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Food Chemistry 84 (2004) 23–28

Food Chemistry

www.elsevier.com/locate/foodchem

Rapid communication

Anthocyanin antioxidants from edible fruits

Linda S. Einbond[a,1], Kurt A. Reynertson[a], Xiao-Dong Luo[a,2], Margaret J. Basile[b], Edward J. Kennelly[a,*]

[a]*Department of Biological Sciences, Lehman College, and The Graduate Center, City University of New York, 250 Bedford Park Boulevard West, Bronx, NY 10468, USA*

[b]*Department of Neurology, University of Miami School of Medicine, 1501 NW 9th Avenue, Miami, FL 33136, USA*

Received 6 November 2002; received in revised form 24 March 2003; accepted 24 March 2003

Abstract

The edible fruits of 12 plants were extracted in methanol and subjected to solvent–solvent partitioning to yield three fractions, hexane, ethyl acetate, and aqueous. The semi-purified aqueous fractions were separated over Diaion HP-20SS resin to remove sugars and ascorbic acid. These fractions were then screened for antioxidant activity using the 1,1-diphenyl-2-picrylhydrazyl assay. Nine of the semi-purified fractions exhibited high antioxidant capacity. Cyanidin-3-*O*-β-glucopyranoside, an anthocyanin antioxidant, was identified from semi-purified aqueous fractions of the tropical fruit star apple (*Chrysophyllum cainito*), Surinam cherry (*Eugenia uniflora*), and jaboticaba (*Myrciaria cauliflora*). Delphinidin-3-*O*-β-glucopyranoside was identified from *E. uniflora*.

Keywords: 1,1-Diphenyl-2-picrylhydrazyl, cyanidin-3-*O*-β-glucopyranoside; Delphinidin-3-*O*-β-glucopyranoside; HPLC

1. Introduction

Oxidative damage in the human body plays an important causative role in disease initiation and progression (Jacob & Burri, 1996; Kelly, 1998). Damage from free radicals and reactive oxygen species has been linked to some neurodegenerative disorders (Floyd, 1999; Youdim & Joseph, 2001) and cancers (Goodwin & Brodwick, 1995), and oxidation of low-density lipoprotein is a major factor in the promotion of coronary heart disease (CHD) and atherosclerosis (Frankel, Kanner, German, Parks, & Kinsella, 1993; Steinberg, 1997). Diets high in fruits and vegetables and low in cholesterol and fats are inversely correlated with the incidence of CHD and cancer (Hertog, Feskens, Hollman, Katan, & Kromhout, 1993; Hertog et al., 1995; Knekt, Järvinen, Reunanen, & Maatela, 1996). Natural antioxidants from fruits and vegetables provide a measure of protection that slows the process of oxidative damage (Jacob & Burri, 1996). Recent studies have shown that many flavonoids and related polyphenols contribute significantly to the total antioxidant activity of many fruits and vegetables (Luo, Basile, & Kennelly, 2002; Vinson et al., 1999). Fruits and vegetables are high in flavonoid content; it is estimated that humans consume between a few hundred milligrams and one gram of flavonoids every day (Hollman & Katan, 1999; Pietta, 2000). Human studies have found that flavonoids appear in blood plasma, at pharmacologically active levels, after eating certain foods but do not accumulate in the plasma (Cao, Booth, Sadowski, & Prior, 1998; Hollman & Katan, 1999). Certain flavonoids are excreted in urine within 4 h of ingestion (Milbury, Cao, Prior, & Blumberg, 2002). Regular consumption of flavonoids may increase longevity by reducing inflammation and contributing to a reduction in CHD (Frankel et al., 1993). There are over 4000 naturally occurring flavonoids (Harborne & Baxter, 1999). The anthocyanins, a subclass of flavonoids, are important flower and fruit pigments. They attract pollinators and seed dispersers and protect plant tissues from photoinhibition and oxidation resulting from photosynthesis (Gould & Lee, 2002).

Anthocyanins contribute greatly to the antioxidant properties of certain colourful foods, such as grapes and

* Corresponding author. Tel.: +1-718-960-1105; fax +1-718-960-8236.

E-mail address: kennelly@lehman.cuny.edu (E.J. Kennelly).

[1] Present address: Center for Complementary and Alternative Medicine Research in Aging and Women's Health, Columbia University College of Physicians and Surgeons, New York, NY 10032, USA.

