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# Vitamin C Equivalent Antioxidant Capacity (VCEAC) of Phenolic Phytochemicals

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To express the antioxidant capacity of plant foods in a more familiar and easily understood manner (equivalent to vitamin C mg/100 g), two stable radical species, $ABTS^{\bullet-}$ and $DPPH^{\bullet}$, commonly used for antioxidant activity measurements, were employed independently to evaluate their efficacies using apple polyphenolic extracts and seven polyphenolic standards including synthetic Trolox. Their antioxidant activities were expressed as vitamin C equivalent antioxidant capacity (VCEAC) in mg/100 g apple or mg/100 mL of the reference chemical compounds in 10 and 30 min using the $ABTS^{\bullet-}$ and $DPPH^{\bullet}$ scavenging assays, respectively. The antioxidant capacity of Gala apples and seven phenolic standards, determined by both $ABTS^{\bullet-}$ and $DPPH^{\bullet}$ scavenging assays, showed a dose−response of the first-order. Fresh Gala apples had a VCEAC of 205.4 ± 5.6 mg/100 g using the ABTS assay, and the relative VCEACs of phenolic standards were as follows: gallic acid > quercetin > epicatechin > catechin > vitamin C > rutin > chlorogenic acid > Trolox. With the DPPH radical assay, the VCEAC of fresh Gala apples was 136.0 ± 6.6 mg/100 g, and the relative VCEACs of seven phenolic standards were, in decreasing order, as follows: gallic acid > quercetin > epicatechin > catechin ≥ vitamin C > Trolox > rutin > chlorogenic acid. Because the ABTS assay can be used in both organic and aqueous solvent systems, employs a specific absorbance at a wavelength remote from the visible region, and requires a short reaction time, it is a more desirable method than the DPPH assay. Therefore, it is recommended that antioxidant capacity be expressed as vitamin C mg/100 g equivalent (VCEAC) using the ABTS assay.

**KEYWORDS: $ABTS^{\bullet-}$; antioxidant activity; $DPPH^{\bullet}$; free radical scavenging assay; phenolic phytochemicals; vitamin C; vitamin C equivalent antioxidant capacity (VCEAC); weight basis**

## INTRODUCTION

Natural phenolic phytochemicals in fruits and vegetables have been receiving increased interest from consumers and researchers for their beneficial health effects on coronary heart diseases and cancers mainly due to their antioxidant activity (*1*−*5*). There have been numerous methods for the measurement of antioxidant activity of biological materials. Chemical assays among them are mainly based on the ability to scavenge various kinds of free radicals. Most well-known methods usually express total antioxidant capacity of foods or chemicals as Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC) or $IC_{50}$ values based on molar units (*6*−*9*). The TEAC method using Trolox to scavenge free $ABTS^{\bullet-}$ was originally introduced for the clinical investigation of premature infants' low serum antioxidant due to vitamin E deficiency (*10*). Total radical-trapping antioxidant parameter (TRAP) assay, another well-known method for antioxidant capacity measurement, is time-consuming (*11, 12*) and expresses the activities as Trolox equivalents (*13*). The oxygen radical absorbance capacity (ORAC) method has also been widely used to measure total antioxidant potential and uses Trolox equivalents to express antioxidant capacity (*14, 15*).

Trolox, a water-soluble analogue of vitamin E having potent antioxidant activity, is not a natural compound found in foods. Vitamin C, on the other hand, is commonly recognized as a major, naturally occurring nutrient and antioxidant in our daily diet. Recently, vitamin C has also been found to have anticarcinogenic effects (*16*). Total antioxidant capacity results, expressed on a molar basis as either Trolox equivalents or $IC_{50}$ values, are difficult to understand, especially for the layperson. Currently, nutritional labeling on the most foods and dietary supplements is expressed on a weight basis, not as molar units as preferred by scientists. It was suggested that the use of the molar unit expression would widen even further the comprehension gap between the general public and researchers (*17*). In this respect, antioxidant capacity of foods should be measured by a simple, reliable method and reported in familiar terms. Therefore, vitamin C equivalent antioxidant capacity (VCEAC) calculated on a weight basis (mg/100 g or mg/100 mL) to show

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the total antioxidant capacity of a food is more desirable than the TEAC, TRAP, or ORAC assays based on molar units.

