



# How to express the antioxidant properties of substances properly?

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## Abstract

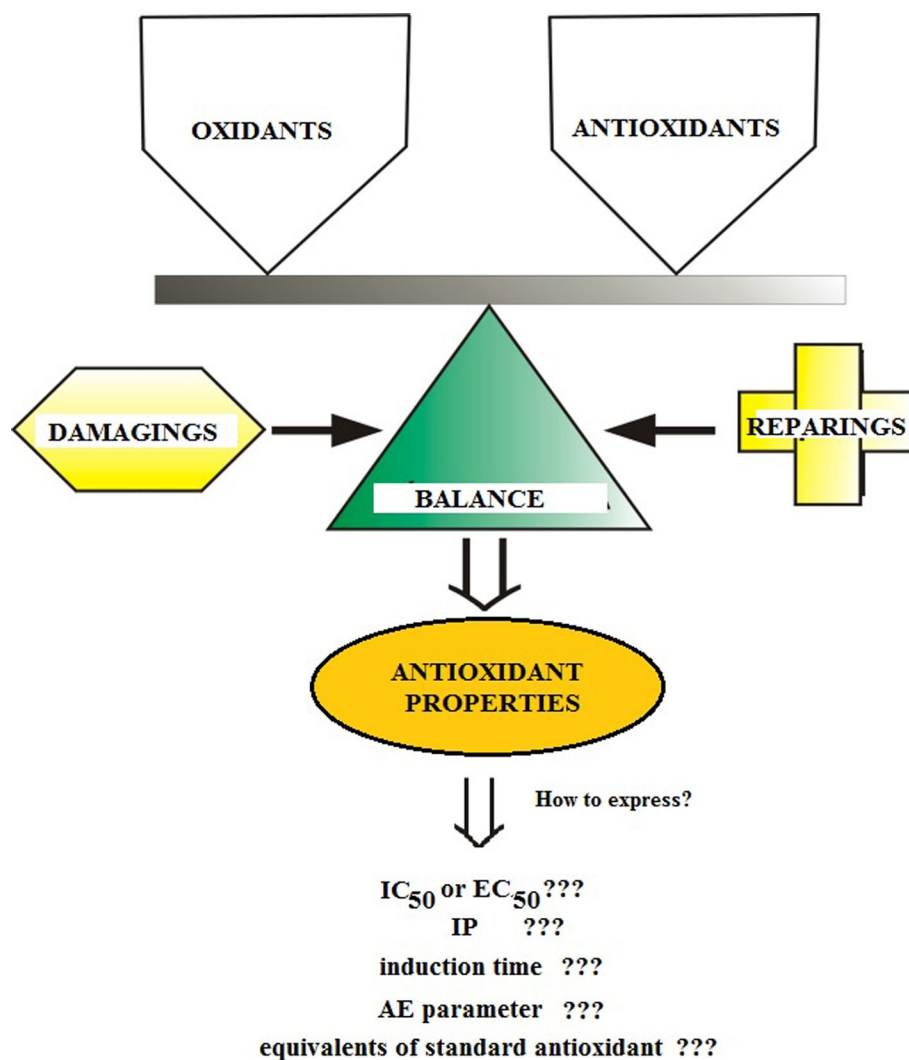
Oxidative stress, associated with an imbalance between the oxidants (reactive oxygen species) and the antioxidants in the body, contributes to the development of many diseases. The body's fight against reactive oxygen species is supported by antioxidants. Nowadays, there are too many analytical methods, but there is no one universal technique for assessing antioxidant properties. Moreover, the applied different ways of expressing the results lead to their incompatibility and unreasonable interpretation. The paper is a literature review concerning the most frequent ways of antioxidant activities expression and for an easy and universal method of the obtained results discussion. This paper is an attempt to point out their disadvantages and advantages. The manuscript can support the searching interpretation of the obtained results which will be a good tool for the development of a number of fields, especially medicine what can help in the future detection and treatment of many serious diseases.

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## Graphic abstract



**Keywords** Antioxidant properties · IC<sub>50</sub> · Inhibition percent · AE parameter · Standard antioxidant

## Introduction

The period of the last 40 years was characterized by search for substances with antioxidant properties and methods for their determination (Bingol et al. 2021; Gülçin 2020). Nowadays there are too many analytical methods, but there is no one universal technique for assessing antioxidant properties and no single available assay provides all required information about the examined antioxidant (ability to neutralize radicals both in the aqueous and lipophilic environments, ability to inhibit and/or delay the oxidation process or protect other important molecules) (Alam et al. 2012). According to Prior et al. (2005), an ideal standardized method should be characterized by: study on chemical

reactions actually occurring in potential applications; utilization of a radical sources which are relevant to biological structure; simplicity; well-defined endpoint and chemical mechanism; availability of the instrumentation, reproducibility within-run and between-day or adaptability for different antioxidants (both: hydrophilic and lipophilic) as well as radicals sources. While the requirements for the standard method are known, there is no information how to interpret the results obtained by it. The question arises how to express antioxidant activities of substances properly by this method and what kind of parameters an ideal standard antioxidant should possess. It is of significant importance because the different ways of expressing the results applied today lead to their incompatibility as well

as to unreasonable interpretation of the results of clinical studies.

Hence, the presented paper is a literature review concerning the most frequent ways of antioxidant activities expression and an attempt to point out their disadvantages and advantages. The manuscript can support searching for an easy and universal way of the obtained results interpretation. Moreover, a clear and comparable way of their expressing will enable reliable assessment of antioxidant properties in the future.

