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COMPARATIVE ANTIOXIDANT PROPERTIES OF CITRUS SPECIES: EVIDENCE FOR POTENT, NON-VITAMIN ANTIOXIDANTS IN C. AURANTIFOLIA

Yu Yao and Amandio Vieira*

Simon Fraser University, Canada

Abstract: Dietary antioxidants can complement the body's endogenous antioxidant activities in moderating oxidative tissue damage. Citrus fruits are widely consumed and are considered an important source of dietary antioxidants, primarily vitamin C. Non-vitamin antioxidants such as flavonids may also contribute to total antioxidant activity of citrus fruits. From a public health perspective, there is growing evidence for decreased risk of major chronic diseases with increased consumption of flavonoid-rich foods such as citrus fruits and other plant-based foods. In this context, we have tested fruit juices from three *Citrus* species, *C. aurantifolia, C. reticulata*, and *C. sinensis*, in a previously standardized oxidation assay with potential pathological relevance. *C. aurantifolia* exhibited the most potent antioxidant activity, approximately 3-fold greater than the other two ($P<0.05$). The antioxidant activity of *C. aurantifolia* was greater than expected if such activity were based only on total phenolic content, or on reported vitamin C and E contents. Overall, this study characterizes the relative antioxidant potency of three *Citrus* species, identifies *C. aurantifolia* as the most potent, and suggests a major contribution of non-vitamin factors to total *in vitro* antioxidant potency of *C. aurantifolia*. Potential antioxidant factors are also suggested based on reported, relative phytochemical levels in the three species.

Keywords: antioxidant; Citrus aurantifolia; Citrus reticulata; Citrus sinensis; eriodityol; flavonoids; lime; nutrients; orange; polyphenols; vitamins

INTRODUCTION

Increased production or deficient elimination of reactive oxygen species (ROS), and other oxidizing agents, leads to oxidative stress and damage to cells and tissues. There is evidence that oxidative damage is an important contributor to aging and various chronic diseases such as cancer and neurodegeneration (Youdim et al., 2004; Scalbert et al., 2005; Nogata et al., 2006). Both dietary antioxidants and those endogenous to the body are involved in controlling oxidative damage. In the context of the nervous system, antioxidants have been shown to improve motor and cognitive functions in

* Corresponding author: Nutrition and Metabolic Research Laboratory, Kin-9600 Sciences, Simon Fraser University, 8888 University Drive, Burnaby, B.C., V5A 1S6 Canada; Tel: 778-782-4251, Fax: 778-782-3040, E-mail: avvieira@sfu.ca

experimental animals and prevent ROS-mediated neuronal death (Youdim et al., 2004; Socci et al., 1995; Joseph et al., 1999; Andres-Lacueva et al., 2005). From a public health perspective, there is much evidence that increased consumption of plant foods, including flavonoid-rich citrus fruits, may decrease the risk of cognitive impairments and neurodegenerative disorders (Dajas et al., 2003; Letenneur et al., 2007; Ng et al., 2008; Scarmeas et al., 2006; Silalahi, 2002). The potential neuro-protective mechanisms are currently being investigated at the molecular and physiological levels; and this current study on citrus antioxidant activities contributes to such research.

Citrus fruits are widely consumed and are recognized as important sources of ascorbate (vitamin C), an antioxidant vitamin. Citrus fruits are also major dietary contributors to total flavonoid intake (Johannot and Somerset, 2006), especially flavanones and flavones (selected flavonoid structures in Figure 1), and are also sources of carotenoids. Several of these compounds are under study in the context of human health; e.g., rutinosyl flavanones such as eriocitrin (eriodictyol 7-*O*-β-rutinoside) and hesperidin (heperetin 7-*O*-β-rutinoside) have been examined for antioxidant activities (Finotti and Dimajo, 2003; Miyake et al., 2000; Areias et al., 2004; Miyake et al., 1998; Minato et al., 2003). Studies on the bioavailability of flavanones such as hesperetin and naringenin have indicated that they can be absorbed by humans and rodents (Erlund et al., 2001; Felgines et al., 2000). Moreover, 2-4 h after administration of eriocitrin to rodents, several metabolites were found in the circulation, and plasma antioxidant activity (but not the levels of vitamins E and C) was significantly increased (Miyake et al., 2000). Thus, there is a possibility that, *in vivo*, citrus fruit flavonoids or their physiological metabolites influence the risk or progression of diseases that involve oxidative and other pathological mechanisms (Nogata et al., 2006; Felgines et al., 2000; Benavente-Garcia et al., 1997).