The methods using ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) or DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging are among the most popular spectrophotometric methods for determining the antioxidant capacity of foods and chemical compounds. These two simple stable radical chromogens (the violet DPPH radicals and the blue-green ABTS radical anions) are easy to use, have a high level of sensitivity, and allow for analysis of a large number of samples in a timely fashion. Numerous analyses using these two chromogens to determine the antioxidant activity of pure compounds, juices, wines, and tea extracts have been reported (*18*−*22*).

The objectives of this investigation were to develop the concept of VCEAC on a weight basis to express total antioxidant capacity of foods and pure polyphenolic chemicals, and to compare ABTS radical anion scavenging activity assay (ABTS assay) and DPPH radical scavenging activity assay (DPPH assay). Gala apple phenolics and pure polyphenolic compounds were evaluated for their VCEAC.

## MATERIALS AND METHODS

**Chemicals.** Gallic acid, ABTS as diammonium salt, (+)-catechin, (−)-epicatechin, chlorogenic acid, quercetin, rutin, DPPH, and Folin−Ciocalteu's phenol reagent were obtained from Sigma Chemical Co. (St. Louis, MO). Trolox was purchased from Aldrich Chemical Co. (Milwaukee, WI). Vitamin C was purchased from Mann Research Laboratories, Inc. (New York). 2,2′-Azobis(2-amidino-propane)dihydrochloride (AAPH) was obtained from Wako Chemicals USA, Inc. (Richmond, VA). All other chemicals used were analytical grade (Fisher, Springfield, NJ).

**Fruits.** The Gala apple cultivar was picked at commercial maturity during the 2001 harvest season at the New York State Agricultural Experiment Station orchard in Geneva, New York. Immediately after harvest, the apples (water content 83.3%) were stored at 2−5 °C in a cold room. Apple slices prepared by hand were frozen and lyophilized. Freeze-dried samples were ground to powder and then stored at − 20 °C.

**Extraction of Phenolics.** The phenolics in Gala apples were extracted from 10 g of ground freeze-dried apples using 100 mL of 80% aqueous methanol in a 250-mL round-bottomed flask. The mixture of apple powder and aqueous methanol was sonicated for 20 min with continual nitrogen gas purging. The mixture was filtered through Whatman #2 filter paper (Whatman International Limited, Kent, England) using a chilled Büchner funnel and rinsed with 50 mL of 100% methanol. The solid filter cake was reextracted by repeating the above steps under the same conditions. The two filtrates were combined and transferred into a 1-L round-bottomed flask with an additional 50 mL of 80% aqueous methanol. The solvent was evaporated using a rotary evaporator under reduced pressure at 40 °C. The phenolic concentrate was dissolved in 50 mL of 100% methanol and made to the final volume of 100 mL with distilled deionized water ($ddH_2O$) obtained with a NANOpure water system (Barnstead, Dubuque, IA). The solution then was centrifuged in a Sorvall RC-5B refrigerated superspeed centrifuge (Du Pont Company, Biomedical Products Department, Wilmington, DE) at 12 000*g* using GSA rotor for 20 min. The final extract solution was stored at − 4 °C until analyzed.

**ABTS Radical Anion Scavenging Activity.** The method developed by van den Berg et al. (*23*) was slightly modified in this experiment. AAPH, 1.0 mM, was mixed with 2.5 mM ABTS as diammonium salt in phosphate buffered saline (PBS) solution (100 mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl). The mixture was heated in a water bath at 68 °C for 13 min. The concentration of the resulting blue-green $ABTS^{\bullet-}$ solution was adjusted to an absorbance of 0.650 ± 0.020 at 734 nm. The sample solution of 20 μL was added to 980 μL of the resulting blue-green ABTS radical solution. The mixture, protected from light, was incubated in a water bath at 37 °C for 10 min. The decrease of absorbance at 734 nm was measured at the endpoint of 10 min. A control consisted of 20 μL of 50% methanol and 980 μL of $ABTS^{\bullet-}$ solution. The stable ABTS radical scavenging activity of apple phenolic extracts and selected pure chemical compounds was expressed as mg/100 g fresh apple and as mg/100 mL standard compounds of VCEAC in 10 min, respectively. The radical stock solution was prepared fresh daily.

