green offshoots in 18th of June 2011 (P 4); flowers in 23th of September 2011 (P 5); fruits in 29th of December 2011 (P 6). The content of plant extracts (w/v; g/ml) was: 1/1 for P1, P3 and P6; 1/1.1 for P2 and P5; 1/1.5 for P4.

1.3. Oxygen radical absorbance capacity (ORAC) assay. A stock solution of 12 mM AAPH in PBS buffer was made. From this solution, an aliquot of 3000 μL was transferred into a 3.5 mL quartz cuvette using a RAININ automatic pipette and was placed in the cuvette holder of a fluorescence spectrophotometer (Perkin Elmer, LS55) which was coupled to a water based thermostat (Julabo, model ED) set at 37°C . The excitation wavelength was 485 nm and the emission intensity was monitored at 515 nm. After 5 min of thermal equilibration, 2 μL of 10 μM fluoresceine (6.6 nM, final concentration) was added and 5 μL of 100 times diluted extract were added in the cuvette and start to monitor the fluorescence intensity at 515 nm for 30 min. A blank sample (without any extract) was also performed.

For the calibration curve, suitable small aliquots (1-16 μL) of trolox standard solution were added in place of extract so that the final concentrations were 0.5, 1, 2, 4, 8 μM trolox. The area under the curve (AUC) was calculated by integration of the kinetic curves using Origin 6.1 software and the net area represents AUC of any sample or standard after subtraction of AUC belonging to the blank. Each experiment was done in duplicates. The calibration curve was obtained by plotting the net area of the standards vs. their concentration and the linear function fitting equation ($R = 0.9983$) was used to express the antioxidant capacity of all tested samples in ORAC units ($\mu\text{moles trolox equivalents (TE)}/100 \text{ g plant}$).

1.4. DPPH bleaching assay. An ethanolic 100 μM DPPH stock solution was prepared and checked for its stability for 30 min by monitoring the absorbance at 517 nm. For each of the six samples, six different aliquots of suitable volumes depending on samples (so as to reach concentrations between 0.6 – 20 mg plant material/mL, depending on samples, commonly between 1 – 20 μL extract) were added to a proper volume of DPPH ethanolic solution so that the final volume was 1000 μL in the quartz cuvette, and the bleaching of the DPPH was kinetically monitored for 30 min at 517 nm using a UV-vis spectrophotometer (Varian, Cary 50) equipped with a multi-cell holder. Typical decay curves were obtained for every sample. The 517 nm absorbance was corrected for dilution effect. The percentage of DPPH remained unbleached after the reaction time was calculated for all tested samples and using a plot of these values vs. extract concentration a curve was generated which was fitted to a first order exponential decay function thereby allowing the calculation of the EC_{50} (efficiency concentration as defined by (Sanchez-Moreno et al. 1998), equivalent of the $T_{1/2}$ value of the curve, calculated in concentration units (mg/mL)) for all samples. The smaller the EC_{50} value the greater the antioxidant capacity. After the EC_{50} parameter was determined, each sample was reassessed in duplicates at the exactly the EC_{50} concentration for 30 min. The kinetic curves were registered and fitted with a second order exponential decay function from which the $T_{\text{EC}_{50}}$ parameter was calculated as the time required for DPPH to accomplish 99.9 % of the reaction. A more complex time-consuming route is to calculate this time parameter for all assessed concentrations and then by interpolation to calculate the reaction time at EC_{50} which represents $T_{\text{EC}_{50}}$. The AE (antioxidant efficiency) parameter was calculated as defined by [20], $\text{AE} = 1/(\text{EC}_{50} \times T_{\text{EC}_{50}})$. For comparison, the percentage of DPPH consumed in 30 min ($\%\text{DPPH}_{30\text{min}}$), at the same sample concentration (3 mg plant/mL) and the quercetin factor (QF) were also experimentally determined as described in (Moř et al. 2011a).

1.5. Trolox equivalent antioxidant capacity (TEAC) assay. The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) radical was enzymatically obtained by 2 h treatment of 2 mM reduced ABTS solution in 5 mM sodium acetate pH 5.5, with 50 nM zucchini peroxidase and 1.3 mM

hydrogen peroxide. The radical was separated from the enzyme using a 10 kDa cut-off Amicon filter. For this assay, end-point experiments were performed, using 10-fold diluted extract samples. In a quartz cuvette, 50 μ L ABTS radical was added along with 950 μ L 5 mM sodium acetate buffer (pH 5.5) and 5 μ L ten times diluted sample. The decrease of the ABTS radical absorbance was monitored at 420 nm for 20 min. Experiments were carried out in triplicates and a reference experiment in which the sample was replaced with 35% ethanol solution was also performed. The percentage of ABTS radical consumption was converted into trolox equivalent (TE) by means of a calibration curve ($R = 0.999$) using trolox standard solutions of 0 – 16 μ M.

