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

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33

34 **Abstract**

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

48

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

51

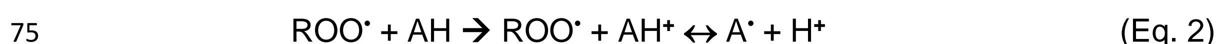
1. Phenolic compounds as antioxidants

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.

101

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

104 Several assays can be used to screen the *in vitro* antioxidant capacity of
105 plant extracts, such as ferrous-ion chelating activity (Carter, 1971), copper
106 chelating activity (Saiga, Tanabe, & Nishimura, 2003), lipid peroxidation inhibition
107 assay (Daker et al., 2008), CUPRAC (Apak et al., 2008), deoxyribose assay
108 (Chen, Zhang, & Xie, 2005), photoreduction of nitro blue tetrazolium assay (Chen,
109 Zhang, & Xie, 2005), superoxide dismutase mimetic activity (Naithani, Nair, &
110 Kakkar, 2006), total reducing capacity using a modified Folin-Ciocalteu assay
111 (Berker et al., 2013), scavenging of hydrogen peroxide (Ruch, Cheng, & Klaunig,
112 1989), and cell-based *in vitro* antioxidant activity (Kellett, Greenspan, & Pegg,
113 2018). Excellent reviews on several chemical *in vitro* and cellular-based assays
114 to assess the antioxidant activity can be found elsewhere (Alves et al., 2010; Niki,
115 2010; López-Alarcón & Denicól, 2013; Shahidi & Zhong, 2015). Without a doubt,
116 the most frequently used methods rely on the use of DPPH, ABTS, FRAP, and
117 ORAC assays (Halliwell, 2012; Schaich, Tian, & Xie, 2015).

118 These methods have many *pros* and *cons*, as any other analytical method,
119 but when the antioxidant activity is evaluated, these methods have particularities
120 in relation to the mechanism of action of the AH, the type of target (*i.e.*, H_2O_2 or
121 DPPH radical), reactional pH, reaction time and temperature, and the use of a
122 standard to build an analytical curve that is used to give a quantitative result in
123 terms of antioxidant activity (Forman et al., 2014). Therefore, no single *in vitro*
124 antioxidant activity assay will reflect the ''total'' antioxidant effect (Apak et al.,
125 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

151 compounds and increase of the antioxidant status in humans (Frankel & German, 152 2006; Saldanha et al., 2016), the search for antioxidants should continue and any 153 allegation on functionality should be supported by preclinical, clinical, and 154 epidemiological studies.

155 As well known, *in vitro* antioxidant methods and the estimation of total 156 phenolic content using colorimetric assays can be used not only to have an idea 157 of the beneficial effects of the food/extract. For quality control of natural products 158 (Guo, Sun, Yu, & Qi, 2017; Lv, Zhang, Shi, & Lin, 2017), the antioxidant activity 159 measured by *in vitro* methods are very useful as a fingerprint of reference 160 materials that can be used for comparison purposes with commercial samples. 161 Therefore, trends are generally very useful for comparative purposes of samples 162 of the same material. In food technology, *in vitro* antioxidant assays together with 163 the total phenolic content may be of importance to assess the best cutting styles 164 of fruits (Li et al., 2017). These examples illustrate the usefulness of *in vitro* 165 methodologies that can be applied in the routine quality control programs of food 166 companies worldwide. Without a doubt, interferences in these nonselective 167 methodologies exist and this fact is well demonstrated when comparing high-168 performance liquid chromatography (HPLC) results with total contents of phenolic 169 compounds. Nevertheless, we need to have something in mind: one cannot rule 170 out the usefulness of *in vitro* results despite their imperfect nature.

171 To date, Williams, Soencer, and Rice-Evans (2004) stated that ‘‘phenolic 172 compounds may exert modulatory actions in cells through actions at protein 173 kinase and lipid kinase signaling pathways A clear understanding of the 174 mechanisms of action of flavonoids, either as antioxidants or modulators of cell 175 signaling, and the influence of their metabolism on these properties are key to

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

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

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

212

213 **3. Finals remarks and conclusions**

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

220 In a perspective, manuscripts on antioxidant properties based solely on
221 colorimetric methods (including the Folin-Ciocalteu assay) will become
222 unacceptable in *Food Chemistry* from now on. Authors are encouraged to assay
223 bioactive compounds using chromatographic techniques (*i.e.,* HPLC/LC-MS)
224 and, preferably, there must be some biological tests using cell lines or simulated
225 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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