In our current study, the in vitro antioxidant potencies of raw juices from three *Citrus* species were evaluated using an assay based on cytochrome c- (Cytc-) enhanced oxidation of N,N,N',N'-tetramethyl-1,4-phenylenediamine (Zhou et al., 2004). This assay may be of relevance to some pathological events involving oxidative stress and mitochondrial dysfunction, cf. (Zhou et

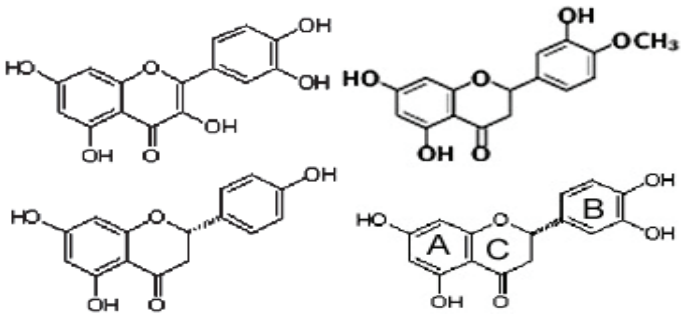

Figure 1. Aglycone structures of flavanones and a flavanol relevant to the comparisons performed in this study. Flavanones: eriodictyol (lower right, with the three rings labeled); hesperetin, (upper right); naringenin, (lower left); Flavonol: quercetin, (upper left).

al., 2004; Hashimoto et al., 1999; Oliver et al., 2005; Clayton et al., 2005; Burkitt et al., 2004; Von Ahsen et al., 2000) which, in turn, may relate to progression of some neurodegenerative disorders (Hashimoto et al., 1999; Oliver et al., 2005; Clayton et al., 2005; Zhou et al., 2004, and references therein). Overall, our results indicate significant differences in the antioxidant potencies of *C. aurantifolia, C. reticulata*, and *C. sinensis* juices, and such differences are related to reported juice phytochemical compositions for the three *Citrus* species.

MATERIALS AND METHODS

Citrus material and reported phytochemical content

Fresh, pulp-free juice from three citrus fruits, *C. aurantifolia* (lime, Mexican), *C. reticulata* (sweet orange, Navel), and *C. sinensis* (mandarin orange), was collected by hand-squeezing the cut fruit, stored frozen (-80 Celsius) and, after centrifugation (14000 rpm for 30 s in table-top Spectrafuge 16M), used for the oxidation assay. The fruits were purchased locally (Vancouver metropolitan area) and had been transported from their production origins in the USA (limes and sweet oranges) and China (mandarin oranges).

Potency of antioxidant activity was obtained using different volumes of the raw juice in the assays (see Figure 1). Reported content of flavonoids (quercetin, hesperidin, eriodictyol, hesperetin, eriocitrin, naringenin) and non-flavonoids (vitamins C and E, beta-carotene) in the raw juice of the three *Citrus* species was used for comparison with antioxidant potency. For each *Citrus* species examined an average of at least 3 (n = 3-10) different reported contents was used. The reported values were pooled to generate a mean and standard deviation irrespective of factors (often not known or reported) such as cultivar, environmental growth conditions, state of maturity.

The data sources for reported phytochemical values were as follows: *C. aurantifolia* (Nogata et al., 2006; Mouly et al., 1994), *C. reticulata* (Nogata et al., 2006; Kanaze et al., 2003; Dhuique-Mayer et al., 2005), and *C. sinensis* (Nogata et al., 2006; Erlund et al., 2001; Mouly et al., 1994; Dhuique-Mayer et al., 2005; Kanaze et al., 2003; Esteve et al., 2005; Rapisarda et al., 1999; Cortes et al., 2004). In addition, the USDA database for the Flavonoid Content of Selected Foods, www.ghbf.com/flav 2003, was a source of data for each of the three *Citrus* species.