### What are the antioxidant properties?

According to the literature (Schaich et al. 2015; Apak et al. 2016; Rubio et al. 2016), antioxidant activities with respect to the methods are defined in different ways as: antioxidant capacity or efficiency or power or parameter or potential or potency, and activity. The three most commonly used terms (antioxidant capacity, antioxidant activity, antioxidant potential) are explained and described below:

- Antioxidant capacity—the entire number of electrons which are donated to the oxidant or target molecules converted per mole of antioxidant during a definite time period. This usually corresponds to the number of phenolic –OH groups in the antioxidant structure, or two electrons per – the OH group, but not always (Gülçin 2009, 2010). The adjective “total” is very often added to the term “antioxidant capacity” which indicates that it refers to all antioxidants which present in the sample. The expression “total antioxidant capacity” (TAC) concerns the resultant action of the whole sample, i.e., serum, blood, urine, etc. TAC is very often used to assess the antioxidant status of biological samples and can evaluate the antioxidant response of organism against the free radicals produced in a given disease (Rubio et al. 2016). This term is originated from chemistry; however, it can be applied to biology and medicine, and further to nutrition and epidemiology. According to Sies (2007), neither the term “total” nor the term “capacity” are applicable to the *in vitro* assays using an artificial, very often non-relevant to the biological system, selected oxidant generator and using quite different conditions than in the biological systems.
- Antioxidant activity—is antioxidant concentration which is required for providing a specified rate or extent of reaction. Hence, this term can be applied when the defined experimental conditions measuring the action of the potential antioxidant are presented (i.e., pressure, temperature, reaction media, coreactants, and reference points). Without these parameters, this term is insignificant. According to Bartosz (2003) “antioxidant activity”, it is the reactivity of the particular antioxidant to

the particular oxidant. In other, this term can be applied with the specific method due to the fact that it expresses the chemical reactivity of antioxidant under the specific conditions applied in this method (Huang et al. 2005).

- Antioxidant potential – this term is used to a lesser degree because it is often confused with the thermodynamic potential. It describes the ability of an antioxidant to neutralize a radical under certain conditions (Schaich et al. 2015).

The other terms listed above are more independent of specific reactions and have similar chemical meanings. According to the data presented in the literature (Brainina et al. 2019), the term “antioxidant activity” is the most commonly used. This phenomenon has been explained by the fact that this term provides direct information about the total concentration of antioxidants/oxidants in the sample.

It is worth mentioning that independently of the applied terms the activities of antioxidants depend not only on their chemical structures but also on many others factors such as concentration, temperature, type of substrate, chemical environment, as well as water content, a type of solvent, a metal and hydrogen ions presence (Gülçin 2012; Olszowy 2019; Olszowy-Tomczyk 2020).

### The ways of expression of antioxidant properties

#### Inhibition percent (% I)

According to the term content in “Compendium of Chemical Terminology (Gold Book),” (IUPAC 1997) the inhibition process is a reduction in the rate of a chemical reaction due to the addition of a substance (inhibitor) affecting the concentration of the reactants, catalyst or intermediate product. The inhibitor is defined as a chemical which decreases a substrate activity. These terms are associated with the antioxidant activity means an antioxidant (inhibitor) and an oxidant (often a reactive radical, a substrate of oxidation process). The effect of the inhibitor action is measured as the percentage decrease of the initial activity of the oxidant at definite time. In the methods using absorbance measurements (for example DPPH, ABTS<sup>+</sup>, O<sub>2</sub><sup>-</sup>, OH, etc.), a percent inhibition is calculated from the changes of the absorbance (of the radical or the measuring system) relative to its initial value after a specified duration reaction (ideally after reaching the reaction equilibrium constant) according to the following equation (Siddhuraju 2007; Olszowy et al. 2019; Kumar et al. 2020):

$$I(\%) = \left( 1 - \frac{A_{60}}{A_0} \right) \cdot 100\%$$

where IP—the percentage of inhibition;  $A_t$ —the absorbance (of the radical) after a specified reaction time  $t$ ;  $A_0$ —the initial (radical) absorbance

A percent inhibition can range from 0 to 100% and depends upon: the concentration of the antioxidant, concentration of the oxidant (radicals), the used solvent, the used reagents ratios incubation time, temperature as well as the presence of metal, hydrogen, water in the measuring systems (Dawidowicz and Olszowy 2011, 2012, 2013; Dawidowicz et al. 2012). The higher value of inhibition percent, the greater antioxidant activity.

It is worth remembering that the value of the percentage of inhibition above 90 may be a subject to error which may be due to the lack of a linear relationship between the measured properties and the increasing concentration of the antioxidant.

Summarizing, this parameter can be used to compare the antioxidant properties of mixtures, extracts, single substances for which measurements were made using the same method under the same measurement conditions (i.e., the same solvent, reagent ratio, time incubation, temp., etc.).

### EC<sub>50</sub> or IC<sub>50</sub>

The term “EC<sub>50</sub>” was associated with the antioxidant properties by Brand-Williams et al. (1995) in the paper concerning the DPPH method. However, according to Sebaugh (2010) EC<sub>50</sub> was used early in the pharmacy sciences to evaluate the suitability and performance of drugs. It is so-called efficient concentration interpreted in chemistry as the concentration of substrate that causes 50% loss of the oxidant’ concentration (for example radical). The concentration of the antioxidant providing 50% inhibition is estimated by plotting the percent of inhibition against different concentrations of the antioxidant (Teixeira et al. 2013). In many papers (Mishra et al. 2012; Wang et al. 2015; Olszowy and Dawidowicz 2016; Muhammad et al. 2017; Rivero-Cruz et al. 2020), the EC<sub>50</sub> value is called also as the IC<sub>50</sub>, which is misused due to the fact that this term is reserved for the life sciences in which it denotes the inhibition concentration of microorganisms. The EC<sub>50</sub> (IC<sub>50</sub>) is the most popular in spectrophotometric methods but also in other methods measuring antioxidant activities, for example using chemiluminescence (Samra et al. 2011).

Although the value EC<sub>50</sub> (IC<sub>50</sub>) is very often used to assess the antioxidant activity, it is not free from drawbacks. Below there are mentioned some drawbacks in the application of EC<sub>50</sub>/IC<sub>50</sub> parameter:

- The lack of the universality. This parameter can be applied only when a decrease of exactly definite known amounts of substrate in a definite reaction time is measured.

It cannot be applied in the methods in which other values are monitored.

- The lack of standardization during its estimation. Different conditions used during the measurements (incubation time, v/v ratio of reagents, different concentrations of reagents, different temperature, different solvent) are responsible for the various EC<sub>50</sub> (or IC<sub>50</sub>) values obtained for the same substance determined by the same assay. The exemplary data of EC<sub>50</sub>, obtained for BHT in DPPH and ABTS assays, which are presented in Table 1, evidently prove this statement. Hence, the comparison of antioxidant activity using EC<sub>50</sub> (IC<sub>50</sub>) determined under different experimental conditions is unreliable. Additionally, it is worth mentioning that the other factors such as: metal ions, hydrogen ions or water contents present in the measuring system can result in differences in the estimation of antioxidant activity and different values of EC<sub>50</sub> (IC<sub>50</sub>) (Dawidowicz and Olszowy 2011, 2012, 2013; Dawidowicz et al. 2012).

