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Functionality of Bioactive Compounds in Brazilian Strawberry (*Fragaria* × *ananassa* Duch.) Cultivars: Evaluation of Hyperglycemia and Hypertension Potential Using in Vitro Models

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Fruits of seven fully ripened strawberry cultivars grown in Brazil (Dover, Camp Dover, Camarosa, Sweet Charlie, Toyonoka, Oso Grande, and Piedade) were evaluated for total phenolics, antioxidant activity based on DPPH radical scavenging assay, and functionality such as inhibition of α-amylase, α-glucosidase, and angiotensin I-converting enzyme (ACE) relevant for potentially managing hyperglycemia and hypertension. The total phenolics content ranged from 966 to 1571 μg of gallic acid/g of fruit fresh weight for Toyonoka and Dover, respectively. No correlation was found between total phenolics and antioxidant activity. The major phenolic compounds in aqueous extracts of strawberries were ellagic acid, quercetin, and chlorogenic acid. Strawberries had high α-glucosidase inhibitory activity. However, α-amylase inhibitory activity was very low in all cultivars. This suggested that strawberries could be considered as a potential dietary source with anti-hyperglycemic potential. The evaluated cultivars had no significant ACE inhibitory activity, reflecting low anti-hypertensive potential.

KEYWORDS: Strawberry cultivars; *Fragaria* × *ananassa* Duch.; antioxidant activity; enzyme inhibition; α-amylase; α-glucosidase; angiotensin I-converting enzyme; phenolic phytochemicals

INTRODUCTION

It is well-known that the consumption of fruits and vegetables could provide health benefits by lowering the risk for chronic diseases such as metabolic syndrome diseases including type 2 diabetes and cardiovascular disease (*1*–*3*). The potential benefits are likely related to the presence of phenolic compounds. Strawberries (*Fragaria* × *ananassa* Duch.) are well-known as good sources of vitamin C and other antioxidant compounds, such as flavonoids and ellagic acid (*4*), and have potential against metabolic syndrome diseases.

Increasingly, the global population including Brazil is facing the challenges from disorders of carbohydrate metabolism and its complications leading to health problems such as obesity and associated type 2 diabetes. The American Diabetes Association (ADA) estimated the national costs of diabetes in the United States alone for 2002 to be around $U.S. 192 billion (*5*).

More than 90% of affected diabetic patients have type 2 diabetes, and this is generally characterized by non-insulin-dependent hyperglycemia in early stages. The therapeutic approach available is by retarding the breakdown of starch and absorption of glucose through the inhibition of α-amylase in the pancreas and α-glucosidase enzymes in the intestinal tract. Examples of these inhibitors in clinical use are acarbose and miglitol (*6*). However, these drugs have side effects such as abdominal distention, flatulence, and possibly diarrhea caused by excessive inhibition of pancreatic α-amylase, which results in the abnormal bacterial fermentation of undigested carbohydrates in the colon (*6*). Natural α-amylase and α-glucosidase inhibitors from fruits and vegetables commonly consumed by a population could offer a good strategy to control postprandial hyperglycemia and provide a benefit without the side effects present in the most available drugs (*7*–*9*).

Khan et al. (*10*) studied common culinary herbs and spices, and they observed improvement in glucose metabolism that apparently was due to phenolic compounds of the extracts. Botanical products can improve glucose metabolism and the overall condition of patients with type 2 diabetes not only by

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direct hypoglycemic effects but also by improving lipid metabolism, antioxidant status, and capillary function (*11*).

One of the long-term complications of type 2 diabetes is high blood pressure, or hypertension (*12*). Hypertension has become the most common serious chronic health problem globally in recent years. According to the World Health Organization (WHO) around 20−45% of a population and nearly 50−60% of elderly people have elevated blood pressure (*13*). Angiotensin I-converting enzyme (ACE) is an important enzyme involved in maintaining vascular tension by two different reactions that it catalyzes: conversion of the inactive angiotensin I into a powerful vasoconstrictor and promoter of sodium retention, angiotensin II, and inactivation of the vasodilator bradykinin, which is conducive to lowering blood pressure (*14*). Inhibition of ACE is considered to be a useful therapy in the control of blood pressure in hypertensive patients, and therefore dietary sources of ACE inhibitors are potentially beneficial (*14*).

The objectives of this study were to characterize seven strawberry cultivars grown in Brazil in relation to the total phenolics, antioxidant activity, and associated inhibition of α-amylase, α-glucosidase, and ACE using in vitro methods. These insights would help target the consumption of strawberries as a part of an overall comprehensive dietary management strategy for type 2 diabetes and related hypertension.