[2] Present address: Kunming Institute of Botany, Kunming 650204,

0308-8146/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0308-8146(03)00162-6

cranberries (Wang, Cao, & Prior, 1997). As pigments, they are almost exclusively responsible for the red, blue and purple colours in fruits. Cyanidin is the most common anthocyanidin, and the 3-glucoside is the most active antioxidant anthocyanin (Wang et al., 1997). Glycosylation and hydroxylation of the anthocyanidin backbone affects antioxidant activity (Wang et al., 1997). It has been estimated that Americans ingest as much as 180–215 mg anthocyanins per day (Kuhnau, 1976). Anthocyanin glycosides remain intact when passing from the digestive tract into the blood circulation of mammals (Miyazawa, Nakagawa, Kudo, Muraishi, & Someya, 1999).

The antioxidant activity of aqueous plant extracts has not been extensively studied, due to the presence of water-soluble antioxidant vitamins and sugars that may mask the activity of polyphenols. The polarity and complexity of water extracts can make it difficult to isolate pure components (Degenhardt, Knapp, & Winterhalter, 2000). Yet the aqueous fruit extracts often contain potent polyphenolic antioxidants, such as anthocyanins and tannins (Wang et al., 1997). The current study is focussed on those polar anthocyanin pigments that are present in water fractions after partitioning methanolic extracts with hexane and ethyl acetate (EtOAc).

2. Materials and methods

2.1. Materials

2.1.1. Fruits

Blighia sapida (Konig), *Chrysophyllum cainito* L., *Eugenia uniflora* L., *Malpighia glabra* Linn., *Mangifera indica* L., *Manilkara zapota* (L.) P. Royen, *Muntingia calabura* L., and *Myrciaria cauliflora* (Berg) O.Berg. were collected at the Fruit and Spice Park in Homestead, Florida and *Mammea americana* L. was collected at The Kampong in Coconut Grove, FL. *Gaultheria shallon* Pursh and *Sambucus caerulea* Raf., were collected in the western United States (Muñoz-Acuña, Atha, Ma, Nee, & Kennelly, 2001). *Theobroma grandiflorum* Schum. was collected in French Guiana. Fruits were frozen immediately after picking and shipped to the laboratory where they were kept in cold (−20 °C) dark storage until processed. Voucher specimens were prepared and deposited in the herbarium of the New York Botanical Garden.

2.1.2. Chemicals and supplies

Diaion HP-20SS resin was purchased from Supelco (PA, USA), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) from Sigma (MO, USA). Sephadex LH-20 (25–100 μm) was manufactured by Pharmacia Fine Chemicals (NJ, USA), reversed-phase C_{18} silica gel (40 μm) by J. T. Baker (NJ, USA), and RP_{18} F_{254} thin-layer chromatography (TLC) plates (1 mm layer thickness) were made by EM Science (Germany).

2.2. Sample preparation

Fruits were deseeded and homogenized in a blender with methanol (MeOH) and extracted exhaustively. Extracts were concentrated in vacuo at temperatures not exceeding 40 °C and resuspended in H_2O. The resulting aqueous solution was then partitioned sequentially with hexane and EtOAc before being separated over Diaion HP-20SS (Fig. 1).

2.3. Hydrophobic interaction chromatography

Diaion HP-20SS resin (4 g) was packed in 12-ml cartridges and conditioned sequentially with MeOH (50 ml, 15 min), and H_2O (50 ml, 10 min). Semi-purified aqueous fractions of each species (200 mg) were resuspended in H_2O (4 ml) and applied to the HP-20SS column and allowed to adsorb on to the resin for 20 min. The column was eluted with 15 ml H_2O (2.7 column volumes) at a rate of 15 ml/h; then sequentially with 9 ml each of H_2O:MeOH (1:1) MeOH; MeOH: acetone (1:1) and acetone.

2.4. DPPH free radical-scavenging assay

The H_2O:MeOH fractions were assayed for free radical scavenging activity by the DPPH assay. The procedure used is an adaptation of those previously described (Smith, Reeves, Dage, & Schnettler, 1987; Yamaguchi, Takamura, Matoba, & Terao, 1998). The H_2O:MeOH fractions were resuspended in MeOH. Ethanolic DPPH (400 μM) was used in the reaction mixture. Serial dilutions of the test sample were combined with the DPPH solution in a 96-well microtitre plate. MeOH was used as a negative control and ascorbic acid and α-tocopherol were used as positive controls. The reaction mixtures were incubated for 30 min at 37 °C and the change in absorbance at 517 nm was measured. Mean values were obtained from triplicate experiments. Inhibition percent was calculated using the equation, $\%\ \text{Inhibition} = [(C - S)/C] \times 100$ where C is the net absorbance of the control and S is the net absorbance of the sample. Percent inhibition was plotted against concentration, and the equation for the line was used to obtain the IC_{50} value. A lower IC_{50} value indicates greater antioxidant activity.