It seems that the EC<sub>50</sub> (IC<sub>50</sub>) parameter, similar to IP, can be used most appropriately in the case of comparing antioxidant properties of mixtures, extracts, single substances for which measurements were made using the same method under the same measurement conditions (i.e., the same solvent, reagent ratio, time incubation, temp., etc.)

- The lower is the value of EC<sub>50</sub>/IC<sub>50</sub>, the higher is the antioxidant activity which can be a disadvantage particularly when for better comparison the obtained results are presented as a bar chart.
- A difficulty with proper estimation of the EC<sub>50</sub>/IC<sub>50</sub> which can result from the lack of a “good” correlation between the percent inhibition values and the EC<sub>50</sub>/IC<sub>50</sub> values with the applied antioxidant concentration (Bellik 2014). It is worth remembering that the higher the concentration is, the lower linear increase of the inhibition percent is observed (Su et al. 2008). In the literature, there seems to be confusion about what kind of correlation to expect (Sebaugh 2010; Gubler et al. 2013).

### T<sub>EC50</sub> and AE parameter

The T<sub>EC50</sub> parameter defines the time needed to reduce the value of the initial concentration of the oxidant (e.g., radical) by 50%. It is established graphically from the reaction kinetics curve. According to this parameter, the kinetic behavior of the antioxidant is classified as follows: fast (T<sub>EC50</sub> < 30 min), medium (T<sub>EC50</sub> 30–60 min) and slow reaction kinetics (T<sub>EC50</sub> > 60 min) (Mahboub and Memmou 2015). In practice, the T<sub>EC50</sub> values (but also EC<sub>50</sub>) should be determined when the neutralization reaction reaches the

**Table 1** Literature data of EC<sub>50</sub> values obtained for BHT

EC <sub>50</sub> (g/mL)	Method	Experimental conditions	References
19.40	DPPH	50 mL of methanolic sample solution 5 mL of methanolic DPPH solution (4 mg/100 mL) Incubation time 30 min Temperature 25 °C	Guangrong et al. 2008
3.65	DPPH	400 L of ethanolic sample solution 400 L of ethanolic DPPH solution (11.8 mg/100 mL) Incubation time 20 min, Temperature 37 °C	Hsu et al. 2012
6.54	DPPH	1 mL of methanolic sample solution 2 mL of methanolic DPPH solution (3.55 mg/100 mL) Incubation time 30 min, Temperature 25 °C	Olszewska 2011
20	DPPH	0.5 mL of methanolic sample solution 3 mL of methanolic DPPH solution (4 mg/100 mL) Incubation time 30 min, Temperature 25 °C	Ceylan et al. 2015
17.78	DPPH	0.1 mL of methanolic sample solution 3.5 mL of methanolic DPPH solution (2.36 mg/100 mL) Incubation time 30 min, Temperature 25 °C	Shi et al. 2015
31.45	DPPH	1.5 mL of ethanolic sample solution 0.5 mL of ethanolic DPPH solution (3.94 mg/100 mL) Incubation time 30 min, Temperature 25 °C	Topal 2019
6.2	ABTS	10 L of ethanolic sample solution 1000 L of ethanolic ABTS <sup>+</sup> solution (A=0.7) Incubation time 6 min, Temperature 30 °C	Capuzzo et al. 2014
19.26	ABTS	1 mL of methanolic sample solution 2 mL of methanolic ABTS <sup>+</sup> solution (A=0.7) Incubation time 15 min Temperature 25 °C	Olszewska 2011
6.14	ABTS	0.15 mL of methanolic sample solution 2.85 mL of methanolic ABTS <sup>+</sup> solution (A=0.7) Incubation time 10 min Temperature 37 °C	Shi et al. 2015
32.36	ABTS	3 mL of ethanolic sample solution 1 mL of ethanolic ABTS <sup>+</sup> solution (A=0.7) Incubation time 30 min, Temperature 25 °C	Topal 2019

equilibrium state (so-called steady state). The  $T_{EC50}$  parameter is dependent on the concentration of oxidant agent and to be used in a meaningful way it has to be correlated not only with the antioxidant concentration but also with the initial amount of measuring oxidant agent (Fadda et al. 2014).

Both factors,  $T_{EC50}$  and  $EC_{50}$ , can be combined in the antiradical efficiency parameter (AE) according to the following equation (Sánchez-Moreno et al. 1998; Villaño et al. 2007; Ahmad et al. 2018):

$$AE = 1/[EC_{50} \times TEC_{50}]$$

As results from the presented equation the lower  $EC_{50}$ , the lower  $TEC_{50}$ , the higher AE value and the higher antioxidant properties.

According to Ahmad et al. (2018), the classification of antiradical efficiency is as follows:

- Low ( $AE < 10^{-3}$ )
- Medium ( $10^{-3} < AE < 5 \cdot 10^{-3}$ )
- High ( $5 \cdot 10^{-3} < AE < 10 \cdot 10^{-3}$ )
- Very high ( $AE > 10 \cdot 10^{-3}$ )

#### Induction time (lag time)

According to the term included in the “Compendium of Chemical Terminology (Gold Book),” (IUPAC 1997) the induction time (also called the lag time) is an initial slow stage of a chemical reaction which lasts for a certain period of time. After the induction, the reaction accelerates. The

induction time is most often expressed in time units (days, hours, minutes). In the antioxidative measurements, the induction time is associated with the oxidation process which is determined from the dependence of the measured value (for example the change in the amount of the oxidation product or substrate) versus time. The induction time (lag time) is established graphically as a segment (relatively constant), between the period for the beginning of the oxidation and the intercept of the tangent for the slope of the propagation phase response curve (Katsube et al. 2004). The measurements of the induction time are performed after the addition of a given amount of the examined or/and the reference antioxidants in both their absence and presence in the measuring system. This time is usually equated to the time during which these additives (antioxidants) are able to reduce the rate of free radical process significantly (Llesuy et al. 2001). Some difficulties may appear in correct determination of the induction time, which is related to the complexity of antioxidants in the tested sample. The measured signal changes slowly, and it is difficult to determine the induction time clearly. This particularly refers to the situation when there are a large number of compounds with very low reactivity in the sample (Llesuy et al. 2001).