1.6. Folin-Ciocalteu reagent based assay. The Folin–Ciocalteu reagent was prepared as described in (Mot et al. 2009). For each sample, 5 μ L of extract were added to 795 μ L water and 50 μ L Folin–Ciocalteu reagent, thus obtaining 850 μ L solution which were incubated in the dark for 5 min. Then, 150 μ L of 20% sodium carbonate solution were added and samples were incubated in the dark for further 30 min. The solution turned deep blue to different degrees, depending on samples. At the same time, gallic acid standards of 1, 2, 4, 8 mg/L final concentration solutions were treated with the Folin–Ciocalteu reagent in the same way as the assayed samples. The absorbances at 750 nm were recorded against the reference solution (zero gallic acid). The measurements were done in duplicates. For the gallic acid standards, a calibration curve was constructed ($R > 0.999$, $p < 0.000$) and the total level of electron-rich components (mainly known as total phenolic content) for each sample was determined in terms of gallic acid equivalents (mg GE /g plant material). For comparison, the phenolic content was also estimated from the UV-vis spectra. The extracts were diluted 100 times in water and for these diluted samples the UV-vis spectra were recorded in duplicates. The absorbance at 280 nm was used for phenolic content estimation using the equation $TPC = Abs_{280nm} \times 100 - 4$.

1.7. Inhibition of induced β -carotene bleaching assay. In a round-bottom flask, 5 mg of β -carotene, 25 μ L linoleic acid and 200 mg tween 20 were dissolved in 3 mL chloroform by sonication. The chloroform from the obtained clear solution was quickly removed under vacuum at 40 $^{\circ}$ C (the complete removal of chloroform is mandatory), the clear residual oily material was dissolved in 50 mL ultra-pure water and the obtained solution was named β -carotene reagent. In each quartz cuvette placed in a multi-cuvette holder attached to a Varian (Cary 50) spectrophotometer and coupled to a water-based thermostat, 950 μ L of β -carotene were added and allowed to stabilize at 50 $^{\circ}$ C for 4 minutes; 50 μ L sample were then added, the cuvettes covered with parafilm, and the absorbance monitored between 350 – 600 nm for 250 min. All extracts were assessed at the same time. For each extract two measurements were performed in two independent experiments. A control experiment, *i.e.* with the sample replaced by 35 % ethanol, was also performed. The kinetic curves at 450 nm were registered and the percentage of

the bleached β -carotene (%B_i) after 250 min was calculated. The degree of inhibition of β -carotene bleaching was calculated by the equation $I\% = (\%B_{\text{control}} - \%B_{\text{sample}})/\%B_{\text{control}}$. Additionally, two solutions of 50 μM rutin and kaempferol respectively were tested. Furthermore, a calibration curve with rutin standard solution between 5 – 70 μM ($I\%$ vs. [rutin]) was built.

1.8. Inhibition of induced peroxidation of liposomes. In a 1.5 mL Eppendorf tube, 5 mg of lecithin were suspended in 1 mL ultra-pure water and sonicated in a water bath for 30 min. In each quartz cuvette placed in a multi-cell holder attached to a Varian (Cary 50) spectrophotometer, 960 μL of 20 mM pH 7.4 sodium phosphate were added, followed by the addition of 30 μL freshly obtained liposome fine suspension, 3 μL 10 times diluted extract and 7 μL horse heart cytochrome *c* (4 μM final concentration). A blank sample (the extract was replaced with solvent) and a positive control (2.2 μM final concentration of quercetin) were also performed. Each tested sample was done in duplicates. The quartz cuvettes were covered with parafilm and monitored at 236 nm for 1000 min. The obtained sigmoidal curves were fitted with a Boltzmann equation and three parameters were calculated for antioxidant capacity estimation (L1, L2, L3), the time where the inflection point is located (L1), calculated from the first derivate function of the curve, the value of the derivative of the curve at the inflection point (L2) and the steepness of the curve after the lag phase (slope of the linear segment) calculated from the Boltzmann equation (L3). The last two parameters bear similar information, but differently calculated.