2.5. Compound isolation and identification

The H_2O:MeOH fractions obtained from Diaion HP-20SS chromatography of Surinam cherry were separated with a Sephadex LH-20 column by eluting with a

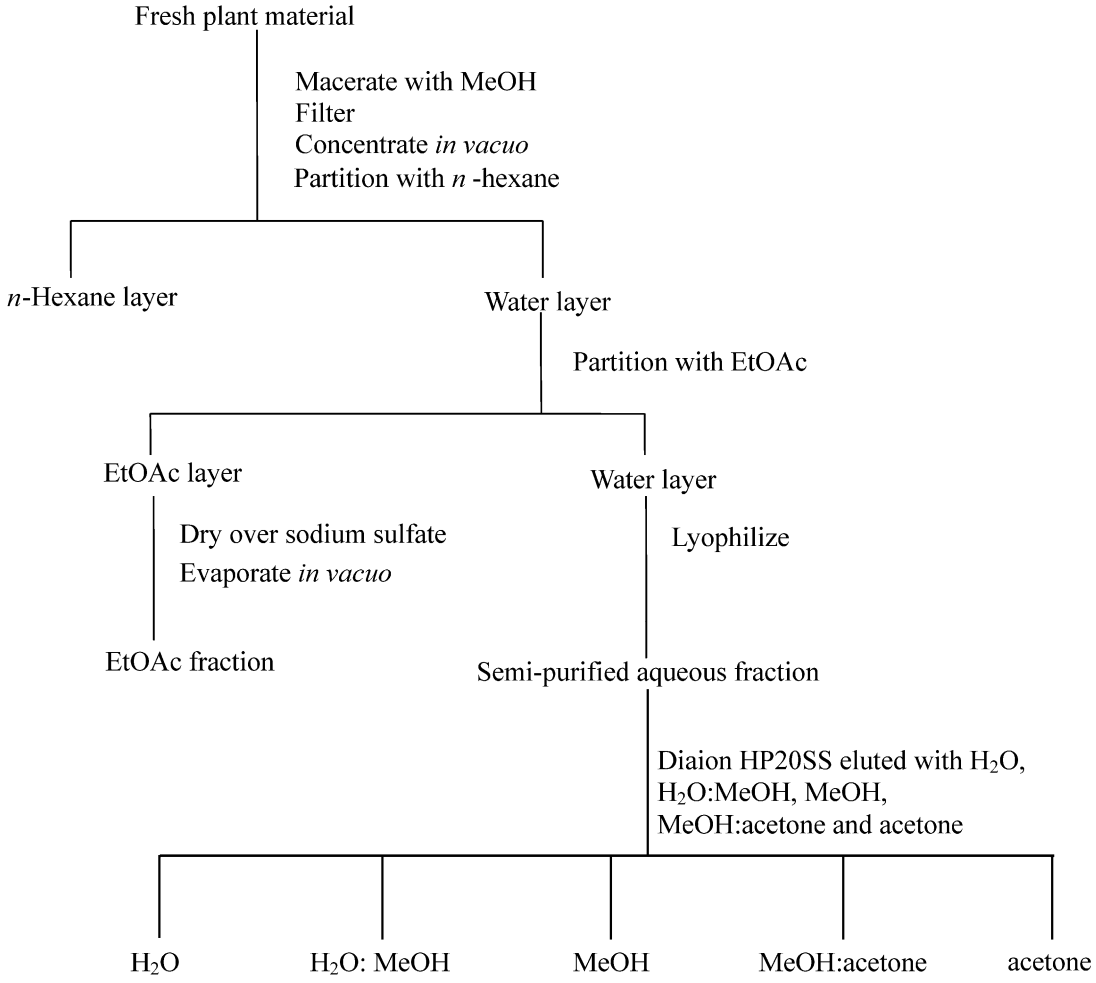

Fig. 1. Partition scheme for fruits.

gradient of MeOH in H_2O. The purple-coloured fractions were further purified on RP_{18} CC silica gel using H_2O:CH_3OH:HCOOH (76:20:4).

