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Antioxidant activity, total phenolics and flavonoids contents: should we ban *in vitro* screening methods?

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Abstract

As many studies are disclosing the association between the ingestion of bioactive compounds and a decreased risk of noncommunicable diseases, the scientific community has shown much interest in these compounds. In addition, as bioactive compounds are regarded as reducing agents, hydrogen donors, singlet oxygen quenchers or metal chelators, the measurement of antioxidant activity by *in vitro* assays has become very popular in the last decades. Measuring the levels of total phenolics, flavonoids, and other (sub)classes using spectrophotometry represents a chemical index but chromatographic techniques are necessary to establish structure-activity. For bioactive purposes, *in vivo* models are recommended or, at very least, different methods that employ distinct mechanisms of action need to be used. In this regard, some comments were made concerning the *in vitro* screening methods that will help one to design future research studies on ‘‘bioactive compounds’’.

Keywords: Folin-Ciocalteu; antioxidants; bioavailability; colorimetric methods; functional properties; *in vivo* studies; HPLC.

1. Phenolic compounds as antioxidants

Halliwell and Gutteridge (2007) state that ‘‘an *antioxidant* is a substance that, when present at a low concentration compared with that of an oxidizable substrate in the medium, inhibits oxidation of the substrate’’. In this classification, phenolic compounds, which are derived from the secondary metabolism of plants, can protect multiple organs from oxidation. Therefore, phenolic compounds are regarded as natural *antioxidants*.

Antioxidants are categorized based on their *Function* (free-radical scavengers, scavengers of non-radical oxidizing agents, compounds that inhibit the generation of oxidants, transition metal chelating agents, and compounds that are able to stimulate the production of endogenous antioxidant compounds); *Polarity* (water-soluble and liposoluble); *Source*: (*exogenous* or *endogenous*); *Mechanism*: Antioxidants can neutralize the deleterious action of reactive species of cell membranes mainly by three mechanisms: hydrogen atom transfer (HAT), electron transfer (ET), and the ability to chelate transition metals (Prior et al., 2005; Brewer, 2011). In this sense, the HAT mechanism measures the ability of an antioxidant (AH) to quench free radicals (*i.e*., peroxyl radical - $ROO^{\bullet}$) by hydrogen donation stabilizing the peroxyl radical by resonance according to the Equation (1):

$$AH + ROO^{\bullet} \rightarrow ROOH + A^{\bullet} \quad \text{(Eq. 1)}$$

The ET-based assays measure the ability of AH to transfer one electron to reduce free radicals, pro-oxidant metals and carbonyls, which are based on Equation (2) (Huang et al., 2005; Apak et al., 2013):

$$ROO^{\bullet} + AH \rightarrow ROO^{\bullet} + AH^{+} \leftrightarrow A^{\bullet} + H^{+} \quad \text{(Eq. 2)}$$

HAT assays include the oxygen radical absorbance capacity (ORAC), inhibition of lipoperoxidation, crocin bleaching assay, and β-carotene bleaching assay. Similarly, ET methods are composed of cupric-ion reducing antioxidant capacity (CUPRAC), Folin-Ciocalteu's phenol reagent reducing ability, scavenging effects in relation to 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), among others (Shahidi & Zhong, 2015).

Some criticisms related to these *in vitro* chemical assays are based on the inexistence of such free radicals (DPPH/ABTS) in humans and the complexity of the mechanism of reaction. In addition, a high *in vitro* antioxidant activity cannot be translated into ''treatment/cure'' of illnesses. For instance, in the ferric reducing ability of plasma (FRAP) assay, as the reaction is performed at low pH values (3.6), much criticism is made on the translation of this method into *in vivo* effectiveness and, therefore, it can only be considered a screening method to have an idea of the antioxidant capacity of the sample (Schaich, Tian, & Xie, 2015). Undoubtedly, as these chemical assays are low-cost, easy to perform, do not require ultra-sensitive equipment, they are used to assess both isolated compounds and extracts from complex food matrices.

The antioxidant activity of phenolic compounds has been studied using a wide variety of methods, including *in vitro*, *ex vivo*, and *in vivo* protocols. Usually, authors find a high degree of correlation between *in vitro* antioxidant activity and the total phenolic content and/or individual phenolics (Rodrigo et al., 2005). However, the association between *in vitro* and *in vivo* antioxidant methods is still debatable and the opinion of experts in the field is divided into the usefulness of such *in vitro* methods.

