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Açai (*Euterpe oleracea* Mart.) Polyphenolics in Their Glycoside and Aglycone Forms Induce Apoptosis of HL-60 Leukemia Cells

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The effects of açai polyphenolics on the antiproliferation and induction of apoptosis in HL-60 human leukemia cells were investigated. Interactions between anthocyanins and non-anthocyanin-polyphenolics in both their glycosidic and their aglycone forms were also investigated to determine additive or nonadditive responses. Polyphenolic fractions at 0.17−10.7 μM were found to reduce cell proliferation from 56 to 86% likely due to caspase-3 activation (apoptosis). Anthocyanin and polyphenolic fractions were nonadditive in their contribution to the cell antiproliferation activity. At equimolar concentrations, the glycosidic forms of phenolic acids and flavonoids induced a higher magnitude of change in cell parameters (proliferation and apoptosis) than their respective aglycone forms, while the opposite trend was observed for anthocyanin aglycones. This study demonstrated that açai offers a rich source of bioactive polyphenolics and confirmed the importance of investigating whole food systems when evaluating the potential health benefits of individual phytochemical compounds.

KEYWORDS: Açai; HL-60 cells; phytochemical interactions; antiproliferation; apoptosis

INTRODUCTION

Dietary intake of naturally occurring polyphenolics has been suggested to contribute to the prevention of many chronic diseases including hyperlipidemia, cardiovascular disease, and cancer (*1*−*5*). Their chemopreventive effects have not only been linked to their antioxidant activity but also to their ability to mediate other physiological functions related to cancer suppression such as antiproliferative and antimutagenic activity, inhibition of enzymes, and induction of detoxification enzymes (*1*−*8*). Flavonoids were shown to induce cell cycle arrest due to their ability to inhibit various biological processes involving mitogen signaling or DNA synthesis (*3*, *4*, *6*, *7*). Various in vitro and in vivo systems have also demonstrated how individual or simple mixtures of phytochemical compounds can influence biological systems (*3*, *4*, *7*). However, only a few studies have investigated how complex mixtures of polyphenolics might interact and influence the bioactive properties of a certain fruit or vegetables (*3*, *5*, *6*). Furthermore, metabolites of the naturally occurring polyphenolics may be responsible for observed bioactivity through the formation of aglycone, sulfonated, gluconated, or methylated derivatives (*1*, *2*).

Euterpe oleracea Mart. (açai) is a native Amazonian palm fruit that was recently characterized to have relatively high amounts of antioxidant polyphenolics (*9*). However, its bioactive properties have yet to be investigated in detail. Therefore, the present study evaluated the antiproliferative and pro-apoptotic activities of açai whole fruit pulp along with its phytochemical fractions in HL-60 human promyelocytic leukemia cells. Phytochemicals present in the fruit pulp were extracted and fractionated based on solubility and affinity characteristics to C_{18} columns to study the relative bioactivity of each fraction. Potential interactions among phytochemical fractions were also investigated. Moreover, polyphenolic fractions were acid hydrolyzed into aglycones for the purpose of investigating their antiproliferative activity in relation to their naturally occurring forms since these compounds could also present bioactive activity in vivo as reported in previous studies (*1*, *2*). The antioxidant and bioactive properties were evaluated within a range of physiological concentrations and were based on the metabolism of cyanidin 3-glucoside (*1*, *11*−*13*), the major polyphenolic compound found in açai. This study demonstrated that açai is a rich source of bioactive polyphenolics that contains pro-apoptotic and antiproliferative activities against HL-60 leukemia cancer cells. We also confirmed the importance of investigating whole food systems when evaluating the potential health benefits of these compounds due to their nonadditive response in cell culture models.

MATERIALS AND METHODS

Materials. Pasteurized, frozen açai pulp was kindly donated by Amazon Energy, LLC (Greeley, CO) and was shipped overnight to the Department of Food Science and Human Nutrition at the University of Florida. Purification and fractionation of açai phytochemicals based

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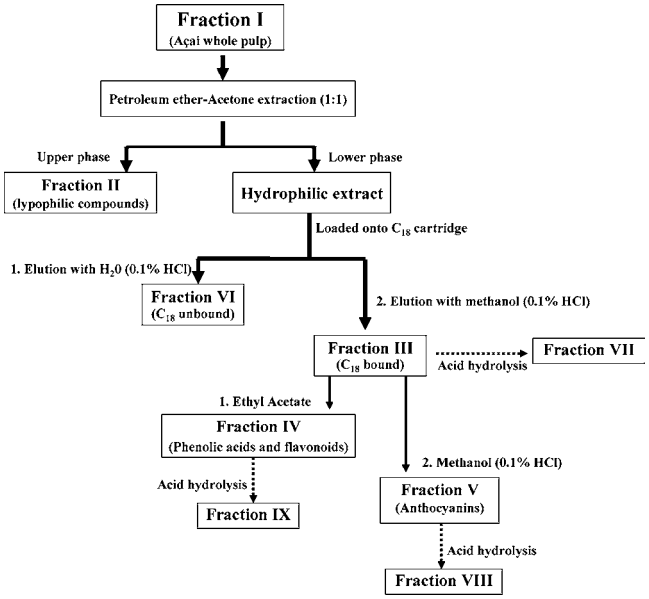