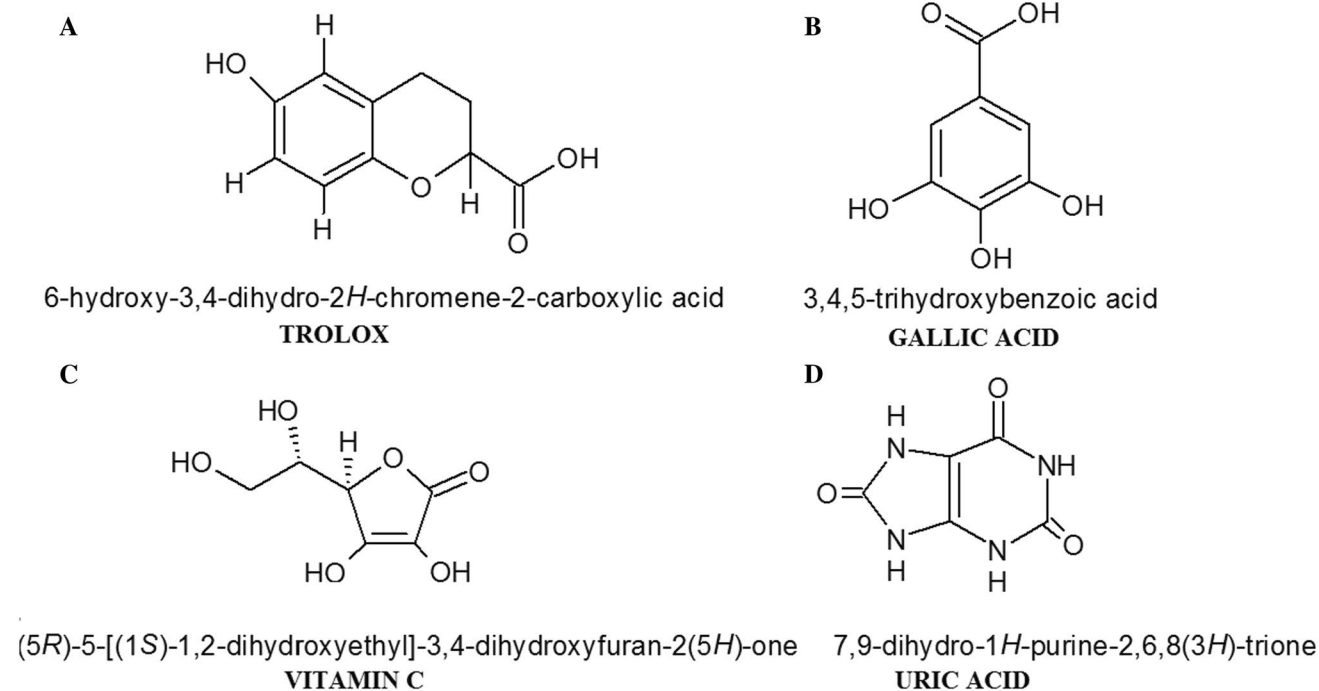
### Antioxidant standards

In many studies, the antioxidant properties of the test sample are compared to those of the standard antioxidants. Many

compounds can be used as standards, but the criteria of their choice are connected with the stability, price and solubility of standard antioxidant in the solvent (Erugur et al. 2019). The most important feature is composition of the examined sample because the antioxidant standard used in the determination should be very similar to the examined compounds. This paper describes the four substances most commonly used as standard antioxidants. Their structural formulae are presented in Fig. 1.

### Trolox

Trolox is a water-soluble analog of vitamin E. As an antioxidant, it is applied in biological and biochemical systems to reduce oxidative stress or damage. It might be used for determination of antioxidant activity of both single compounds and their mixture (Taslimi et al. 2020; Türkan et al. 2020). Additionally, for this compound a small impact of environmental conditions on the number of exchanged electron in the reaction is observed. The antioxidant value corresponding to Trolox is expressed in units known as Trolox Equivalents Antioxidant Capacity (TEAC) which are calculated from the ratio of the test compound reaction (measured as inhibition for example) to that of Trolox reaction. It is worth mentioning that the measurements for both Trolox and the sample should be performed under the same conditions and the examined solutions should have the same concentrations.



**Fig. 1** Structural formulae of: **a** Trolox, **b** gallic acid, **c** uric acid, **d** ascorbic acid

According to Apak et al. (2013), the measurements of TEAC values should be based on the following concept:

$$\text{TEAC value (unitless)} = \frac{\text{inhibition the test compounds}}{\text{inhibition by Trolox}}$$

Sometimes the  $IC_{50}$  values of the examined sample compared to  $IC_{50}$  of Trolox are used instead of inhibition (Xiao et al. 2020).

In practice, one can observe:

- The lack of standardization during the TEAC estimation. The TEAC values are calculated using different conditions during the measurements (incubation time, v/v ratio of reagents, different concentrations of reagents, different temperature, different solvent) which is responsible for the differences in the values obtained by different laboratories.
- The lack of units unification (if TEAC values are expressed in any units). The sample size can be expressed in the units of weight (gram of dry weight or 100 g or wet weight) or in the units of volume (in L, ml, etc.) resulting in the TEAC values as follows:  $\mu\text{M Trolox/g dry weight}$  or  $\mu\text{M Trolox/g wet weight}$  or per liter of extract. It seems that there is a need to normalize with regard to the mass or volume of the sample used for testing. However, in the case of solid matrices (e.g., plant matrices), some extraction technique must be used to bring the sample into solution. The extraction of the same material and the same amount but under different conditions will result in obtaining different extracts with various antioxidant properties. It would seem that the dose is the same but the result expressed per gram of sample will be different. Additionally, the amount of Trolox itself corresponding to the antioxidant properties of a given amount of sample can be reported in various units: in moles (e.g., mM) or in units of mass (e.g., g, mg). This depends on the concentration units used to create the calibration curve needed to relate the antioxidant properties of the test sample to the properties of the Trolox (Le Grandois et al. 2017; Kubilienė et al. 2020).

Most commonly, the antioxidant measuring method, in which the antioxidant activity of a given substance or a mixture is compared to the Trolox, is the ABTS assay. This method is very often called as the TEAC method (Arts et al. 2004). The other antioxidant capacity assays which apply Trolox as a standard include DPPH, ORAC and FRAP (Abramovič et al. 2018).

Summarizing, Trolox is nowadays generally accepted as the reference compound in an attempt to support a common value to be used to compare the results from different laboratories for various samples having similar effects. However,

this compound exhibits some drawbacks as a standard antioxidant: It is not the most efficient, has no physiologi-

cal relevance and has unsuitable solubility characteristics especially in the assessment of oils antioxidant properties (Litescu et al. 2014).