MATERIALS AND METHODS

Materials. Fully ripened strawberry (*Fragaria* × *ananassa* Duch.) fruits that were ready for consumption of the cultivars Dover, Camp Dover, Camarosa, Sweet Charlie, Toyonoka, Oso Grande, and Piedade grown in Sao Paulo state (winter season of 2005) were harvested in Atibaia (Sao Paulo State, Brazil). Two kilograms of each cultivar, grown at the same place and in the same conditions, were cut into pieces, immediately frozen in liquid nitrogen, lyophilized, and stored at −20 °C until analyses. Porcine pancreatic α-amylase (EC 3.2.1.1), rat intestinal α-glucosidase (EC 3.2.1.20), rabbit lung ACE (EC 3.4.15.1), hippuric acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), chlorogenic acid, quercetin, and ellagic acid were purchased from Sigma Chemical Co. (St. Louis, MO).

Sample Preparation. Samples of lyophilized strawberry powder (5 g) were extracted in 100 mL of distilled water under reflux at 95 °C for 30 min. The samples were filtered through a Whatman no. 1 filter paper and then stored in a refrigerator at −20 °C until analysis for a period of no more than 1 week.

Total Phenolics Assay. The total phenolics in aqueous strawberry extracts were determined by using a method modified by Shetty et al. (*15*). Briefly, 0.5 mL of the aqueous extract was added to a test tube and mixed with 0.5 mL of 95% ethanol and 5 mL of distilled water. To each sample was added 0.5 mL of 50% (v/v) Folin−Ciocalteu reagent and mixed. The absorbance was read at 725 nm using a spectrophotometer (Genesys UV−visible, Milton Roy, Inc., Rochester, NY). Different concentrations of gallic acid solution were used to develop a standard curve. Results were expressed as micrograms of gallic acid per gram of fruit fresh weight (FW).

Antioxidant Capacity by the DPPH Radical Inhibition Assay. The antioxidant capacity was determined by the DPPH radical-scavenging method as modified from Kwon et al. (*8*). A 250 μL aliquot of the aqueous strawberry extract was mixed with 1250 μL of DPPH (60 μM in ethanol). The mixture was centrifuged at 13000*g* for 1 min, and after this time, the absorbance was measured at 517 nm using a spectrophotometer (Genesys UV−visible, Milton Roy, Inc.). The readings were compared with the controls, containing 95% ethanol instead of sample extract. The percentage inhibition was calculated by the equation

$$\%\ \text{inhibition} = \frac{\text{Abs}^{\text{control}} - \text{Abs}^{\text{extract}} \times 100}{\text{Abs}^{\text{control}}} \quad (1)$$

α-Amylase Inhibition Assay. The α-amylase inhibitory activity was determined according to an assay modified from the *Worthington Enzyme Manual* (*16*). Porcine pancreatic α-amylase (1 unit liberates 1.0 mg of maltose from starch in 3 min at pH 6.9 at 20 °C) was purchased from Sigma. A total of 500 μL of aqueous strawberry extract and 500 μL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing α-amylase solution (0.5 mg/mL) were incubated at 25 °C for 10 min. After preincubation, 500 μL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube at timed intervals. The reaction was stopped with 1.0 mL of dinitrosalicylic acid color reagent. The test tubes were then incubated in boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after the addition of 15 mL of distilled water, and absorbance was measured at 540 nm using a spectrophotometer (Genesys UV−visible, Milton Roy, Inc.). The readings were compared with the controls, containing buffer instead of sample extract. The results were expressed as percent α-amylase inhibition and calculated according to eq 1.

α-Glucosidase Inhibition Assay. The assay was performed according to the *Worthington Enzyme Manual* (*17*), with some modifications (*18*). α-Glucosidase (1 unit/mL) was assayed by using 50 μL of aqueous strawberry extracts and 100 μL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution and was incubated in 96-well plates at 25 °C for 10 min. After preincubation, 50 μL of 5 mM *p*-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25 °C for 5 min. Before and after incubation, absorbance readings were recorded at 405 nm by microplate reader (Thermomax, Molecular Device Co., Sunnyvale, CA) and compared to a control that had 50 μL of buffer solution in place of the extract. The results were expressed as percent of α-glucosidase inhibition and calculated according to eq 1.