2. Should we ban *in vitro* screening method to assess the antioxidant activity?

Several assays can be used to screen the *in vitro* antioxidant capacity of plant extracts, such as ferrous-ion chelating activity (Carter, 1971), copper chelating activity (Saiga, Tanabe, & Nishimura, 2003), lipid peroxidation inhibition assay (Daker et al., 2008), CUPRAC (Apak et al., 2008), deoxyribose assay (Chen, Zhang, & Xie, 2005), photoreduction of nitro blue tetrazolium assay (Chen, Zhang, & Xie, 2005), superoxide dismutase mimetic activity (Naithani, Nair, & Kakkar, 2006), total reducing capacity using a modified Folin-Ciocalteu assay (Berker et al., 2013), scavenging of hydrogen peroxide (Ruch, Cheng, & Klaunig, 1989), and cell-based *in vitro* antioxidant activity (Kellett, Greenspan, & Pegg, 2018). Excellent reviews on several chemical *in vitro* and cellular-based assays to assess the antioxidant activity can be found elsewhere (Alves et al., 2010; Niki, 2010; López-Alarcón & Denicól, 2013; Shahidi & Zhong, 2015). Without a doubt, the most frequently used methods rely on the use of DPPH, ABTS, FRAP, and ORAC assays (Halliwell, 2012; Schaich, Tian, & Xie, 2015).

These methods have many *pros* and *cons*, as any other analytical method, but when the antioxidant activity is evaluated, these methods have particularities in relation to the mechanism of action of the AH, the type of target (*i.e.*, H_2O_2 or DPPH radical), reactional pH, reaction time and temperature, and the use of a standard to build an analytical curve that is used to give a quantitative result in terms of antioxidant activity (Forman et al., 2014). Therefore, no single *in vitro* antioxidant activity assay will reflect the ‘‘total’’ antioxidant effect (Apak et al., 2013; Berker et al., 2013).

Recently, Harnly (2017) stated that studies regarding the measurement of *in vitro* antioxidant activity and total phenolic content using the Folin-Ciocalteu reagent is not appropriate. The reasons are:

1. There is currently no accepted standard mechanism or method to measure the antioxidant activity;

2. Only state-of-the-art techniques to identify antioxidants (*i.e.,* flavonoids) should be used in scientific research;

3. Results of a method *X* (*i.e*., FRAP) are (usually) not comparable with data obtained using the method *Y* (*i.e*., DPPH) or even between laboratories; and

4. *Antioxidant* is a marketing term of questionable health and analytical value as epidemiological studies are inconsistent.

In this regard, it is unquestionable that ‘‘state-of-the-art’’ techniques, such as liquid chromatography-mass spectroscopy (LC-MS), to identify and quantify phenolic compounds in foods, beverages, and herbal extracts have high accuracy and precision. However, screening spectrophotometric methods should also be used to characterize these materials and have an idea of the total content of phenolic compounds in the matrix (Granato, Santos, Maciel, & Nunes, 2016).

Halliwell (2012) stated that ‘‘the consumption of mega-doses of antioxidants (*i.e.,* pills) have also generally failed to prevent human disease, in part because they do not decrease oxidative damage *in vivo*’’. Individuality (*i.e.,* genetics, gender, and body mass index) and life habits (*i.e.,* exercising, drugs/alcohol abuse, and smoking) also play an important role in the oxidative status of humans. Although some studies show discrepancies and inconsistencies to show a clear association between consumption of phenolic

compounds and increase of the antioxidant status in humans (Frankel & German, 2006; Saldanha et al., 2016), the search for antioxidants should continue and any allegation on functionality should be supported by preclinical, clinical, and epidemiological studies.