### Gallic acid

In many papers on the antioxidant properties of substances or mixtures, gallic acid is applied as a standard antioxidant (Sharma et al. 2011; Dontha 2016; Noreen et al. 2017; Abramovič et al. 2018). The gallic acid is mainly used as a standard antioxidant in the DPPH method (Pyrzyńska and Pękal 2013), in the process of determination of total phenolic compounds (Folin–Ciocalteu method) (Prior et al. 2005; Gaba and Malik 2015; Aryal et al. 2019). The antioxidant activity of the examined sample is expressed, similar to Trolox, as the gallic acid equivalent (GAE) in the units, for example mol/g sample (Sirivibulkovit et al. 2018) or mg gallic acid equivalents (GAE)/100 g sample (dry weight) (Wang et al. 2019). In all cases, the results were calculated according to the standard curve of gallic acid (Boutennoun et al. 2017; Zhijing et al. 2018). Similarly to the TEAC value, determining the GAE value is related to the lack of standardization of its determination and the unification of units. However, its advantage over Trolox is its better antioxidative response. Gallic acid is frequently used as the standard antioxidant because in many methods it gives the best response of all tested, standard compounds. For example, Antolovich et al. (2002) reported that when the antioxidant activity of four standard antioxidants (gallic acid, uric acid, Trolox and ascorbic acid) was compared using ABTS, TRAP (total radical-trapping antioxidant parameter) assays and the LDL (low-density lipoprotein) oxidation, the results were not comparable. Moreover, gallic acid was the strongest antioxidant in all three systems, but the relative activity of the remaining compounds depended on the system.

### Uric acid

Uric acid can be used as the standard antioxidant owing to its natural presence in human fluids (urine, serum, blood and saliva) (Koracevic et al. 2001; Cybul and Nowak 2008; Gülçin et al. 2008). This compound can be also found in the body of birds, reptiles and some primate species. The end product of purine degradation is found in all mentioned organisms (Settle and Klandorf 2014). Its antioxidant properties are associated mainly with its ability to scavenge

peroxynitrite and other free radicals. Additionally, it is responsible for protecting DNA against single-strand breaks caused by reactive oxygen species. This role is of significant importance in the neurodegenerative diseases (Settle and Klandorf 2014).

Antioxidant capacity using uric acid as the standard antioxidant is expressed in terms of the amount of the so-called equivalents of uric acid in the sample. For this purpose, a graph of the dependence of the uric acid response to the measured value versus its concentration is constructed. This dependence should be linear in the applied concentration range. For example, in the CUPRAC method, the graph of the absorbance relationship of the Cu (I) complex with neocupreine is linear in the uric acid concentration range of 0.05–2 mM (Apak et al. 2005), whereas the range of its concentrations 0.5–2.5 mM caused linear inhibition of thiobarbituric acid reactive substances production (TBARS) (Koracevic et al. 2001). In the latter, the authors noticed that increasing the uric acid concentration did not cause 100% inhibition of the free radical reaction. The maximal inhibition of TBARS production by uric acid was 80%.

Compared to Trolox, uric acid is characterized by worse activity in neutralizing  $H_2O_2$ , ABTS cation radical and exhibits a smaller ability of metal chelating. However, it is a better antioxidant in the DPPH scavenging process (Apak et al. 2005). Similarly to the TEAC value and the GAE value, the lack of universal conditions of its application is observed for the uric acid equivalents. This is associated with the lack of standardization of methods which are applied for the antioxidant activities determination. On one hand, uric acid appears to be an excellent standard antioxidant because it is an antioxidant that occurs naturally in the body. On the other hand, its use is limited due to its hydrophilic nature, which makes it not effective in all reaction environments. It loses an ability to scavenge lipophilic radicals and cannot break the radical chain propagation within the lipid membranes (Sautin and Johnson 2008) which is probably a major limitation of its antioxidant function. Additionally, the prooxidant activity of uric acid, primarily within the cell, was reported which can occur in the cardiovascular disease and may have a contributory role in its pathogenesis (Sautin and Johnson 2008).

### Ascorbic acid

Ascorbic acid (vitamin C) is regarded as a naturally occurring effective antioxidant (Khatoun et al. 2013) although the capacity of its biosynthesis does not occur in a number of species (including primates, guinea pigs, teleost fishes, bats, and birds) (Lachapelle and Drouin, 2011). Its antioxidant properties are associated with its ability of the hydrogen atom donation and the formation a relatively stable ascorbyl free radical. As a scavenger of reactive oxygen and nitrogen

species, ascorbic acid has proved to be effective against the superoxide radical ion, hydrogen peroxide, the hydroxyl radical and singlet oxygen (Moreira et al. 2012). The antioxidant activity of the examined sample (in the measuring system in which ascorbic acid is used as a standard antioxidant), known as vitamin C equivalent antioxidant capacity (VCEAC), is expressed as  $\mu\text{g}$  of ascorbic acid equivalents (AAE) per mL (Ahmed et al. 2015) or M ascorbic acid equivalent AAE/100 g of dried weight (dw) (Kim et al. 2002; Al-Laith et al. 2019). Similar to the other antioxidant standards, a graph of the dependence of the ascorbic acid response to measure the value versus its concentration is constructed. This dependence should be linear in the applied concentration range which can vary depending on many factors (method, incubation time, volume ratio of the reagents, etc.). This is related to the lack of standardization of methods for testing antioxidant properties. As the standard antioxidant, ascorbic acid is typically used in the hydrophilic antioxidant methods in which it exhibits large antioxidant properties (Prior et al. 2005). According to Moreira et al. (2012) in FRAP, TEAC, TRAP and ORAC, the ascorbic acid standard produced generally higher values than the other standards which can be associated with its highest hydrophilic character in comparison with Trolox and gallic acid as well as the aqueous environment of antioxidant reaction.