ACE Inhibition Assay. ACE inhibition was assayed according to a method modified by Kwon et al. (*8*). The substrate hippuryl-histidyl-leucine (HHL) and ACE-I from rabbit lung (1 unit produces 1.0 μmol of hippuric acid from HHL per minute in 50 mM HEPES and 300 mM NaCl at pH 8.3 at 37 °C) were used. Fifty microliters of water-soluble supernatant of water extracts was incubated with 100 μL of 1 M NaCl−borate buffer (pH 8.3) containing 2 milliunits of ACE-I solution at 37 °C for 10 min. After preincubation, 100 μL of a 5 milliunit substrate (HHL) solution was added to the reaction mixture. Test solutions were incubated at 37 °C for 1 h. The reaction was stopped with 150 μL of 0.5 N HCl. The hippuric acid formed was detected; the spectra were confirmed and quantified by high-performance liquid chromatography (HPLC). Five microliters of the sample was injected using an Agilent ALS 1100 autosampler into an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a DAD 1100 diode array detector. The solvents used for the gradient were (1) 10 mM phosphoric acid (pH 2.5) and (2) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% for 5 min and then was decreased to 0% for the next 5 min (18 min total run time). The analytical column used was a Nucleosil 100-5C18, 250 × 4.6 mm i.d., with packing material of 5 μm particle size at a flow rate of 1 mL/min at ambient temperature. During each run, the absorbance was recorded at 228 nm and the related chromatogram was integrated using Agilent Chemstation (Agilent Technologies) enhanced integrator for detection of liberated hippuric acid. Pure hippuric acid (purchased from Sigma Chemical Co.) was used to calibrate the standard curve and retention time. The percent inhibition was calculated according to eq 2:

$$\%\ \text{inhibition} = \frac{E^{\text{control}} - E^{\text{extract}} \times 100}{E^{\text{control}} - E^{\text{blank}}} \quad (2)$$

HPLC Analysis of Phenolic Profiles. Two milliliters of aqueous strawberry extracts was filtered through a 0.2 μm filter. A 5 μL volume of sample was injected using an Agilent ALS 1100 autosampler into an Agilent 1100 series HPLC (Agilent Technologies) equipped with a DAD 1100 diode array detector. The solvents used for gradient elution were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% over the next 7 min and then decreased to 0% for the next 3 min and maintained for the next 7 min (total run time = 25 min). The analytical column used was an Agilent Zorbax SB-C18, 250