1.9. Inhibition of hemoglobin ascorbate peroxidase activity (HAPX) assay. The HAPX assay was run according to two procedures, first (HAPX1, kinetic procedure) as described in (Mot et al. 2009) and second (HAPX2, new chronometric procedure) as detailed bellow, after several preliminary experiments. In a quartz cuvette 979 μL of 50 mM sodium acetate pH 5.5 were added, followed by addition of 4 μL 50 mM ascorbic acid, 4 μL 120 mM hydrogen peroxide, and 5 μL extract – after which the absorbance changes were monitored between 300 – 700 nm for 60 s. The reaction was triggered by the addition of 8 μL 1.6 mM met hemoglobin (metHb) and further monitored for 20 min. Control experiments (no extract) were also performed. Each experiment was done in triplicates. From the triggering, the reaction was monitored at both 575 nm and Soret band (405 nm) until sudden changes at these wavelengths started; the time interval required for the lag phase was registered (T_r). A calibration curve based on T_r at several rutin concentrations (3, 6, 10, 13 μM) was used for converting the antioxidant capacity, according to this assay, in rutin equivalents (RE) ($\mu\text{mol RE}/100\text{ g plant}$).

1.10. High-Performance liquid chromatography (HPLC) measurements and analysis. The experiment was carried out using an Agilent 1100 HPLC Series system equipped with an autosampler G1311A. For the separation, a reversed-phase Zorbax SB-C18 analytical column (100 $\times$ 3.0 mm, 3.5 mm

particle) was used. The column was operated at 48 °C in a G1316A oven. For the gradient elution, a degasser G1322A and quaternary gradient pump G1311A were employed. The detection of all the compounds was performed at 330 and 370 nm using G1316A diode array detector system, and the chromatographic data were processed with a Chemstation software (Agilent, USA). The mobile phase was prepared from methanol and acetic acid 0.1 % (v/v). The elution began with a linear gradient (started at 5 to 42% methanol) for the first 35 min, followed by isocratic elution with 42 % methanol for the next 3 min. The flow rate was 1 mL min⁻¹ and the injection volume was 5 µL. All solvents were filtered through 0.5 µm filters (Sartorius) and degassed in an ultrasonic bath. For liquid chromatography (LC) electrospray ionisation (ESI) mass spectrometry (MS) analysis, an Agilent Ion Trap 1100 SL instrument was used. The MS was equipped with Turbo-Ionspray (electrospray ionisation) interface, negative ion mode. ESI settings were: negative ionisation, ion source temperature: 350 °C, gas: nitrogen at 12 L min⁻¹, nebuliser: 70 psi. A high performance liquid chromatographic method has been previously developed and successfully applies for the determination of phenolic compounds from 6 extracts of ivy. The simultaneous analysis of different classes of polyphenols was performed by a single column pass, and the separation of all examined compounds was carried out in 35 min. In order to obtain more accurate data on flavonoid glycosides and aglycones concentration, and to estimate the nature of hydrolyzed compounds, each sample was analyzed before and after acid hydrolysis. The concentrations of identified polyphenolic compounds in all samples before and after acid hydrolysis were determined. Two milliliters of extractive solution were treated with 2 mL 2 M hydrochloric acid and 0.2 mL ascorbic acid solution 100 mg mL⁻¹; the mixtures were heated at 80 °C on a water bath for 30 min, sonicated for 15 min and heated for another 30 min at 80 °C. During the heating, 1 mL methanol was added to the extraction mixture for every 10 min, in order to ensure the permanent presence of methanol. The mixtures were centrifuged at 4000 rpm and the solutions were diluted with distilled water in a 10 mL volumetric flask and filtered through a 0.45 µm filter before injection.

1.11. Statistical analysis. All the measurements were done in multiple replicates and the deviation standard and standard error of the mean was calculated for evaluation of the precision of the measurement. Statistical analysis was performed using Statistica 7.0 for Windows (Stat-Soft, Inc., USA). Box and Whisker plot and Pearson correlation were used to examine the strength of associations between the results. The experimental data were evaluated using the classical ANOVA one-way analysis of variance. All the statistical results confirm the hypothesis that the differences between the results are highly significant ($p < 0.000$). Multivariate data analysis was performed on the entire eighteen antioxidant parameters using PCA (Principal Component Analysis) incorporated in Statistica software. The main purpose of PCA is to conveniently represent the location of the objects (samples) in a reduced

coordinate system where, instead of m -axes (corresponding to m variables), only p ($p < m$) are used to describe the data set with the maximum possible information (in well models, two or three components contain almost all information from the primary matrix).