**DPPH Radical Scavenging Activity.** $DPPH^{\bullet}$ scavenging activity was determined using a modified method of Brand-Williams et al. (*24*). One hundred μM $DPPH^{\bullet}$ was dissolved in 80% aqueous methanol. The apple extracts or pure polyphenolic solutions, 0.1 mL, were added to 2.9 mL of the methanolic $DPPH^{\bullet}$ solution. The mixture was shaken vigorously and allowed to stand at 23 °C in the dark for 30 min. The decrease in absorbance of the resulting solution was monitored at 517 nm at 30 min. A control consisted of 0.1 mL of 50% aqueous methanol and 2.9 mL of $DPPH^{\bullet}$ solution. The DPPH radical scavenging activity of apple phenolic extracts and selected pure chemical compounds was expressed as mg/100 g fresh apple and mg/100 mL standard compounds of VCEAC in 30 min reaction time, respectively. The radical stock solution was prepared fresh daily.

**VCEAC of Polyphenolics and Apples.** Vitamin C standard curves that relate the concentration of vitamin C and the amount of absorbance reduction caused by vitamin C were obtained using the ABTS assay and the DPPH assay (**Figures 1A** and **2A**). The absorbance reduction at 734 and 517 nm of seven polyphenolics (gallic acid, quercetin, epicatechin, catechin, rutin, chlorogenic acid, and Trolox) was also measured at various concentrations by ABTS assay and DPPH assay, respectively. The calculation of VCEAC of each compound at the various concentration levels was made using vitamin C standard curves (**Figures 1B** and **2B**). For Gala apples, properly diluted extract was assayed by both the ABTS and DPPH procedures. The amount of absorbance reduction of Gala apple phenolics was also related to that of vitamin C standard, and the result was calculated as vitamin C equivalents in mg/100 g.

## RESULTS AND DISCUSSION

AAPH, a thermolabile water-soluble radical initiator, oxidized $ABTS^{2-}$ to ABTS radical anion (*25*). The generation of the ABTS radical anion before the addition of antioxidants was reported to eliminate the interference of compounds affecting radical production (*7, 23*). The reduction of ABTS radical chromogen by an antioxidant decreases absorbance at 734 nm. The calibration curve showed a linear relationship ($r^2 = 0.990$) between vitamin C concentration and absorbance reduction at 734 nm (**Figure 1A**). First order of linear regression in relation to absorbance reduction vs various concentrations of seven phenolic compounds (catechin, epicatechin, rutin, chlorogenic acid, quercetin, gallic acid, and Trolox) was attained, of which the correlation coefficient ($r^2$) was found to be higher than 0.990 (**Figure 1A**). The standard curve that relates the concentration of vitamin C and the amount of absorbance reduction caused by vitamin C was employed to calculate VCEACs based on the absorbance reduction of seven tested polyphenolic compounds. Antioxidant capacity of seven polyphenolics also showed an apparent first-order relationship with VCEAC (**Figure 1B**). From **Figure 1B**, the antioxidant capacity of any reference compound, including Trolox, can easily be converted to VCEAC on a weight basis. As the three plotted lines of chlorogenic acid, rutin, and Trolox in **Figure 1B** are below the tangential line of 45 degrees, their antioxidant capacities are lower than that of vitamin C; whereas gallic acid, quercetin, epicatechin, and catechin have much higher antioxidant potential than vitamin C on weight basis. The overall relative antioxidant capacity of phenolic compounds in VCEAC evaluated by ABTS assay was in decreasing order as follows: gallic acid > quercetin > epicatechin > catechin > vitamin C > rutin > chlorogenic acid > Trolox.