Analytical HPLC was performed with a Waters 2690 Separations Module and a Waters 996 photo-diode array detector operating from 190 to 700 nm controlled by Millenium^{32} software. Compounds were separated over a Phenomenex (Torrance, CA) C_{18} Aqua column (4.6 × 250 mm, 5 μm). The solvent system was a linear gradient from 100% A (17.6% formic acid) to 50% A in 15 min, followed by 5 min of 100% B (acetonitrile) at a flow rate of 1 ml/min. The solvent for reversed-phase TLC was H_2O:MeOH (1:1 or 2:1).

^1H and ^{13}C NMR spectra were measured on a JEOL 400 instrument, operating at 400 and 100 MHz, respectively. Compounds were measured in CD_3OD with 5% TFA. Mass spectra were obtained on a Finnigan LCQ Deca spectrometer from Thermoquest (CA, USA) by electrospray ionization (ESIMS) in the negative mode. ESIMS was performed by direct injection. Capillary voltage was at 10 V, the spray voltage was 4.5 kV, and the tube lens was offset at 0 V. The capillary temperature was 230 °C.

3. Results and discussion

3.1. Removal of sugars and ascorbic acid

Many of the 12 fruits in this study have a high sugar content, with carbohydrates representing, on average, 14% of the fresh fruit by weight (Morton, 1987). In the extraction and partition scheme used in this study (Fig. 1), carbohydrates partition into the aqueous fractions. Sugars and ascorbic acid may be present in these semi-purified aqueous fractions and can mask the antioxidant activity of polyphenols that may be present in the fraction. For this reason, vitamin C and carbohydrates were removed from these semi-purified aqueous fractions, using the Diaion HP-20SS resin. Sugars and vitamin C are eluted in the H_2O fraction of the Diaion HP-20SS column and polyphenols are eluted in the H_2O:MeOH and MeOH fractions.

In all 12 semi-purified aqueous fractions examined, the major pigmented constituents eluted from the Diaion HP-20SS column with the H_2O:MeOH eluate. Generally, the mass of the extracts recovered from the Diaion HP-20SS H_2O fractions was higher than from

the other fractions. Approximately 90% w/w of the *T. grandiflorum* semi-purified aqueous fraction eluted in the Diaion HP-20SS H_2O fraction due to a high sugar content of this species (Morton, 1987). *B. sapida* fruit, which is not known for a high sugar content, eluted 43% w/w of the semi-purified aqueous fraction in the Diaion HP-20SS H_2O fraction. The yield of the Diaion HP-20SS H_2O fraction of *G. shallon* was the lowest, with only 24% of the semi-purified aqueous fractions eluted in the Diaion HP-20SS H_2O fraction. Diaion HP-20SS is a simple and effective method to separate sugars and vitamin C from the polyphenolic constituents of aqueous fractions of fruit extracts.

3.2. DPPH radical scavenging assay

The IC_{50} values of the H_2O:MeOH Diaion HP-20SS fractions ranged from 4.0 μg/ml for Surinam cherry to 176 μg/ml for cupuaçu (Table 1). The IC_{50} value for vitamin C in MeOH is 7.9±0.5 μg/ml. The H_2O:MeOH fraction from Diaion HP-20SS for *C. cainito*, *G. shallon*, *M. glabra*, *M. cauliflora*, and *S. caerulea* were highly active (Table 1) and red-purple in colour, suggesting the presence of anthocyanins. Certain extracts without a red-purple colour, such as *M. americana*, also displayed high activity in the DPPH assay (Table 1). The activity of *M. americana* may be the result of a high tannin content. The Diaion HP-20SS H_2O fraction of *M. glabra* exhibited a high antioxidant activity (IC_{50} = 30 μg/ml), which is consistent with the known high vitamin C content of the fruit (Morton, 1987). The colour of the aqueous extract may be useful for identifying fruits with high antioxidant activity due to anthocyanins, but there are examples of active extracts which are not red-purple. Anthocyanins are the most common red-purple fruit pigments, and have been extensively studied for their antioxidant anthocyanin contents. Fruits that are not red-purple may contain other potent polyphenolic antioxidants.

3.3. Isolation and identification of anthocyanins

UV–Vis PDA spectra suggested that several of the semi-purified aqueous fractions contained anthocyanins (Catalano, Fossen, & Andersen, 1998). The constituents of *E. uniflora* that eluted from Diaion HP-20SS with H_2O:MeOH were purified by reversed-phase column chromatography to yield cyanidin-3-glucoside and delphinidin-3-glucoside (Fig. 2), as confirmed by HPLC, NMR and LC-MS analyses (Ikuta, Fukai, Nomura, & Uzawa, 1985; Strack & Wray, 1994).