However, it is worth remembering that:

- Despite the fact that solid vitamin C is relatively stable, its solutions in water are unstable. Ascorbic acid (vitamin C) used as the control standard antioxidant should be dissolved in alcohol (for example ethanol or methanol) because in the aqueous environment it undergoes a decomposition process. The factors such as pH, temperature, oxygen and the presence of catalysts (iron, copper) influence this process (Dolińska et al. 2012).
- Under certain conditions (high concentration and presence of metal ions, such as iron and copper), ascorbic acid can act as a pro-oxidant which can limit its application as the standard antioxidant (Timoshnikov et al. 2020)

### Conclusions

At present, there is no one universal method for assessing antioxidant properties and no single available assay provides all of the required information about the examined antioxidant. Thus, assessing overall antioxidant activity requires multiple tests to generate a “universal antioxidant profile” that will reflect the actual “picture” of antioxidant activity in many respects. While the requirements for the standard method are known, there is no information on how to interpret the results obtained in it. The question arises how to



express antioxidant activities of substances properly and what kind of parameters an ideal standard antioxidant should possess. It is of significant importance because the present different ways of expressing the results lead to their incompatibility as well as unreasonable interpretation of the results of clinical studies. Only standardization of the methods and the universal way of interpretation of the results obtained by them will allow for a credible and reliable assessment of the antioxidant properties. The proper interpretation of the obtained results will be a good tool for the development of a number of fields like medicine, sports, food, pharmacology, cosmetology and others. The research in these area particularly in medicine will be useful for detection and treatment of many diseases associated with oxidative stress.

## Declarations

**Conflict of interest** There is no conflict of interest.

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## References

- Abramovič H, Grobin B, Poklar Ulrih N, Cigič B (2018) Relevance and standardization of *in vitro* antioxidant assays: ABTS DPPH, and Folin-Ciocalteu. *J Chem* 2018:1–9
- Ahmad NA, Jumbri K, Ramli A, Ghani NA, Ahmad H, Lim JW (2018) A kinetic approach of DPPH free radical assay of ferulate-based protic ionic liquids (PILs). *Molecules* 23:3201–3213
- Ahmed D, Khan MM, Saeed R (2015) Comparative analysis of phenolics, flavonoids, and antioxidant and antibacterial potential of methanolic, hexanic and aqueous extracts from *Adiantum caudatum* leaves. *Antioxidants* 4:394–409
- Alam MN, Bristi NJ, Rafiquzzaman M (2012) Review on *in vivo* and *in vitro* methods evaluation of antioxidant activity. *Saudi Pharm J* 21:143–152
- Al-Laith AA, Alkhuzai J, Freije A (2019) Assessment of antioxidant activities of three wild medicinal plants from Bahrain. *Arab J Chem* 12:2365–2371
- Antolovich M, Prenzler PD, Patsalides E, McDonald S, Roberts K (2002) Methods for testing antioxidant activity. *Analyst* 127:183–198
- Apak R, Gorinstein S, Böhm V, Schaich KM, Özyürek M, Güçlü K, (2013) Methods of measurement and evaluation of natural antioxidant capacity/activity) (IUPAC technical report). *Pure Appl Chem* 85(5):957–998
- Apak R, Güçlü K, Özyürek M, Karademir SE, Altun M (2005) Total antioxidant capacity assay of human serum using copper(II)-neocuproine as chromogenic oxidant: the CUPRAC method. *Free Radical Res* 39(9):949–961
- Apak R, Özyürek M, Guclu K, Capanoglu E (2016) Antioxidant activity/capacity measurement. 1. Classification, physicochemical principles, mechanisms, and electron transfer (ET)-based assays. *J Agric Food Chem* 64:997–1027
- Arts MJTJ, Haenen GRMM, Vost HP, Bast A (2004) Antioxidant capacity of reaction products limits the applicability of the trolox equivalent antioxidant capacity (TEAC) assay. *Food Chem Toxicol* 42:45–49
- Aryal S, Baniya MK, Danekhu K, Kunwar P, Gurung R, Koirala N (2019) Total phenolic content, flavonoid content and antioxidant potential of wild vegetables from western Nepal. *Plants* 8:1–12
- Bartosz G (2003) *Druga twarz tlenu. Wolne rodniki w przyrodzie*. Wydawnictwo Naukowe PWN, Warszawa
- Bellik Y (2014) Total antioxidant activity and antimicrobial potency of the essential oil and oleoresin of *Zingiber officinale Roscoe*. *Asian Pac J Trop Dis* 4(1):40–44
- Bingol Z, Kızıldağ H, Gören AC, Polat Kosee L, Topal M, Durmaz L, Alwasel SH, Gülçin İ (2021) Antidiabetic, anticholinergic and antioxidant activities of aerial parts of shaggy bindweed (*Convolvulus betonicifolia* Miller subsp.)—Profiling of phenolic compounds by LC-HRMS, Heliyon, 7 (5):e06986.
- Boutennoun H, Boussouf L, Rawashdeh A, Al-Qaoud K, Abdelhazef S, Kebieche M, Madani K (2017) *In vitro* cytotoxic and antioxidant activities of phenolic components of Algerian *Achillea odorata* leaves. *Arab J Chem* 10:403–409
- Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology* 28(1):25–30. [https://doi.org/10.1016/S0023-6438\(95\)80008-5](https://doi.org/10.1016/S0023-6438(95)80008-5)
- Brainina K, Stozhko N, Vidrevich M (2019) Antioxidants: terminology, methods, and future considerations. *Antioxidants* 8:297–304
- Capuzzo A, Occhipinti A, Maffei ME (2014) Antioxidant and radical scavenging activities of chamazulene. *Nat Prod Res* 24:2321–2323
- Ceylan Y, Usta K, Usta A, Maltasc AE, Yildiz S (2015) Evaluation of antioxidant activity, phytochemicals and ESR analysis of *Lavandula Stoechas*. *Acta Phys Pol A* 128:483–487
- Cybul M, Nowak R (2008) Przegląd metod stosowanych w analizie właściwości antyoksydacyjnych wyciągów roślinnych. *Herba Pol* 54:68–78
- Dawidowicz AL, Olszowy M (2011) Antioxidant properties of BHT estimated by ABTS assay in systems differing in pH or metal ion or water concentration. *Eur Food Res Technol* 232:837–842
- Dawidowicz AL, Olszowy M (2012) Mechanism change in estimating of antioxidant activity of phenolic compounds. *Talanta* 97:312–317
- Dawidowicz AL, Olszowy M (2013) The importance of solvent type in estimating antioxidant properties of phenolic compounds by ABTS assay. *Eur Food Res Technol* 236:1099–1105
- Dawidowicz AL, Wianowska D, Olszowy M (2012) On practical problems in estimation of antioxidant activity of compounds by DPPH method (problems in estimation of antioxidant activity). *Food Chem* 131(3):1037–1043
- Dolińska B, Ostróżka-Cieślak A, Caban A, Rimantas K, Leszczyńska L, Ryszka F (2012) Influence of trace elements on stabilization of aqueous solutions of ascorbic acid. *Biol Trace Elem Res* 150:509–512
- Dontha S (2016) A review on antioxidant methods. *Asian J Pharm Clin Res* 9:14–32
- Eruygur N, Koçyiğit UM, Taslimi P, Ataş M, Tekin M, Gülçin İ (2019) Screening the *in vitro* antioxidant, antimicrobial, anticholinesterase, antidiabetic activities of endemic *Achillea cucullata* (Asteraceae) ethanol extract. *S Afr J Bot* 120:141–145