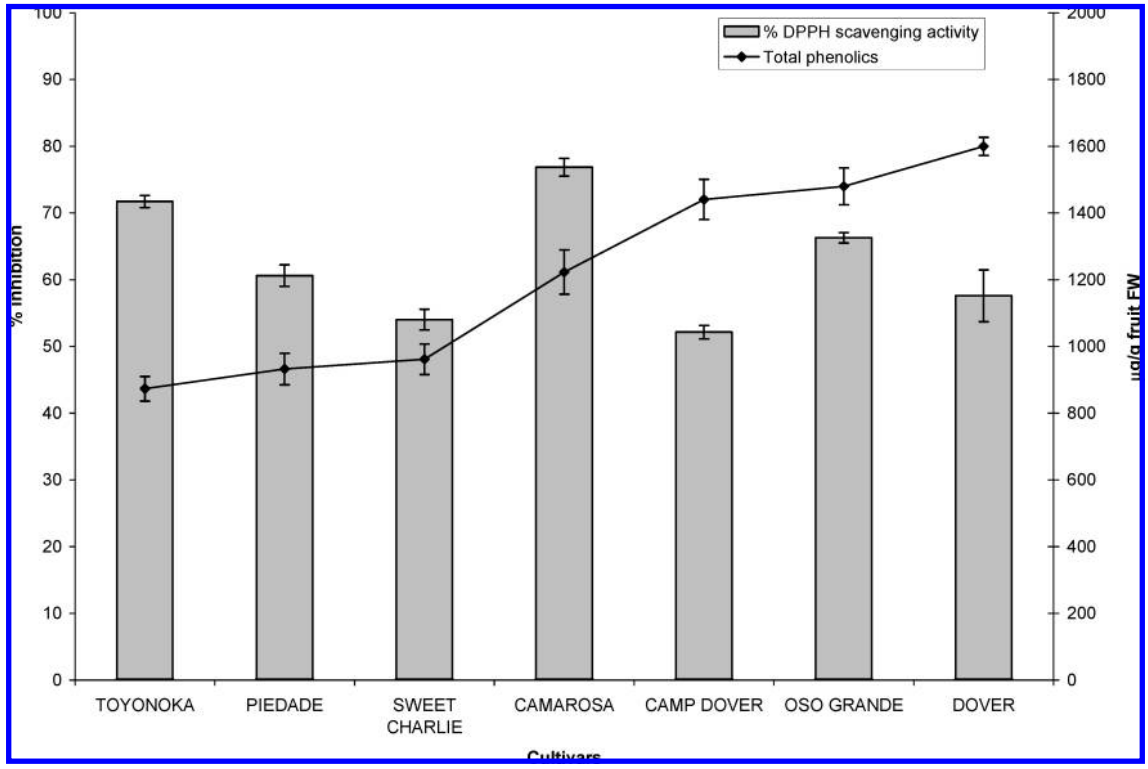

Figure 1. Total phenolic content (μg of gallic acid/g of fruit FW) and DPPH radical scavenging activity (%) in aqueous extracts of different strawberry cultivars.

$\times$ 4.6 mm i.d., with packing material of 5 μm particle size at a flow rate of 1 mL/min at ambient temperature. During each run the absorbance was recorded at 306 and 333 nm, and the chromatogram was integrated using Agilent Chemstation enhanced integrator. Pure standards of ellagic acid, quercetin, and chlorogenic acid (purchased from Sigma Chemical Co.) in 100% methanol were used to calibrate the standard curve and retention times. Results were expressed as micrograms per gram of fruit FW.

Statistical Analysis. All analyses were run at least in triplicate and were expressed as mean $\pm$ standard deviation (SD). Statistical analysis was done by using the Statistic software package version 5.0 (StatSoft, Inc., Tulsa, OK). Differences between means were first analyzed by ANOVA test and then least significant difference (LSD) test ($p < 0.05$).

RESULTS AND DISCUSSION

Total Phenolics and Antioxidant Activity. Strawberries are well-known as good sources of vitamin C and polyphenolics such as flavonoids and ellagic acid, which have potential health benefits (*4*). Aqueous extracts from seven of the most common strawberry cultivars grown in Brazil were evaluated in this study to investigate the effect of water-soluble phenolic compounds in relation to the antioxidant activity and hyperglycemia-relevant functionality. **Figure 1** shows the results for the total phenolics and antioxidant activity in the strawberry cultivars.

The results indicated that there is a significant difference among all cultivars. The total phenolic content measured by the Folin−Ciocalteu assay ranged from 966 to 1571 μg/g of FW. The cultivars Dover and Oso Grande had the highest total phenolic contents. The antioxidant activity of the strawberry cultivars was evaluated by the DPPH radical inhibition assay. However, no correlation between total phenolics and antioxidant activity was observed ($r = 0.1561$). These results for strawberry cultivars suggested that the free radical linked antioxidant activity of these samples could be associated not only with the total phenolic content but with other compounds. For example, the cultivar Sweet Charlie had one of the lowest total phenolic content but had the highest antioxidant activity among all cultivars.

Hassimotto et al. (*19*) found no correlation between total phenolic content and antioxidant activity, assessed by liposome oxidation and β-carotene bleaching methods, of fruits, vegetables, and commercial frozen pulps consumed in the Brazilian diet. This study suggested that the antioxidant activity is a result of the combination of different compounds having synergistic and antagonistic effects (*19*).

Wang et al. (*20*) reported that among 12 fruits analyzed, strawberries showed the highest antioxidant activity assessed by the oxygen radical absorbance capacity (ORAC) assay. In raspberry fruits the main antioxidant compounds observed were anthocyanins, ellagitannins, and proanthocyanidins (*21*). Among these compounds, ellagitannins were the principal antioxidants found in these fruits, contributing 30−60% of the total antioxidant activity measured by the ABTS method. However, according to Heinonen (*22*) the antioxidant effect of berry phenolics is strongly dependent on the raw material, and a great diversity of methods have been applied to study the antioxidant activity of these compounds, resulting in differences in the results.

From the current study we cannot draw conclusion about the relevance of total phenolic content to antioxidant activity. Therefore, the phenolic profile in each strawberry cultivar is important to explain the difference found in relation to the antioxidant activity and possibly the health-relevant functionality.