Antioxidant assays

The assay with TMPD (N, N, N', N'-Tetramethyl-1-4-phenylenediamine dihydrochloride; Sigma-Aldrich) as the indicator for oxidative activity has been previously detailed and was performed as described (Zhou et al., 2004). The TMPD stock solution was prepared in phosphate buffer, pH 7.4, and flushed through with argon gas as described (Zhou et al., 2004). The putative antioxidants to be tested (see volumes of raw juice in figure legends) were added to the reaction buffer in a cuvette containing preincubated Cytc (equine heart, Sigma-Aldrich) and hydrogen peroxide. The cuvette contents were flushed with argon before addition of TMPD. Based on total amounts of reagents added to the assay, the final concentrations of TMPD, Cytc, and hydrogen peroxide in the assay were 400 μM, 10 μM, and 5 mM, respectively. Progression of the oxidation reactions was followed spectrophotometrically at 630 nm. Typically, the 20 μl juice volume values for antioxidant activity (Figure 2) were used for the comparative studies (Figures 3 and 4). The relative antioxidant activities for this volume were

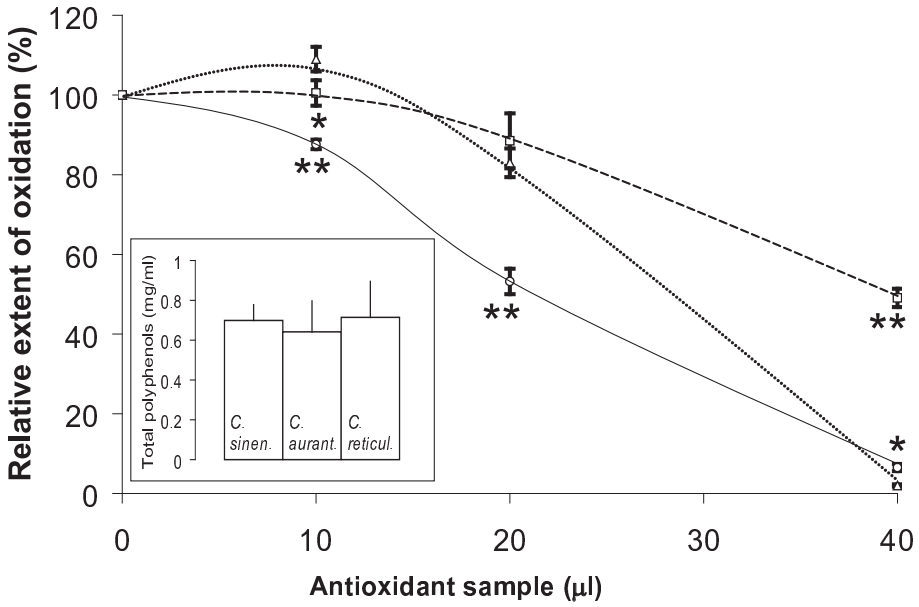

Figure 2. Comparison of the antioxidant activities of juices from three *Citrus* species. Raw juices (volume used is indicated in µl) from C. *aurantifolia* (solid), C. *sinensis* (dotted), and C. *reticulata* (dashed) were tested in cytochrome c-enhanced oxidation assay. Inhibition of 6-OH dopamine (TMPD) oxidation by the three C*itrus* species is shown. The statistical significance indicated for C. *aurantifolia* and C. *reticulata* is defined relative to C. *sinensis*. **Insert** shows total phenol content (mg/ml of gallic acid equivalents; see Materials and Methods) of the raw juice of the three C*itrus* species. None of the differences in total phenols was statistically significant (error bars indicate SEM).

similar to those obtained from an average of antioxidant activities expressed per µl juice (see Results).

Content of total phenols

The assays were performed using Folin-Ciocalteu reagent (Sigma-Aldrich) following typical protocols (Heimler et al., 2005) with minor modifications, as follows: to the centrifuged (see above) raw citrus juices added at different volumes to microplate (Nunc Maxisorp) wells, water was added to bring the total well volume of 50 µl, followed by an equal volume of Folin-Ciocalteu reagent. An equal volume (100 µl) of 20% sodium carbonate in water (BA Allied) was then added. Absorbance was measured after an incubation period of approximately 10 min at room temperature. Measurements were performed on three different fruits, with *n* of 4-6 for each juice volume tested. A standard curve was obtained using gallic acid (Sigma), and total polyphenol content of the citrus samples was expressed as mg gallic acid equivalents/ml (Figure 2, inset).