- Fadda A, Serra M, Molinu MG, Azara M, Barberis A, Sanna A (2014) Reaction time and DPPH concentration influence antioxidant activity and kinetic parameters of bioactive molecules and plant extracts in their reaction with the DPPH radical. *J Food Compos Anal* 35:112–119
- Gaba SA, Malik SA (2015) Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of *Arisaema jacquemontii* Blume. *J Taibah Univ Sci* 9:449–454
- Guangrong H, Jiabin J, Dehui D (2008) Antioxidative and antibacterial activity of the methanol extract of *Artemisia anomala* S. Moore. *Afr J Biotechnol* 7(9):1335–1338
- Gubler H, Schopfer U, Jacoby E (2013) Theoretical and experimental relationships between percent inhibition and IC<sub>50</sub> data observed in high-throughput screening. *J Biomol Screen* 18(1):1–13
- Gülçin I (2009) Antioxidant activity of L-adrenaline: a structure-activity insight. *Chemico-Biol Interact* 179(2–3):71–80
- Gülçin I (2010) Antioxidant properties of resveratrol: a structure-activity insight. *Innov Food Sci Emerg Technol* 11:210–218
- Gülçin I (2012) Antioxidant activity of food constituents: an overview. *Arch Toxicol* 86:345–391
- Gülçin I (2020) Antioxidants and antioxidant methods: an updated overview. *Arch Toxicol* 94(3):651–715
- Gülçin I, Oktay M, Köksal E, Serbetci H, Beydemir S, Küfrevioğlu ÖI (2008) Antioxidant and radical scavenging activities of uric acid. *Asian J Chem* 20(3):2079–2090
- Hsu FL, Huang WJ, Wu TH, Lee MH, Chen LC, Lu HJ, Hou WC, Lin MH (2012) Evaluation of antioxidant and free radical scavenging capacities of polyphenolics from pods of *Caesalpinia pulcherrima*. *Int J Mol Sci* 13:6073–6088.
- Huang D, Ou B, Prior RL (2005) The chemistry behind antioxidant capacity assays. *J Agric Food Chem* 53(6):1841–1856
- IUPAC (1997) Compendium of Chemical Terminology, 2nd ed. (the “Gold Book”). Compiled by A D McNaught and A Wilkinson. Blackwell Scientific Publications, Oxford, Online version (2019) created by SJ Chalk. ISBN 0–9678550–9–8.
- Katsube T, Tabata H, Ohta Y, Yamasaki Y, Anuurad E, Shiwaku K, Yamane Y (2004) Screening for antioxidant activity in edible plant products: comparison of low-density lipoprotein oxidation assay DPPH radical scavenging assay, and Folin-Ciocalteu assay. *J Agric Food Chem* 52:2391–2396
- Khatoun M, Islam E, Islam R, Aziz Abdur Rahman AA, Alam AHMK, Khondkar P, Rashid M, Parvin S (2013) Estimation of total phenol and *in vitro* antioxidant activity of *Albizia procera* leaves. *BMC Res Notes* 6:121–128
- Kim DO, Lee KW, Lee HJ, Lee ChY (2002) Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. *J Agric Food Chem* 50:3713–3717
- Koracevic D, Koracevic G, Djordjevic V, Andrejevic S, Cosic V (2001) Method for the measurement of antioxidant activity in human fluids. *J Clin Pathol* 54:356–361
- Kubilienė A, Marksa M, Baranauskaitė J, Ragažinskienė O, Ivanauskas L (2020) Comparative evaluation of antioxidant activity of *Cannabis sativa* L. using FRAP and CUPRAC assays. *Chemija* 31:156–161
- Kumar J, Kumar N, Sati N, Kumar Hota P (2020) Antioxidant properties of ethenyl indole: DPPH assay and TDDFT studies. *New J Chem* 44:8960–8970
- Lachapelle MY, Drouin G (2011) Inactivation dates of the human and guinea pig vitamin C genes. *Genetica* 139:199–207
- Le Grandois J, Guffond D, Hamon E, Marchioni E, Werner D (2017) Combined microplate-ABTS and HPLC-ABTS analysis of tomato and pepper extracts reveals synergetic and antagonist effects of their lipophilic antioxidative components. *Food Chem* 223:62–71
- Litescu SC, Eremia SAV, Tache A, Vasilescu I, Radu GL (2014) The use of oxygen radical absorbance capacity (ORAC) and trolox equivalent antioxidant capacity (TEAC) Assays in the assessment of beverages’ antioxidant properties, In Processing and impact on antioxidants in Beverages, 245–251.
- Llesuy S, Evelson P, Campos AM, Lissi E (2001) Methodologies for evaluation of total antioxidant activities in complex mixtures. *Crit Rev Biol Res* 34(2):51–73
- Mahboub M, Memmou F (2015) Antioxidant activity and kinetics studies of eugenol and 6-bromoeugenol. *Nat Prod Res* 29:966–971
- Mishra K, Himanshu O, Chaudhury NK (2012) Estimation of antiradical properties of antioxidants using DPPH assay: a critical review and results. *Food Chem* 130:1036–1043
- Moreira FTC, Guerreiro JRL, Barros R, Goreti M, Sales F (2012) The effect of method, standard and sample components on the total antioxidant capacity of commercial waters assessed by optical conventional assays. *Food Chem* 134:564–571
- Muhammad DRA, Praseptianga D, Van de Walle D (2017) Interaction between antioxidants derived from cinnamon and cocoa in binary and complex mixtures. *Food Chem* 231:356–364
- Noreen H, Semmar N, Farman M, McCullagh JSO (2017) Measurement of total phenolic content and antioxidant activity of aerial parts of medicinal plant *Coronopus didymus*. *Asian Pac J Trop Med* 10(8):792–801
- Olzewska MA (2011) *In vitro* antioxidant activity and total phenolic content of the inflorescences, leaves and fruits of (*Sorbus Torminalis* L.) Crantz. *Acta Poloniae Pharm Drug Res* 68(6):945–953
- Olzowy M (2019) What is responsible for antioxidant activity of polyphenolic compounds from plants? *Plant Physiol Biochem* 144:135–143
- Olzowy M, Dawidowicz AL (2016) Essential oils as antioxidants: their evaluation by DPPH ABTS, FRAP, CUPRAC, and  $\beta$ -Carotene bleaching methods. *Monatsh Chem Chem Mon* 147:2083–2091
- Olzowy M, Dawidowicz AL, Józwick-Dolęba M (2019) Are mutual interactions between antioxidants the only factors responsible for antagonistic antioxidant effect of their mixtures? Additive and antagonistic antioxidant effects. *Eur Food Res Technol* 245(7):1473–1485
- Olzowy-Tomczyk M (2020) Synergistic, antagonistic and additive antioxidant effects in the binary mixtures. *Phytochem Rev* 19:63–103
- Prior RL, Wu X, Schaich K (2005) Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J Agric Food Chem* 53:4290–4302
- Pyrzyńska K, Pękal A (2013) Application of free radical diphenylpicrylhydrazyl (DPPH) to estimate the antioxidant capacity of food samples. *Anal Methods* 5:4288–4295
- Rivero-Cruz JF, Granados-Pineda J, Pedraza-Chaverri J, Pérez-Rojas JM, Kumar-Passari A, Diaz-Ruiz G, Rivero-Cruz BE (2020) Phytochemical constituents, antioxidant, cytotoxic, and antimicrobial activities of the ethanolic extract of Mexican brown propolis. *Antioxidants* 9:70–81
- Rubio CP, Hernández-Ruiz J, Martínez-Subiela S, Tvarijonaviciute A, Ceron JJ (2016) Spectrophotometric assays for total antioxidant capacity (TAC) in dog serum: an update. *BMC Vet Res* 12:166–173
- Samra MA, Chedea VS, Economou A, Calokerinos A, Kefalas P (2011) Antioxidant/prooxidant properties of model phenolic compounds: part I studies on equimolar mixtures by chemiluminescence and cyclic voltammetry. *Food Chem* 125:622–629
- Sánchez-Moreno C, Larrauri JA, Saura-Calixto F (1998) A procedure to measure the antiradical efficiency of polyphenols. *J Sci Food Agric* 76:270–276
- Sautin YY, Johnson RJ (2008) Uric acid: the oxidant-antioxidant paradox. *Nucleosides Nucleotides Nucl Acids* 27(6):608–619