HPLC Analysis. Figure 2 shows the major phenolic compounds present in the aqueous extract from strawberries. Quercetin, chlorogenic acid, and ellagic acid were the major phenolic compounds in strawberries. Extracts of strawberry cultivars had an ellagic acid content ranging from 6 to 20 μg/g of FW. Quercetin content in these fruits ranged from 16 to 55

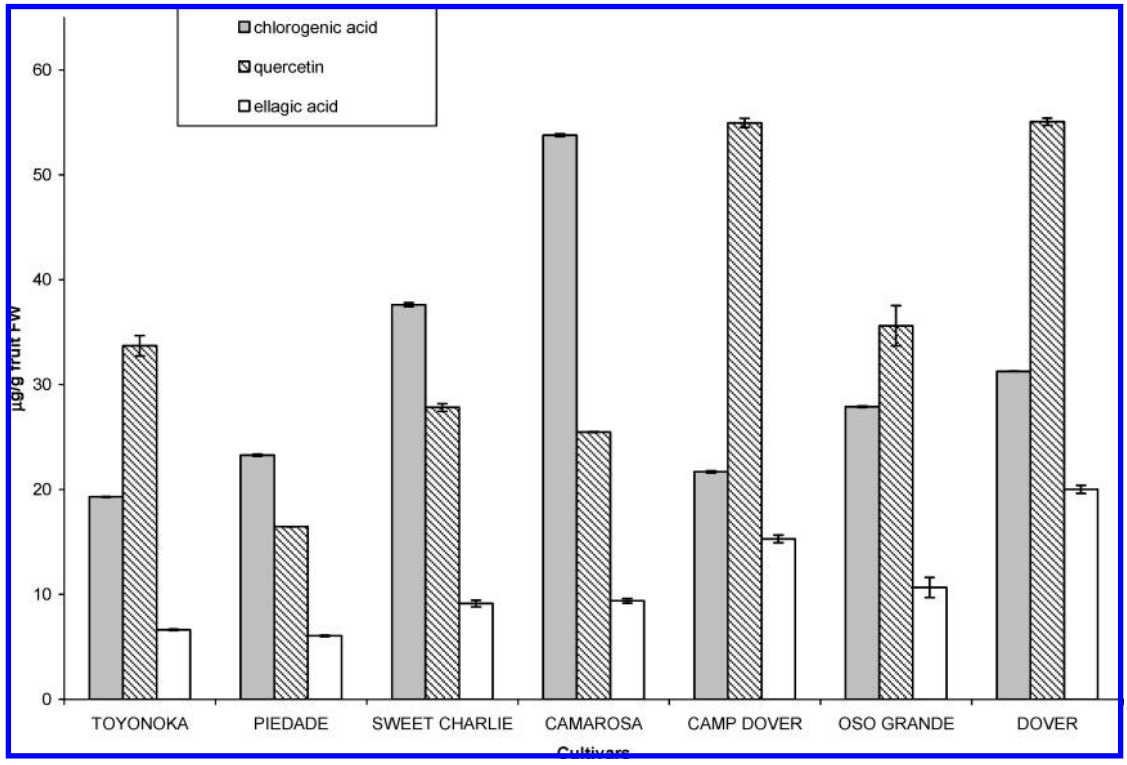

Figure 2. HPLC analysis of individual phenolic compounds (μg/g of fruit FW) in aqueous extracts of different strawberry cultivars.