Statistics

Results are presented as the group mean ± standard error of the mean (SEM) for each experimental group, unless noted otherwise. The alpha-level for statistical

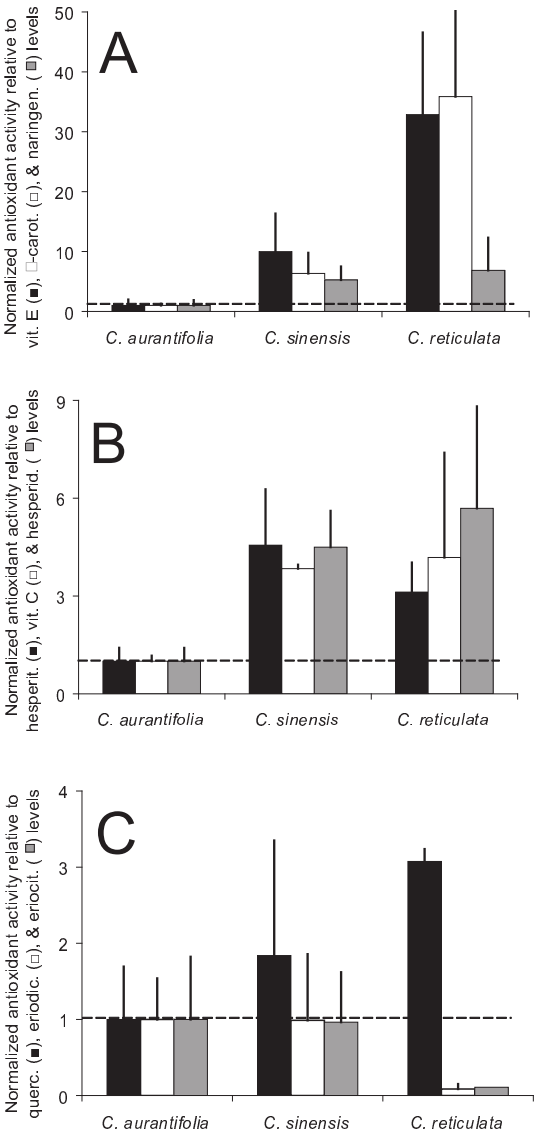

Figure 3. Relative contribution of various putative antioxidants to the antioxidant activities of three *Citrus* species. Raw juices from *C. aurantifolia*, *C. sinensis*, and *C. reticulata* were tested in the cytochrome *c*-enhanced TMPD oxidation assay. In each case, inhibition of oxidation was expressed relative to the reported (see Results section for references) content of the putative antioxidants indicated: vit. E (vitamin E), β-carot. (beta-carotene), naringen. (narigenin), hesperet. (hesperetin), vit. C (vitamin C), hesperid. (hesperidin), querc. (quercetin), eriodic. (eriodictyol), eriocit. (eriocitrin). The putative antioxidants were placed into three groups, **A**, **B**, **C**, based on similar extents of overall antioxidant effects (i.e., to minimize difference in *x* axis values between highest and lowest activities).