- Schaich KM, Tian X, Xie J (2015) Hurdles and pitfalls in measuring antioxidant efficacy: a critical evaluation of ABTS DPPH, and ORAC assays. *J Funct Foods* 14:111–125
- Sebaugh JL (2010) Guidelines for accurate  $EC_{50}/IC_{50}$  estimation. *Pharm Stat* 10:128–134
- Settle T, Klandorf H (2014) The role of uric acid as an antioxidant in selected neurodegenerative disease pathogenesis: a short review. *Brain Disord Therapy* 3:129–134
- Sharma GN, Dubey SK, Sati N, Sanadya J (2011) Phytochemical screening and estimation of total phenolic content in *Aegle marmelos* seeds. *Int J Pharm Clin Res* 3(2):27–29
- Shi L, Ji ZG, Li YM, Li YY, Guo GS (2015) Antioxidant and antibacterial activity of *Toddalia Asiatica* (Lin) Lam root extracts. *Afr J Tradit Complement Altern Med* 12(6):169–179
- Siddhuraju P (2007) Antioxidant activity of polyphenolic compounds extracted from defatted raw and dry heated *Tamarindus indica* seed coat. *LWT* 40:982–990
- Sies H (2007) Total antioxidant capacity: appraisal of a concept. *J Nutr* 13:1493–1495
- Srivibulkovit K, Nouanthavong S, Sameenoi Y (2018) Paper-based DPPH assay for antioxidant activity analysis. *Anal Sci* 34:795–800
- Su MS, Shyu YT, Chien PJ (2008) Antioxidant activity of citrus herbal product extracts. *Food Chem* 111:892–896
- Taslimi P, Köksal E, Gören AC, Bursal E, Aras A, Kılıç Ö, Alwasel S, Gülçini İ (2020) Anti-Alzheimer, antidiabetic and antioxidant potential of *Satureja cuneifolia* and analysis of its phenolic contents by LC-MS/MS. *Arab J Chem* 13:4528–4537
- Teixeira B, Marques A, Ramos C, Serrano C, Neng NR, Nogueira JMF, Saraiva JA, Nunes ML (2013) Chemical composition and antibacterial and antioxidant properties of commercial essential oils. *Ind Crops Prod* 43:587–595
- Timoshnikov VA, Kobzeva TV, Polyakov NE, Kontoghiorghes GJ (2020) Redox interactions of vitamin C and iron: inhibition of the pro-oxidant activity by deferiprone. *Int J Mol Sci* 21:3967–3983
- Topal F (2019) Determination of antioxidant capacity of 2,6-quinolinediol. *J Inst Sci Technol* 9(3):1520–1527
- Türkan F, Atalar MN, Aras A, Gülçini İ, Bursal E (2020) ICP-MS and HPLC analyses, enzyme inhibition and antioxidant potential of *Achillea schischkinii* Sosn., *Bioorganic Chem*, 94: 103333.
- Villaño D, Fernandez-Pachón MS, Moyá ML, Troncoso AM, García-Parrilla MC (2007) Radical scavenging ability of polyphenolic compounds towards DPPH free radical. *Talanta* 71:230–235
- Wang S, Wang D, Liu Z (2015) Synergistic, additive and antagonistic effects of *Potentilla fruticosa* combined with EGb761 on antioxidant capacities and the possible mechanism. *Ind Crops Prod* 67:227–238
- Wang H, Qiu C, Chen L, Abbasi AM, Guo X, Liu RH (2019) Comparative study of phenolic profiles, antioxidant and antiproliferative activities in different vegetative parts of ramie (*Boehmeria nivea* L.). *Molecules* 24:1–13
- Xiao F, Xu T, Lu B, Liu R (2020) Guidelines for antioxidant assays for food components. *Food Front* 1:60–69
- Zhijing Y, Shavandi A, Harrision R, Bekhit AEDA (2018) Characterization of phenolic compounds in wine lees. *Antioxidants* 7(48):1–13

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