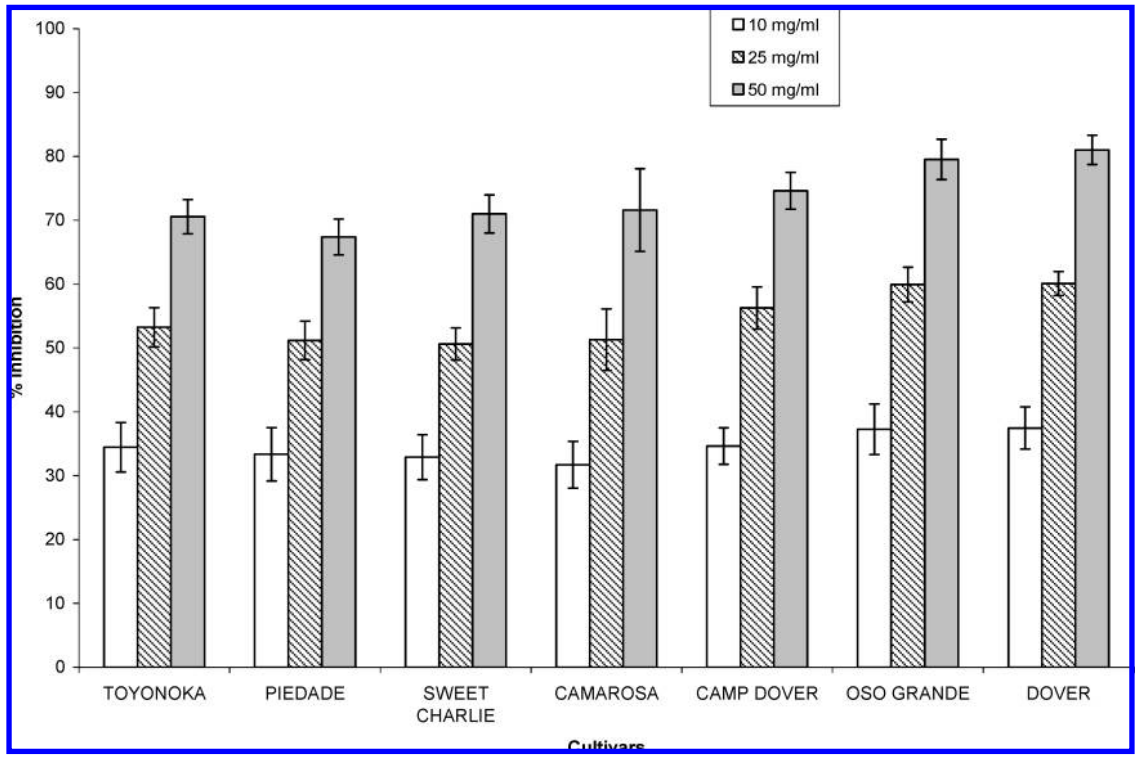

Figure 3. Dose-dependent changes in α-glucosidase inhibitory activity (%) in aqueous extracts of different strawberry cultivars (sample concentrations on a total dry weight basis = 10, 25, and 50 mg/mL).

μg/g of FW. The cultivars Camp Dover and Dover had the highest quercetin and ellagic acid contents. Also, the results indicated that the quercetin content was much higher than the ellagic acid content. Chlorogenic acid content in strawberries ranged from 19 (Toyonoka) to 54 (Camarosa) μg/g of FW.

From the HPLC analysis specific phenolics were higher in cultivars with high total phenolics but did not correlate to DPPH radical scavenging linked antioxidant activity. This indicates that Sweet Charlie cultivar, which had among the lowest phenolic profiles, had the highest antioxidant activity, indicating additional factors contributing to this functionality. Further studies on the classification of antioxidant function to specific phenolics are essential. In addition, cell culture and animal studies are necessary to determine the antioxidant potential of these compounds because the in vitro antioxidant activity would not reflect in vivo biological activity (*23*).