The stable ABTS radical anions presented a biphasic kinetic response with apple phenolics and vitamin C (**Figure 3**). There

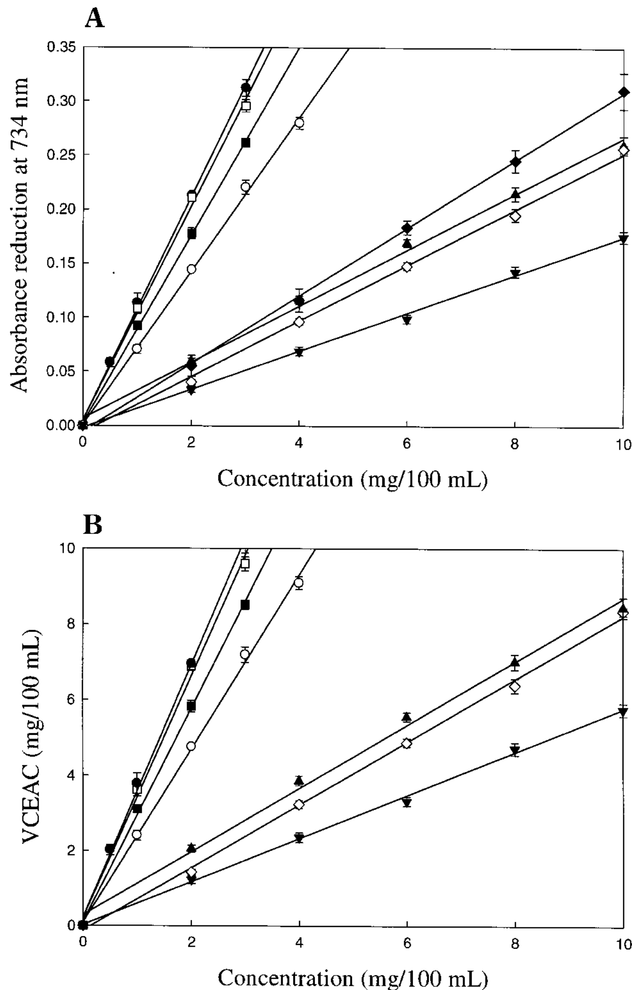


**Figure 1.** (A) Relationship between vitamin C or pure polyphenolics and absorbance reduction of blue-green ABTS radical anions at 10 min and 37 °C. Error bar = standard deviation; $n = 22$ for vitamin C; $n = 7$ for polyphenolics; ● gallic acid ($r^2 = 0.997$); □ quercetin ($r^2 = 0.996$); ■ epicatechin ($r^2 = 0.999$); ○ catechin ($r^2 = 0.997$); ◆ vitamin C ($r^2 = 0.990$); ▲ rutin ($r^2 = 0.994$); ◇ chlorogenic acid ($r^2 = 0.996$); ▼ Trolox ($r^2 = 0.994$). (B) Conversion of antioxidant potential of seven polyphenolics into vitamin C equivalent antioxidant capacity (VCEAC) by ABTS assay (symbols are the same as those listed above for **Figure 1A**).