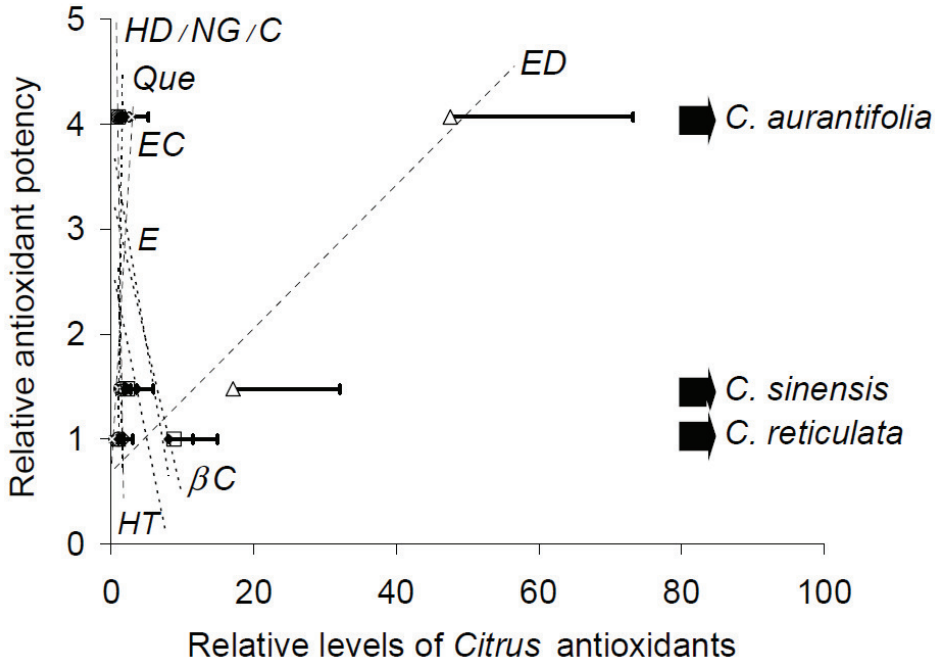

Figure 4. Relative antioxidant activities of juices from three *Citrus* species correlated to reported content of putative antioxidants. Raw juices from *C. aurantifolia*, *C. sinensis*, and *C. reticulata* were tested in cytochrome *c*-enhanced oxidation assay for 6-OH dopamine, and the results were normalized such that the lowest antioxidant potency (i.e., *C. reticulata*) had unitary value. The same normalization was performed for the reported contents of flavonoids and antioxidant vitamins/provitamins; in this case the normalization resulted in unitary value assigned to the lowest level for each phytochemical. See Results section for references of reported values, as well as all the correlation coefficients. Lines are labeled for clarity in cases where individual points cannot be distinguished. The two strongest correlations ($r^2 > 0.9$) were EC (eriocitrin) and ED (eriodictyol). The black arrows and names of the *Citrus* species indicate the three levels of relative antioxidant potency. Error bars for each symbol indicate standard deviations in reported antioxidant levels. The other data point symbols used are as follows: Que, quercetin; E, vitamin E; HT, hesperetin; βC, β-carotene; HD, hesperidin; NG, naringenin; C, vitamin C. The latter three are labeled jointly because they had very similar correlations (see Results section).

significance was 0.05. Independent t-tests (two-tailed) were performed on the data for the comparison of two samples. Two P-value levels—P less than or equal to 0.05 but greater than or equal to 0.01, and P less than 0.01—are indicated in the figure legend with * or **, respectively, for statistical significance.

RESULTS

Relative antioxidant potencies and total phenolic content of three Citrus species

Raw juices from *C. aurantifolia*, *C. sinensis*, and *C. reticulata* were tested in the cyto-

chrome c-enhanced oxidation assay. Figure 2 shows the relation between inhibition of TMPD oxidation and the amount (µl) of juice used from each of the *Citrus* species. Comparing the intermediate (20 µl) juice volume, *C. aurantifolia* is typically 2.7 and 4.1 times more potent as an antioxidant than *C. sinensis* and *C. reticulata*, respectively (these are also the relative antioxidant levels shown in Figure 4). At the highest volume tested, *C. aurantifolia* and *C. sinensis* display similar antioxidant potencies in this assay. For each citrus fruit, the three antioxidant potencies at each volume (i.e., 10, 20, and 40 µl) were also used to calculate an average antioxidant potency per µl; these relative values for *C. aurantifolia*, *C. sinensis,* and *C. reticulata*, are 3.3, 1.3, 1.0, respectively, and are similar (e.g., lime is 3.3/1.3 or 2.5-fold more potent than orange) to those presented above for 20 µl.

Total polyphenol content is shown in the inset of Figure 2. The values range from 0.64 mg gallic acid equivalents/ml for *C. aurantifolia* to 0.71 mg gallic acid equivalents/ml for *C. reticulata.* Differences in total polyphenol content among the three *Citrus* species were not statistically significant (P values ranged from 0.33 to 0.96). The values that we obtained for total phenol content of *C. sinensis* were similar to those previously reported, 0.4-1.1 mg/ml, for the same *Citrus* species (Rapisarda et al., 1999; Baghurst, 2003).

Antioxidant activities of the three Citrus species correlated to reported content of some flavonoids and nutrients

Figure 3 shows the relative contribution of various putative antioxidants to the antioxidant activities of the three *Citrus* species. In all cases, the ratio of antioxidant activity to levels of putative antioxidant was set at 1 for *C. aurantifolia*, the most potent antioxidant *Citrus* species tested. While eriocitrin and eriodictyol contribute relatively more to the antioxidant potency of *C. aurantifolia* (relative to *C. reticulata*, and *C. sinensis*), all other flavonoids and vitamins E and C contribute more to the antioxidant potency of *C. reticulata* and *C. sinensis* (relative to *C. aurantifolia*). Of all the compounds examined, β-carotene and vitamin E (Fig. 3A) are likely to have made the smallest relative contribution to the antioxidant activity of *C. aurantifolia*.