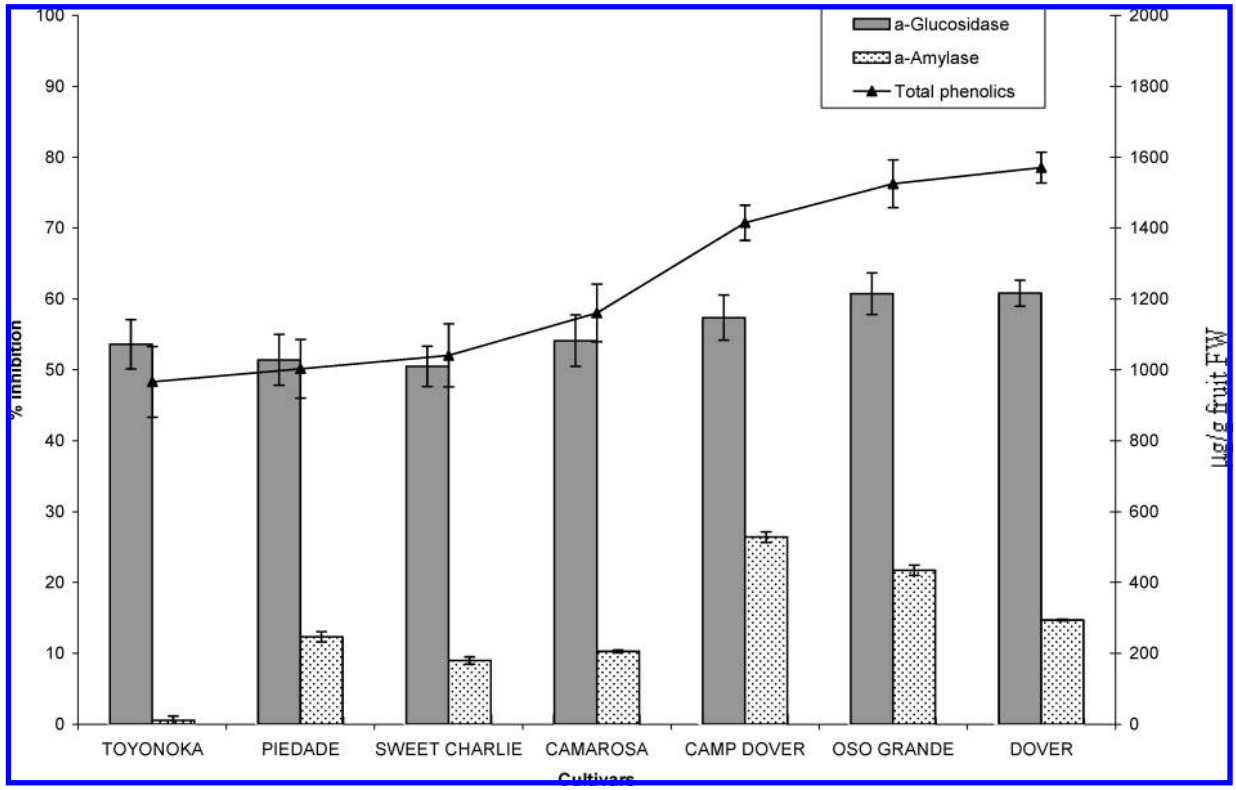

Figure 4. Comparison of α-amylase and α-glucosidase inhibition (sample concentration on a total dry weight basis = 25 mg/mL) and total phenolic content (μg of gallic acid/g of fruit FW) in aqueous extracts of different strawberry cultivars.

There is a particular interest in the amount of ellagic acid in fruits because of increasing evidence of its chemopreventive and antioxidant effects (*24*). This compound exists in plants in many derivative forms that differ in solubility, mobility, and reactivity in plant as well as in animal systems. However, most of the ellagic acid in berries is present as an ellagitannin esterified with glucose, requiring an acid hydrolysis step to liberate it (*25*). There are several in vivo and in vitro studies showing that the ellagitannins can be hydrolyzed, yielding ellagic acid, but there is no information if it occurs at physiological pH or mainly modified by colon microflora (*24*).

Ellagic acid is found in nuts and berries such as strawberry, raspberry, and blackberry (*24*). Strawberries represent the main source of ellagic acid derivatives in the Brazilian diet, corresponding to >50% of all phenolic compounds found in the fruit (*25*).

Many studies reported the ellagic acid content of different cultivars of strawberries, but significant differences were observed among them, ranging from 20 to 522 μg/g of FW (*4, 24–26*). This fact could be attributed not only to differences in the cultivar studied but also to the method chosen for the determination because free ellagic acid levels are generally low and substantial quantities of this compound are detected only after acid hydrolysis of extracts, as a product of ellagitannin breakdown (*24, 25*).

Despite several studies showing the in vitro antioxidant capacity of the ellagitannins in several model systems, it is not clear if this property was evident in vivo because a compound that showed high antioxidant activity could show low absorption, high metabolic turnover, or be quickly eliminated, resulting in low biological activity (*27*). In addition, such phenolic compounds have other functional properties for disease management (*23*).

α-Glucosidase and α-Amylase Inhibition Assays. α-Glucosidase and α-amylase are well-known enzymes in the management of hyperglycemia linked to type 2 diabetes (*6*). In this study, different strawberry cultivars were evaluated in relation to the possible inhibition of these two enzymes using in vitro assays. **Figure 3** shows the dose-dependent response of α-glucosidase to the strawberry extracts of different evaluated cultivars at different sample dry weights.

The cultivars Dover and Oso Grande had the highest α-glucosidase inhibitory activity among strawberry cultivars. As shown in **Figure 1** these cultivars had also the highest total phenolic contents. Therefore, good correlations were observed between α-glucosidase inhibitory activity and total phenolic content ($r = 0.9474$), ellagic acid ($r = 0.7463$), and quercetin ($r = 0.7414$). However, high free radical linked antioxidant activity could not be used as a predictor of α-glucosidase inhibitory activity because no correlation was found ($r = -0.0227$). Also, no correlation was observed between α-glucosidase inhibitory activity and chlorogenic acid ($r = -0.1423$).