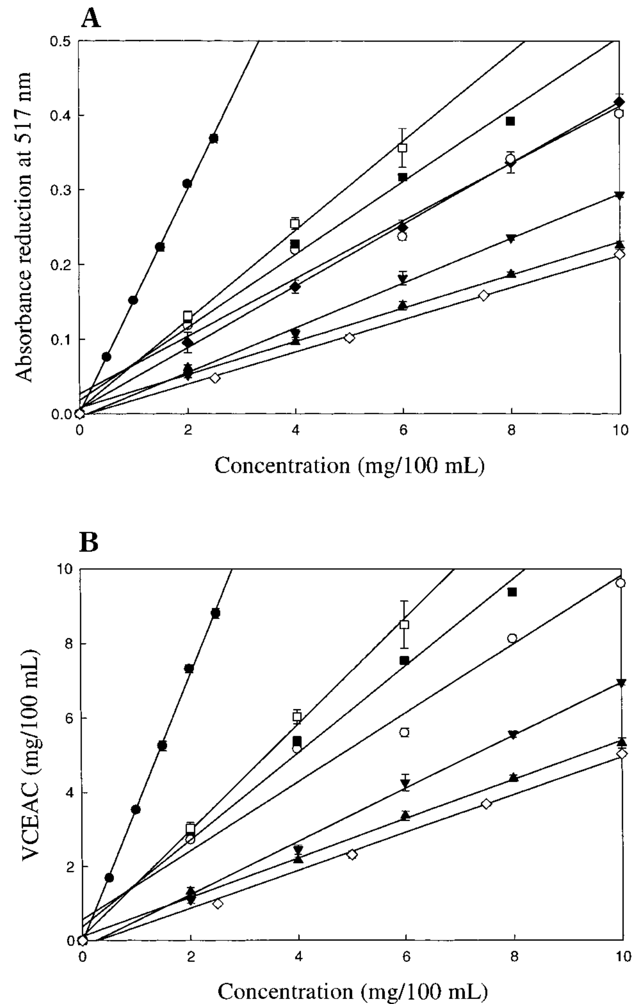


**Figure 2.** (A) Relationship between vitamin C or pure polyphenolics and absorbance reduction of violet DPPH radicals at 30 min and 23 °C. Error bar = standard deviation; $n = 15$ for vitamin C; $n = 5$ for polyphenolics; ● gallic acid ($r^2 = 0.998$); □ quercetin ($r^2 = 0.987$); ■ epicatechin ($r^2 = 0.989$); ○ catechin ($r^2 = 0.972$); ◆ vitamin C ($r^2 = 0.993$); ▼ Trolox ($r^2 = 0.996$); ▲ rutin ($r^2 = 0.993$); ◇ chlorogenic acid ($r^2 = 0.998$). (B) Conversion of antioxidant potential of seven polyphenolics into vitamin C equivalent antioxidant capacity (VCEAC) by DPPH assay (symbols are the same as those listed above for **Figure 2A**).

have been several similar reports of biphasic responses with various phytochemicals (*7, 23, 26*). The reaction occurred rapidly during the initial 10 s and then slowed. At the onset, the ABTS radical scavenging of fresh Gala apples (1.2 g/100 mL) that rapidly took place amounted to 55.0% of the total activity of the entire 10 min reaction time, whereas, at 10 s, the ABTS radical scavenging of 2 mg/100 mL vitamin C amounted to 70.1% of the total absorbance reduction of the 10-min reaction (**Figure 3**).

The antioxidant potential of the same Gala apple phenolics and pure phenolic compounds used in the ABTS assay were also evaluated by using violet DPPH radical. The relationship between absorbance reduction at 517 nm and ascorbic acid concentration showed a linear dose−response relationship ($r^2 = 0.993$) (**Figure 2A**) similar to that observed with the ABTS radical assay (**Figure 1A**). Likewise, all phenolic compounds tested displayed a linear relationship between the concentration (mg/100 mL) and absorbance reduction at 517 nm as shown in **Figure 2A**. The relative antioxidant capacity of these compounds evaluated by DPPH assay was as follows: gallic acid > quercetin > epicatechin > catechin ≥ vitamin C > Trolox > rutin > chlorogenic acid. This order of relative antioxidant capacity is similar to that observed by the ABTS assay except for Trolox. Also, three tested compounds (rutin, chlorogenic acid, and Trolox) exhibited lower antioxidant capacity than vitamin C. As noticed in the ABTS assay, the DPPH radical chromogen demonstrated the same biphasic kinetics with the apple phenolics but at a slower rate (**Figure 4**).

It was noticed that epicatechin had a higher VCEAC than its isomer, catechin, and that rutin (quercetin-3-*O*-rutinose) showed lower antioxidant capacity than its aglycon, quercetin, in both ABTS and DPPH assays. The ABTS radical assay showed rutin and chlorogenic acid with higher VCEAC than Trolox, whereas in the DPPH radical assay, those particular phenolics had lower VCEACs than Trolox. This may be due to the fact that the phenolic compounds on the free radicals reacted differently between aqueous phase (ABTS assay system) and organic phase (DPPH assay system).

Based on the absorbance reduction in the ABTS assay and DPPH assay due to apple phenolics in relation to that of the vitamin C standard, Gala apples showed 205.4 ± 5.6 mg/100 g