Supplementary results and discussions

The scientific interest in the antioxidant activity of numerous synthetic and natural products, both *in vitro* and *in vivo*, as well as development of standardized assays for determination of their antioxidant capacity continues to be high in the last decades. As this domain expands and the chemistry and the mechanisms behind the antioxidant activity and related directions continue to gain new insights, the necessity to strengthen more relevant methods and to correct the shortcomings of others is more obvious (Huang et al. 2005; Prior et al. 2005; Jones 2006; Niki 2010). There is a growing claim that the *in vitro* antioxidant activity assays poorly describe the real antioxidant properties of natural products and the need to develop more physiological relevant and *in vivo* methods becomes a real necessity (Frankel & Finley 2008; López-Alarcón & Denicola 2013).

Since different scientists from various fields of research, with different views and with vast types of trainings used the numerous classic and new proposed methods for antioxidant activity evaluation, the standardization and comparison between the published results are still difficult tasks. The known antioxidant capacity evaluation assays involve different mechanisms of action, different pH values, solvent, different chemistry behind the result - and may reflect to different degrees the quantity, reactivity and kinetic properties of the assessed sample. Since the sample in interest is usually a very complex mixture, sometimes poorly known in terms of chemical composition, the choice of a *most suitable* method to assess its antioxidant capacity is a difficult (if not avoidable) task, and most of the times several methods are recommended to be performed, bringing a “holistic” picture of the sample in terms of antioxidant activity. For these considerations and many others, a recent excellent IUPAC technical report is available (Apak et al. 2013). In the present study we present and discuss the results for each method (group of methods), after which a comparative discussion is presented.

The key chemical components from *Hedera helix* extract, which are thought to be responsible for these effects are triterpene saponins, flavonoids, coumarins, phenolic acids, alkaloids, sterols (Trute & Nahrstedt 1997; Bedir et al. 2000). Systematic studies on antioxidant activity of whole *H. helix* extracts are not available, much less seasonal variation of this property, despite the fact that this is thought to be responsible for some of the biological activities (Liu & Liu 1997).

2.1. ORAC assay

The ORAC assay is one of the most popular assays and also known to nutritionists and common people, thus its usage is a need when the results are wanted to be compared to other food/nutritional supplements. ORAC measures antioxidant inhibition of peroxy radical induced oxidations and thus reflects classical radical chain breaking antioxidant activity by H atom transfer (HAT mechanism) (Prior et al. 2005). Using high quality calibration curves and good accuracy for sample measurements of the kinetic curves (Figures S3 and S4), the ORAC results for the studied extracts are presented in Figure S5, and they place the *H. helix* extracts in the medium-high range of common foods (Apak et al. 2013). According to this assay, the leaves are highest in antioxidant activity in spring-early summer, followed by winter-time leaves, and then with values reduced to half in the fall leaves. While the young offshoots have much poorer antioxidant capacity, *H. helix* flowers poses high antioxidant activity whereas the fruit has less activity. From the shape of the kinetic curves in the ORAC assay one may infer either quantitative information (the amount of the antioxidants present in the sample) or the reactivity, depending on the reference probe and the sample (Niki 2010). In our case, the shapes of the decay curves (Figure S4) present distinct lag phases, which means that the reactivity of the antioxidants towards *in situ* generated peroxy radicals in the sample is higher than the one of the fluoresceine and thus the total concentrations of antioxidants can be accurately measured.

2.2. DPPH bleaching assay

Despite important drawbacks, the DPPH assay continues to be highly used for biologically relevant antioxidant capacity evaluation, due to its simplicity and low cost (Prior et al. 2005; Apak et al. 2013). The simple end-time measurement of DPPH percentage bleached at a given concentration of a natural extract cannot be accepted as a measure for its antioxidant capacity. However, when a large number of samples are to be measured, for a fast estimation of their antioxidant capacity, the parameter QF (quercetin factor) which is based on the entire profile of kinetic curve for DPPH bleaching process and a calibration curve of a standard (quercetin) is a suitable choice (Moř et al. 2011b). The use of this parameter is limited due to the need of chemometric processing of the curves (Principal Component Analysis). The QF values for the studied extracts are found in Figure S6. The most known parameters obtained from the DPPH assay are antiradical efficiency (AE), effective concentration (EC_{50}) and its corresponding time needed to reach the steady state ($T_{EC_{50}}$) (Sánchez-Moreno et al. 1998; Karadag et al. 2009). From the curves of the dependence of percentage of unbleached DPPH after 30 min upon extract concentration (Figure S7), the EC_{50} was calculated according to the definition (Gurumoorthi, 2012). All