The remaining 11 H_2O:MeOH fractions were screened for anthocyanins using TLC and HPLC. *M. cauliflora*, *G. shallon*, *S. caerulea*, *M. glabra*, and *C. cainito* all appear to contain anthocyanin compounds, based on UV–Vis absorbance patterns; the DPPH

Table 1
DPPH results of H_2O:MeOH fractions

Latin binomial	Common name	Family	IC_{50} (μg/ml ±SD)
Blighia sapida	Akee	Sapindaceae	6.6±1.1
Chrysophyllum cainito	Star-apple	Sapotaceae	7.9±0.3
Eugenia uniflora	Surinam cherry	Myrtaceae	4.0±2.2
Gaultheria shallon	Salal	Ericaceae	5.9±0.3
Malpighia glabra	Barbados cherry	Malpighiaceae	13.9±1.3
Mammea americana	Mammee apple	Clusiaceae	7.9±2.7
Mangifera indica	Mango	Anacardiaceae	145±20
Manilkara zapota	Sapodilla	Sapotaceae	50.8±4.5
Muntingia calabura	Jamaica cherry	Elaeocarpaceae	6.5±0.6
Myrciaria cauliflora	Jaboticaba	Myrtaceae	6.2±0.7
Sambucus caerulea	Blue elder	Caprifoliaceae	16.9±0.6
Theobroma grandiflorum	Cupuaçu	Sterculiaceae	177.0±12

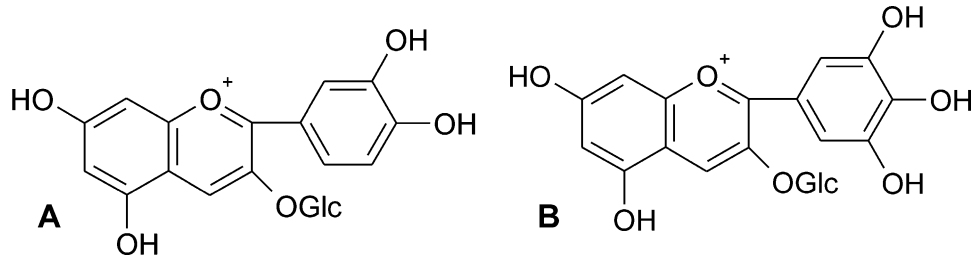

Fig. 2. Cyanidin-3-*O*-β-glucopyranoside (A) and delphinidin-3-*O*-β-glucopyranoside (B).