McDougall et al. (*9*) studied high-anthocyanin-containing fruit extracts from blueberry, currant, raspberry, and strawberry and observed good α-glucosidase inhibition by these extracts. However, Cheplick et al. (*28*) reported high α-glucosidase inhibitory activity for a yellow raspberry cultivar among red, black, and yellow raspberries, suggesting that the α-glucosidase may be influenced more by specific anthocyanins rather than the actual amount of the overall total plant phenolics.

Figure 4 shows the comparison of α-amylase and α-glucosidase inhibitory activities at the same sample dry weight and variable total phenolic content of strawberry cultivars. The results indicated that strawberry cultivars had very low α-amylase inhibitory activity when compared to α-glucosidase inhibition. Also, a good correlation was observed between α-amylase and total phenolics ($r = 0.7577$) even at these low levels of α-amylase inhibitory activity. These results showed that the antioxidant activity does not reflect the structure−function

Table 1. Angiotensin I-Converting Enzyme (ACE) Inhibitory Activity in Aqueous Extracts of Different Strawberry Cultivars

cultivar	% ACE inhibition
Toyonoka	11 ± 2
Piedade	9 ± 2
Sweet Charlie	9 ± 2
Camarosa	nd[a]
Camp Dover	nd
Oso Grande	nd
Dover	nd

[a] Not detected.

relevance of these compounds in the context of α-amylase and α-glucosidase inhibitory activities.

Cheplick et al. (*28*) also reported that when comparing different raspberry cultivars for α-amylase inhibitory and antioxidant activity no correlation was found. These authors suggested that probably the α-amylase inhibitory activity might be due to some specific phenolics. Many fruits including strawberry, raspberry, and grape are known to contain high levels of soluble tannins, and these fruits have α-amylase inhibitory properties (*9*). However, these studies observed that neither ellagic acid nor gallic acid inhibited α-amylase (*9*).

It is known that dietary management of hyperglycemia linked to type 2 diabetes can be targeted through whole foods that have high α-glucosidase and moderate α-amylase inhibition (*7, 8, 23*). Excessive α-amylase inhibition can lead to undigested starch in the intestines and consequent stomach distention and discomfort (*6*). As a consequence, strawberry cultivars such as Dover and Oso Grande with high α-glucosidase and low α-amylase inhibitory activities could be considered potential candidates for further in vivo studies as part of more comprehensive dietary designs to manage the early stages of hyperglycemia linked to type 2 diabetes.

ACE Inhibition. Hypertension is a related long-term macrovascular complication of diabetes. All of the strawberry cultivars were evaluated for possible inhibitory activity on ACE (**Table 1**). No significant inhibitory activity was observed for all strawberry cultivars investigated in the present study. Kwon et al. (*8*) tested several purified compounds in relation to the ACE inhibition, and they observed that among them quercetin, chlorogenic acid, and ellagic acid had no inhibitory activity. As observed in **Figure 2** the main phenolic compounds in aqueous extracts of strawberries were quercetin, chlorogenic acid, and ellagic acid. However, Suzuki et al. (*29*) reported an ACE inhibitory activity of chlorogenic acid in hypertensive rats with an improvement on the vasodilation. Also, water-soluble extracts of green coffee beans, the main component of which was chlorogenic acid, lowered blood pressure in mildly hypertensive patients (*30*). In the present study, chlorogenic acid was a major phenolic found in the strawberry cultivars. However, no in vitro inhibitory activity was observed. It is possible that in vivo responses may be supported by overall intracellular redox responses and therefore more efficient in producing benefits. Therefore, better in vivo cell culture or mice models reflecting potential in vivo benefits are needed.

Another possibility is that other compounds present in food are likely to be responsible for the inhibitory activity because there is evidence that ACE inhibition could be due to the presence of some water-soluble compounds other than the phenolics, which may be low in these strawberry cultivars (*28*). This is supported further by the findings of Kwon et al. (*8*) that the ACE inhibitory activity in water extracts of lemon balm, rosemary, and raspberry did not correlate to the total phenolic content or even specific phenolics present in these samples. There is evidence that some peptides naturally present in foods could be responsible for such inhibitory activity (*31, 32*).

In conclusion, there are considerable differences in the contents of bioactive compounds and functionality among strawberry cultivars grown in Brazil. Also, specific strawberry cultivars could be selected for contribution to an overall healthy diet for the management of postprandial hyperglycemia through their capacity to inhibit α-glucosidase concurrently with low inhibition of α-amylase, likely resulting in fewer undesirable side effects.

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