As well known, *in vitro* antioxidant methods and the estimation of total phenolic content using colorimetric assays can be used not only to have an idea of the beneficial effects of the food/extract. For quality control of natural products (Guo, Sun, Yu, & Qi, 2017; Lv, Zhang, Shi, & Lin, 2017), the antioxidant activity measured by *in vitro* methods are very useful as a fingerprint of reference materials that can be used for comparison purposes with commercial samples. Therefore, trends are generally very useful for comparative purposes of samples of the same material. In food technology, *in vitro* antioxidant assays together with the total phenolic content may be of importance to assess the best cutting styles of fruits (Li et al., 2017). These examples illustrate the usefulness of *in vitro* methodologies that can be applied in the routine quality control programs of food companies worldwide. Without a doubt, interferences in these nonselective methodologies exist and this fact is well demonstrated when comparing high-performance liquid chromatography (HPLC) results with total contents of phenolic compounds. Nevertheless, we need to have something in mind: one cannot rule out the usefulness of *in vitro* results despite their imperfect nature.

To date, Williams, Soencer, and Rice-Evans (2004) stated that ‘‘phenolic compounds may exert modulatory actions in cells through actions at protein kinase and lipid kinase signaling pathways A clear understanding of the mechanisms of action of flavonoids, either as antioxidants or modulators of cell signaling, and the influence of their metabolism on these properties are key to

the evaluation of these potent biomolecules as anticancer agents, cardioprotectants, and inhibitors of neurodegeneration''. In addition, Alam, Bristi, & Rafiquzzaman (2013) stated that ''antioxidants may be of great benefit in improving the quality of life by preventing or postponing the onset of non-communicable diseases''.

In recent studies, the antioxidant activity of bioactive compounds measured by *in vitro* and *in vivo* models are associated in a way that, depending on the biomarker used to assess the oxidative stress, interesting conclusions with practical applications arise (Macedo et al., 2013; Yan, Chen, & Zheng, 2017; Sun et al., 2017; Villa-Hernández et al., 2017; Aouachria et al., 2017; Naeimi & Alizadeh, 2017; Donado-Pestana et al., 2018). Obviously, there is a need to demonstrate the mechanistic approach behind the antioxidant activity of polyphenols *in vivo*. Animal models (*i.e.,* rat, mouse, rabbit, and dog) and human studies (*i.e.,* preclinical and randomized double-blind placebo-controlled clinical trials) are more appropriate but also more expensive, complex, and time-consuming compared to chemical and cellular-based methods (Thompson, Pederick, Singh, & Santhakumar, 2017). The assessment of *in vivo* antioxidant activity should include the measurement the activity of endogenous enzymes and antioxidant gene expression compared to a placebo, for instance. The bioaccessibility of phenolic compounds should also be studied in detail during and, principally, after the gastrointestinal digestion because the bioavailability of antioxidants, such as polyphenols, is generally very low. If these antioxidants could be absorbed, there is sometimes an insufficient concentration of the antioxidants in target tissues for the activity to be the prevalent protective mechanism (Huang et al., 2017).

Another point of consideration is as follows: what is measured in the food is not fully representative for what is active in humans. As well stressed by Espín, González-Sarrías, and Tomás-Barberán (2017) and Granado-Lorencio, Blanco-Navarro, Pérez-Sacristán, and Hernández-Álvarez (2017), ‘‘the type and quantity of the carotenoid/phenolic compounds metabolites produced in humans depend on the gut microbiota composition and function.The beneficial effect biological upon carotenoid/polyphenols intervention varies considerably and the chronic use of large doses may lead to saturation effects and the loss of linearity in the response.Therefore, the final health effects of dietary polyphenols/carotenoids depend on the gut microbiota composition’’. As the microbiota of each individual is unique, we cannot assume ‘‘functionality’’ based only on *in vitro* tests.

3. Finals remarks and conclusions

As a conclusion of this viewpoint, although there will be divergent opinions in the scientific community based on thousands of studies available, we cannot close our eyes to dietary antioxidants and ignore some *in vitro* screening methods (*i.e*., total phenolic/total flavonoids contents and antioxidant activity measurements) as low-cost, high-throughput tools to discover potential antioxidant sources for human consumption.

In a perspective, manuscripts on antioxidant properties based solely on colorimetric methods (including the Folin-Ciocalteu assay) will become unacceptable in *Food Chemistry* from now on. Authors are encouraged to assay bioactive compounds using chromatographic techniques (*i.e.,* HPLC/LC-MS) and, preferably, there must be some biological tests using cell lines or simulated digestion, or at the very least, measurement of bioactivity (*i.e.,* antioxidant effect)

using multiple assays that employ different mechanisms of action (*i.e.,* HAT, ET, and metal chelation property).

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