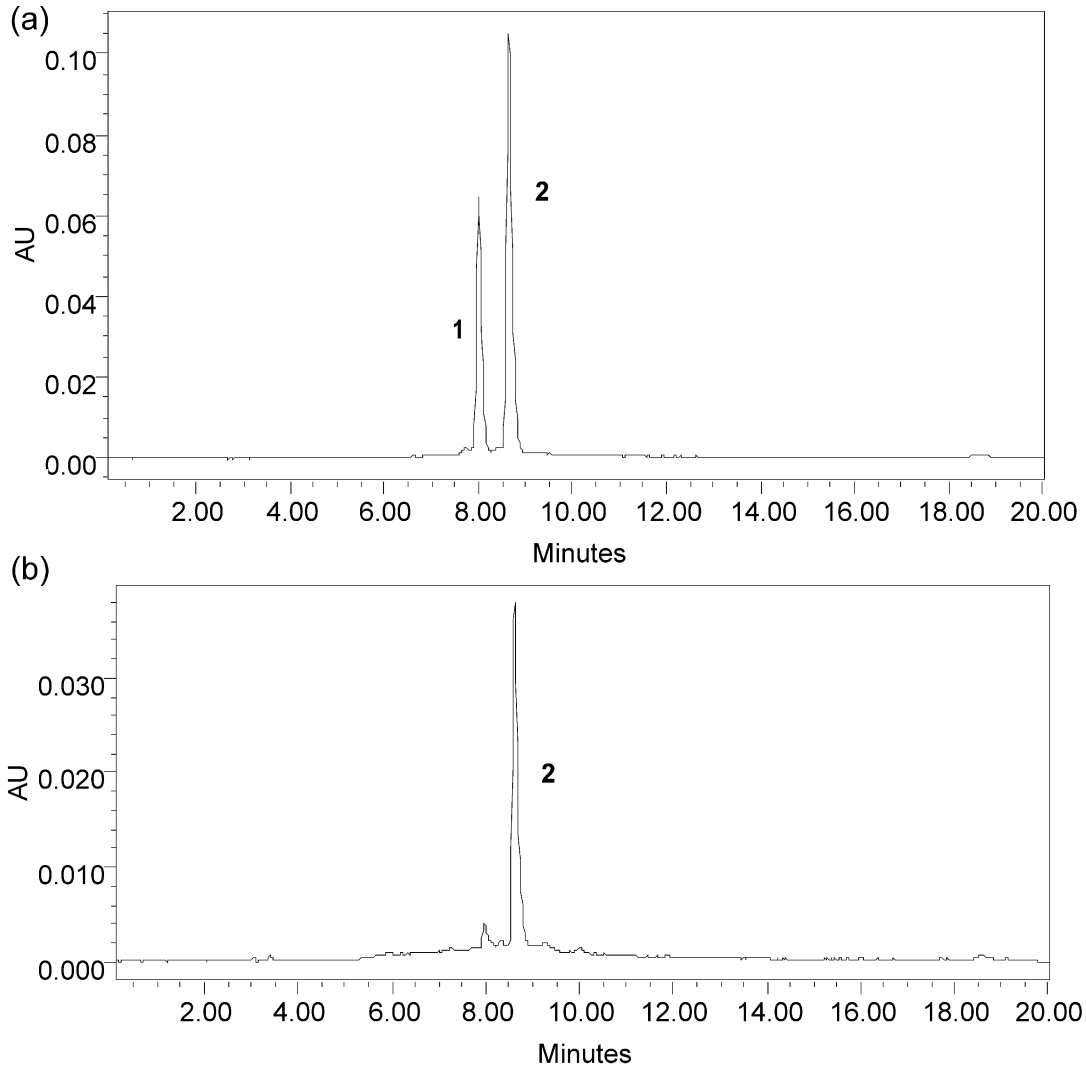

Fig. 3. (a) HPLC chromatogram at 520 nm of A standard mixture of delphinidin-3-glucoside (**1**) and cyanidin-3-glucoside (**2**). (b) *Chrysophyllum cainito* extract showing (**2**). The solvent system is 100% A (17.6% formic acid) to 50% A in 15 min, followed by 5 min of 100% B (acetonitrile) with a flow rate of 1 ml/min.

activities of these extracts are comparable with ascorbic acid (Table 1) and previously published results of anthocyanins (Wang et al., 1997). HPLC retention time and UV–Vis absorbance profiles confirmed the presence of cyanidin-3-glucoside in *M. cauliflora* and *C. cainito* (Fig. 3). Only *E. uniflora* contained delphinidin-3-glucoside. Isolation work will continue to determine which anthocyanins contribute to the antioxidant activity of the fruits. The antioxidant constituents of *M. americana*, which do not appear to be anthocyanins, are currently being isolated and identified by our laboratory.

4. Conclusion

Diaion HP-20SS resin provides an effective method for removing sugars and ascorbic acid from polyphenolic antioxidants in aqueous plant extracts and serves as a first step in purifying the polyphenols. The procedure has resulted in the identification of the anthocyanin, cyanidin-3-glucoside, from the semi-purified aqueous fractions of Surinam cherry, star apple and jaboticaba and delphinidin-3-glucoside, from Surinam cherry. Future plans include identification of the remaining antioxidant constituents in the semi-purified aqueous fractions, and study of the anticancer effects of these aqueous extracts and purified components in vitro, using cell proliferation and cell cycle analysis, as well as biochemical receptor assays.

Acknowledgements

We thank Chris Rollins (Director, Fruit and Spice Park) and Larry Schokman (Director, The Kampong) for allowing us to collect fruits, Dr. Scott Mori and

Vanessa Hequet (The New York Botanical Garden) for collecting the cupuaçu, Scott Baggett, Yongli Gao, Jun Ma, Ulyana Muñoz-Acuña and Ainsley Parkinson for fruit extracts, and Dr. Lining Cai and Dr. Baoliang Cui for their advice and assistance.

This work was supported by PSC-CUNY Award No. 669662 and the National Institutes of Health—National Institute of General Medical Sciences SCORE grant S06GM08225-16S1. K.A. Reynertson is supported by The NIH—National Center for Complementary and Alternative Medicine National Research Service Award F31AT00801.

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