As shown in Figure 4, there is a strong, positive correlation ($r^2 \sim 0.96$, $P < 0.05$) of antioxidant activity of the three *Citrus* species with their reported eriodictyol contents (from USDA source and articles referenced in 'Materials and Methods'). There is also a suggestion of a strong positive correlation ($r^2 \sim 0.97$) with eriocitrin; but the few data points available in the literature do not allow for statistical significance ($P > 0.05$). The strongest negative correlation ($r^2 \sim 0.86$, $P > 0.05$) is observed between antioxidant activity and content of narigenin. As can be seen from the correlation values below, hesperetin and vitamin C exhibit the weakest correlations with antioxidant potency in the three *Citrus* species. Only reported *Citrus* levels of quercetin, eriodictyol, and eriocitrin were positively correlated with antioxidant potencies in our *in vitro* assay.

The following is a list of all the equations for linear regression and correlation coefficients (r^2), respectively, related to Figure 4: Que, quercetin (y = 5.9029x - 4.9549; 0.847); E, vitamin E (y = -0.3992x + 3.8732; 0.7479); HT, hesperetin (y = -0.3362x + 2.683; 0.015); βC, β-carotene (y = -0.2898x + 3.3534; 0.5398); HD, hesperidin (y = -4.4627x + 8.1806; 0.7503); NG, naringenin (y = -3.2251x + 7.1224; 0.8576); C, vitamin C (y = -3.1963x + 5.829; 0.182); EC, eriocitrin

(y = 1.1455x + 0.6807; 0.9655); ED, eriodictyol (y = 0.0684x + 0.6856; 0.96). The contents of these flavonoids in the three *Citrus* species were obtained from, or calculated based on, reported values (see Materials and Methods section for references).

DISCUSSION

In the current study, the relative in vitro antioxidant potency of raw juice from three *Citrus* fruits has been characterized. The results indicate that Mexican lime, *C. aurantifolia*, is the most potent of the three. From a comparison of reported citrus contents of some flavonoids and vitamins/provitamins, the results suggest a significant contribution of non-vitamin antioxidant factors, to total antioxidant potency of *C. aurantifolia*; in particular, there were strong correlations of antioxidant activity with reported content of eriodictyols.

We observed a poor, negative correlation (r^2 = 0.18) of antioxidant activity with reported ascorbate (vitamin C) content for the three *Citrus* species. Similar poor correlations with vitamin C have been reported for citrus (e.g., correlation coefficients between 0.065 and 0.33 for four different antioxidant assay methods (Rapisarda et al., 1999)) and other fruits (Zhou et al., 2004 and references therein). Ascorbate, an important physiological antioxidant, as well as many flavonoids and other phytochemicals often referred to as antioxidants, can display prooxidant activities under specific conditions (Chan et al., 1999; Song et al., 1999). Naringenin, the flavanone displaying the strongest negative correlation with antioxidant potency in Figure 4, has been shown (along with other flavanones including hesperid(t)in) to have significant prooxidant activity in assays involving NADH oxidation and lipoprotein lipid peroxidation (Chan et al., 1999; Miranda et al., 2000).

Eriocitrin and other eriodictyol-based flavanones are potential candidates for the relatively higher antioxidant potency of lime in the current assay. Their levels in all three *Citrus* species are strongly and positively correlated with antioxidant potency (Figures 3 and 4); and other studies have also provided evidence for their potent antioxidant activities (Minato et al., 2003; Yao et al., 2006; Es-Safi et al., 2005; Edenharder and Grunhage 2003). Moreover, eriodictyol has been reported to be more potent as an antioxidant than quercetin in an iron-ascorbate oxidative stress assay (Areias et al., 2004). Other candidates for the relatively higher antioxidant potency of lime in the current assay are, for example, the flavones isorhoifolin and diosmin; both of these are much more abundant in the juice of *C. aurantifolia* relative to the juices of *C. reticulata* and *C. sinensis* (Areias et al., 2004). It should be noted that, although differences in phytochemical contents between these *Citrus* species may provide an indication of the most effective in vitro antioxidants, there is likely to be a complex balance of pro- and anti-oxidant factors in the whole, raw juices tested in the Cytc-TMPD assay. Thus, comparison of purified compounds and their physiological metabolites in multiple, pathologically relevant assays is important for future studies related to citrus phytochemicals and health.

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BIOGRAPHY

Dr. Amandio Vieira, Associate Professor, is director of the Nutrition and Metabolism Research Laboratory at Simon Fraser University. He is also Head of the Nutrition

Program in the School of Kinesiology at the same university. His research is funded by NSERC Canada. **Ms. Yu Yao**, Research Assistant in the Nutrition and Metabolism Research Laboratory, performed the experiments for this study. She was funded in part by FJST2000, a BC Government Science and Technology program.

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