these three parameters can be found in Figure S9. The EC₅₀ values are slightly higher than the typical ones for plant extracts but the T_{EC50} values classify these extracts in terms of kinetics as rapid except for the *flower* extract which is intermediate (Sánchez-Moreno et al. 1998; Karadag et al. 2009). Based on the DPPH results (AE parameter), leaves from September are about double fold more antioxidant than those from June and December which are comparable to *flower* extract while offshoot and fruit extracts are less antioxidant. The poor correlation between DPPH results and others (*vide infra*) can be explained by the fact that this assay's parameters do not contain information regarding the reactivity of the antioxidants or the reaction stoichiometry, and are also strongly influenced by steric effects (Niki, 2010 and Apak et al. 2013). The mechanism of action in this assay is nowadays accepted to be a mixture of HAT and ET, mainly dominated by the last one.

2.3. TEAC and Folin-Ciocalteu reagent based assays

In 2005, Prior and coworkers, after a consistent discussion upon the antioxidant evaluation assays available at that time, suggested that besides ORAC assay, TEAC and Folin-Ciocalteu method (abbreviated here GAE, gallic acid equivalents) should be considered for standardization (Prior et al. 2005). According to their opinion, up to that time, these three methods appeared to be the most accurate, reproducible, robust and with well known chemistry and mechanism, thus being the only good candidates for standardization and common use. However, in the last decade, numerous papers describing several types of methods for antioxidant capacity evaluation with different methodologies and views were published. The main possible reasons for this might be the correction of the shortcomings of other available methods and the proposals of more physiological relevant conditions as well as development of other assays more compatible with living cells phenomena. Despite their excellent importance, the TAEC and Folin-Ciocalteu methods are far for reproducing biological systems and have numerous compounds which may act as interferents such as sugars, thiols, aromatic amines for Folin-Ciocalteu reagent and formation of strongly coloring ABTS-phenolic compounds/tyrosine residues adducts in the TEAC assay, a less known and mentioned fact (Osman et al. 2006; Akerström et al. 2007).

The TEAC and GAE results appear to be well correlated for our analysed samples (Figure S12). Both methods are based on ET mechanisms and are employed in aqueous systems. A very good correlation between the results of these assays and the ORAC assay may be observed except for the *Leaf June* extract, which behaves as an outlier (is much more antioxidant in ORAC assay than estimated by TEAC and GAE). A possible explanation is that this extract may have some components which are strong peroxy scavengers (thus well detected by ORAC) but poor electron donors thus weak

contributors in the TEAC and GAE assays. This situation is a good illustration for the need to employ more assays in order to have a well-defined picture regarding the antioxidant capacity of a given extract.

There are natural extracts for which the UV-vis spectra, besides their valuable qualitative information, might be used for rapid quantitative estimation of phenolic compounds (Moț et al. 2011b), though this is not possible for other numerous extracts or food material due to complex matrices. It is worth mentioning that in our extracts the absorbance at 280 nm strongly correlates with GAE, TEAC, QF, DPPH_{30min}, ORAC (after *Leaf June* extract removal for the latter one only) results (*vide infra*) and thus can be well used for rapid estimation of antioxidant capacity (phenolic content) in *H. helix* extracts. The UV-vis spectra for the studied extracts can be found in Figure S13.

2.4. Comparison between the assays performed in this study

After applying PCA on a matrix containing all the determined antioxidant parameters, the new variables (called principal components), are represented by a linear combination of the primary variables (in our case the antioxidant parameters). The most important results obtained are loadings and scores. Loadings indicate the relative importance of the corresponding antioxidant parameters in the principal component, and scores represent the new coordinates corresponding to each principal component for every sample (Moț et al. 2010). Usually, when a well model is obtained, it may turn out that two or three principal components provide a most of the information (variance) from the primary matrix - which is also true in this case, as the first three principal components explain 96.00 % of total variance. Scores and loading plots are very useful as a display tool for examining the relationships between the parameters, looking for trends, and sorting out outliers (Sârbu & Moț 2011). The loading plots for the determined antioxidant parameters are presented in Figure S16 and the correlations coefficients are given in Table S2, Supplementary data. The closer are the parameters to the circle, the better are these described by the PCA model and the closer the parameters are to each other, the higher their relationship (correlation) is. Parameters situated at 90° have correlation zero, while two parameters situated at an angle higher than 90° indicate a negative correlation. At first glance, HAPX1 can be noticed to have no correlation with all other parameters and is far from the circle; thus, considering the previous discussions (*vide supra*), in case of these extracts it may be that the degree of interference is very high. A visibly high correlation between ORAC, TEAC, GAE, DPPH (QF, EC₅₀ (at 180°, negative but very high correlation, see Table S2), all ET-based assays, is observed; these assay form a distinct cluster, followed closely by HAPX2. Even though L3 and β-carotene bleaching parameters are also very close to these parameters, in the second plot (PC1 vs. PC3) they are further separated, indicating that they contain different additional information; indeed, these are HAT based assays.