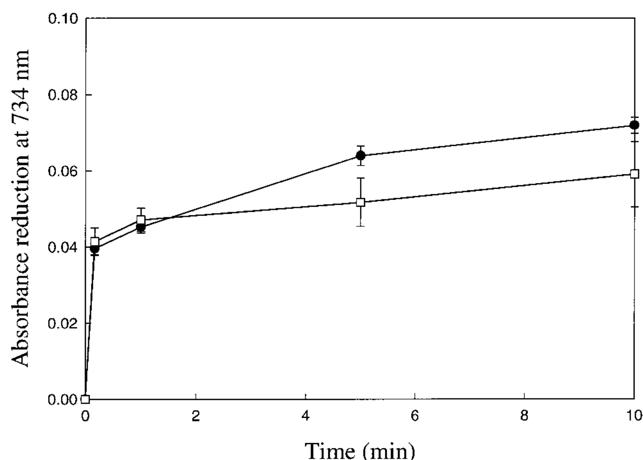


**Figure 3.** Time course of absorbance reduction of Gala apples (1.20 g/100 mL) and vitamin C (2 mg/100 mL) by the decolorization reaction of free ABTS radical anions at 10 min and 37 °C (error bar = standard deviation; $n = 5$ for Gala apples (●); $n = 10$ for vitamin C (□)).

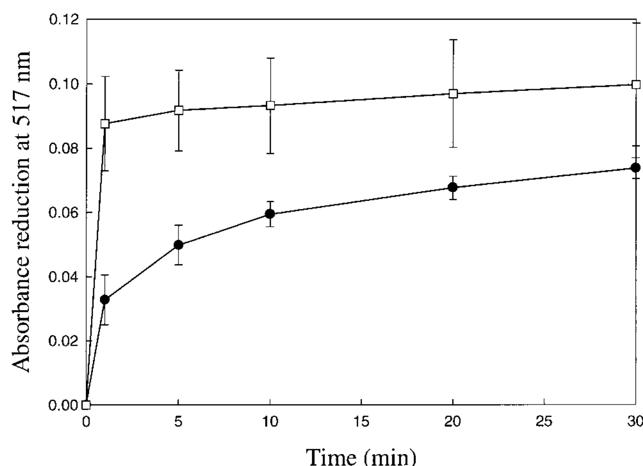


**Figure 4.** Time course of absorbance reduction of Gala apples (1.20 g/100 mL) and vitamin C (2 mg/100 mL) by the decolorization reaction of free violet DPPH radicals at 30 min and 23 °C (error bar = standard deviation; $n = 5$ for Gala apples (●); $n = 10$ for vitamin C (□)).

VCEAC and 136.0 ± 6.6 mg/100 g VCEAC, respectively. The DPPH assay underestimated antioxidant capacity about 33.8% compared to the ABTS assay. A similar methodological difference was reported previously (*22*). This underestimation of VCEAC by DPPH radicals may be due to the interference of other absorbing compounds at 517 nm.

In conclusion, this study presents a new concept of antioxidant capacity expression based on the natural antioxidant, vitamin C, in mg/100 g food using spectrophotometric methods. The most commonly used radicals, $ABTS^{\bullet-}$ and $DPPH^{\bullet}$, were used in this comparative study. VCEAC showed a linear relationship with pure phenolic compounds including Trolox, the most widely used reference compound. The results demonstrated that the existing Trolox equivalent value can be easily converted to a more familiar vitamin C equivalent (**Figures 1B** and **2B**). It was also found that the blue-green ABTS radical chromogens were specifically detected at 734 nm, a wavelength far from the visible region, whereas the DPPH radicals were detected at 517 nm which might be attenuated by possible interference. The radical scavenging time in the ABTS assay is 10 min, whereas the DPPH assay requires 30 min. Another advantage of the ABTS assay is that ABTS radical chromogens can be dissolved not only in aqueous phases but also in organic phases, whereas DPPH radical chromogens can be solubilized only in organic media. This particular characteristic of the $ABTS^{\bullet-}$ chromogen allows for the measurement of total antioxidant activity of any food sample in which the compounds are either lipophilic or hydrophilic (*21, 26*). Hence, it is recommended that total antioxidant capacity be measured by the ABTS radical assay and that total antioxidant capacity be expressed in VCEAC as mg/100 g sample.

## ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidino-propane)dihydrochloride; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; $IC_{50}$, concentration of a polyphenolic in mM required to quench the free radicals by 50%; ORAC, oxygen radical absorbance capacity; PBS, phosphate buffered saline;TEAC, Trolox equivalent antioxidant capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TRAP, total radical-trapping antioxidant parameter; VCEAC, vitamin C equivalent antioxidant capacity.

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