Figure S1. Liposomes oxidation kinetic profiles for the studied *H. helix* extracts as described in *Materials and methods* section. Each curve is an average for two replicates.

Figure S2. Spectral changes of the β -carotene for a control and a sample and their corresponding kinetic profiles in the β -carotene bleaching assay as described in *Materials and methods* section.

Figure S3. Kinetic curves of the standards measurements for the ORAC assay and the final calibration curve. The values associated with the curves represent trolox concentration in the sample (in μM).

Figure S4. Kinetic curves of tested samples for the ORAC assay, in duplicates.

Figure S5. The ORAC values for the studied extracts (ANOVA test, $p < 0.000$).

Figure S6. Percentage of DPPH bleached in 30 min at the same concentration of extract (3 mg plant/mL) and the quercetin factor (QF) for the assessed samples.

Figure S7. Dependence of percentage of unbleached DPPH upon extract concentration for all the studied extracts.

Figure S8 Kinetic profiles of DPPH bleaching for *Leaf June* extract at several concentration and calculation of T_{EC50} .

Figure S9 Antiradical efficiency (AE), effective concentration (EC₅₀) and its corresponding time needed to reach the steady state (TEC₅₀) results obtained from the DPPH bleaching assay for the tested extracts. For the clarity, the error bars are not pictured, the relative standard deviation is less than 10% in every case (ANOVA test, $p < 0.000$).

Figure S10. Calibration curve for Folin-Ciocalteu method. Dotted lines are 95% confidence interval associated to the fitting curve.

Figure S11 Calibration curve for TEAC assay. Dotted lines are 95% confidence interval associated to the fitting curve.

Figure S12. ABTS based assay (TEAC) and Folin-Ciocalteu method (GAE) for the studied extracts (ANOVA test, $p < 0.000$).

Figure S13. UV-vis spectra of the studied extracts in water after 100 times dilution.

Figure S14 Calibration curve using rutin as standard compound for HAPX2 method.

Figure S15 Quantitative profiles of the identified flavonoids in *H. hedera* extracts for as prepared extracts and for the hydrolysed ones (h as left subscript index for latter ones, L refers to left Y axis while R refers to right Y axis (for rutin only)).

Figure S16 Correlation circle (loadings plot) using the first three principal components of the PCA model obtained after applying PCA on the fourteen determined antioxidant parameters for the studied extracts.

Table S1. Quantification of five polyphenolic compounds (out of 18 standards available) in the studied samples (the results are given in $\mu\text{g mL}^{-1}$). Leaf extracts data are in bold.

Samples	non-hydrolyzed samples					hydrolyzed samples			
	caffeic	ferulic	rutin	quercetin	kaempferol	caffeic	ferulic	quercetin	kaempferol

	acid	acid				acid	acid		
Leaf									
June	0.00	0.00	121	1.55	1.28	0.00	0.00	24.1	7.51
Offshoot									
June	0.00	0.00	4.42	0.23	0.00	0.00	0.00	0.61	0.35
Leaf									
Sept.	0.00	0.00	34.0	13.3	4.20	0.50	0.00	18.6	5.26
Flower									
Sept.	1.04	0.51	130	7.11	7.90	1.40	0.96	37.8	20.6
Leaf									
Dec.	0.00	0.00	346	24.7	2.80	0.00	0.00	93.3	6.32
Fruit									
Dec.	0.68	3.23	170	2.16	1.15	0.86	2.93	31.8	3.87

Table S2. Correlation matrix (containing the correlation coefficients) between the antioxidant parameters described in the current paper. Values over 0.900 are bold and colored in blue while those smaller than 0.5 are bold and colored in red.

[illegible]

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