Figure 1. Simplified isolation scheme used to fractionate and purify phytochemicals present in açai whole pulp.

on solubility and affinity characteristics was conducted as previously described (*9*). The isolation scheme used to fractionate açai phytochemical constituents can be observed in **Figure 1** and is briefly described as follows. Lipophilic compounds were partitioned from hydrophilic compounds by the addition of petroleum ether and acetone (1:1). The upper petroleum ether phase was removed and concentrated to dryness under a gentle stream of nitrogen, and the resulting isolate was redissolved in a known volume of dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO). Hydrophilic compounds from the lower phase were recovered by removing acetone under reduced pressure at <40 °C. Polyphenolics from the aqueous phase were subsequently fractionated from polar compounds using C_{18} Sep-Pak Vac 20 cm^3 mini-columns (Waters Corporation, Milford, MA). Phenolic acids and flavonoids were then separated from anthocyanins by eluting with ethyl acetate followed by acidified (0.01% HCl) methanol to remove anthocyanins. Each fraction was evaporated under vacuum at <40 °C and redissolved in DMSO with a final DMSO concentration of 2 mL/L. Polyphenolic recovery was >96% for all fractions. Six fractions were obtained from the extraction of açai that included whole pulp (fraction I), lipophilic fraction (fraction II), C_{18} bound phenolics and anthocyanins (fraction III), ethyl-acetate soluble polyphenolics (fraction IV), isolated anthocyanins (fraction V), and C_{18} nonretained (fraction VI).

To compare the antioxidant and antiproliferative properties of açai polyphenolics in either glycosidic or aglycone forms, compounds present in fractions III−V were acid hydrolyzed into their respective aglycones with 2 N HCl in 50% v/v methanol for 60 min at 90 °C. Following acid hydrolysis, polyphenolics were partitioned from C_{18} columns as previously described, and fractions were redissolved in a known volume of DMSO, creating three additional fractions that included hydrolyzed C_{18} bound phenolics and anthocyanins (fraction VII), hydrolyzed anthocyanins (fraction VIII), and hydrolyzed ethyl-acetate soluble polyphenolics (fraction IX).

The polyphenolic levels chosen for use in cell cultures were derived from those that might reasonably be expected in the blood after consumption of foods rich in cyanidin 3-glucoside (*1*, *11*−*13*). Extracts containing 0−156 μg/L cyanidin (or 0−10.7 μM of total soluble phenolics) were evaluated and compared to that of quercetin aglycone (0−400 μM) (Sigma, St. Louis, MO). Non-hydrolyzed and hydrolyzed fractions were tested at an equivalent polyphenolic and/or anthocyanin concentration as contained in the whole pulp.

Cells and Cell Culture. Human promyelocytic leukemia (HL-60) cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium containing 10% fetal calf serum, 2 μM L-glutamine, 100 000 units/L penicillin, 0.1 g/L streptomycin, 0.25 mg/L fungizone, and 0.05 g/L gentamycin. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2, utilized between passages 45−60, and maintained at a density between 2.5 and 10 × 10^5 cells/mL by resuspending the cells in fresh culture medium. Cell viability as determined by tryphan blue exclusion was ≥95%. For analysis, cells were resuspended at a concentration of 1.33 × 10^7 cells/mL in a 96-well tissue culture microtiter plate and treated with total soluble phenolic concentrations from each açai fraction ranging from 0 to 10.7 μM and quercetin from 0 to 400 μM. Camptotechin (Sigma, St. Louis, MO), a potent topoisomerase-I-inhibitor, was used as a positive control (0−10 μM) to induce apoptosis. A control culture containing DMSO at 2 mL/L was included in all assays. Viability and cell counts were performed by tryphan blue dye exclusion in a Neubauer hemacytomer.

Cell Viability. Cell viability based on mitochondrial functions was assayed by the ability of cells to convert soluble MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma, St. Louis, MO) into an insoluble purple formazan reaction product. After 24 h polyphenolic exposure, cells (1 × 10^6 cells/mL) were transferred to an empty microtiter plate, and 10 μL of sterile-filtered MTT in phosphate-buffered saline (PBS; 1:1) at 5 mg/mL was added to each well. The plate was then placed in a 5% CO_2 incubator at 37 °C for 4 h. Following incubation, 100 μL of acid 2-propanol (0.04 N HCl, 1:300) was added to dissolve the purple crystals. Absorbance was subsequently measured at 540 nm with a reference wavelength of 490 nm on a UV-max microplate reader (Molecular Devices, Menlo Park, CA). Data were expressed as cell mortality with respect to the DMSO control, and corrections were made for background interference due to the presence of anthocyanins using a blank plate without HL-60 cells.

Caspase-3 Activity. Pro-apoptotic activity of each açai phytochemical fraction or pure compound was assessed by measuring caspase-3 activity. In this assay, caspase-3 activation lead to cleavage of a substrate (DEVD), resulting in the release of *p*-nitroaniline (*p*NA), which can be measured at 405 nm. Treated HL-60 cells were collected after different exposure times (2, 3, 4, 5, and 6 h) and triply washed in PBS and centrifuged at 1200 rpm for 10 min at 10 °C. Cells were then resuspended in 100 μL of cell lysis buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 2 mM ethylenediaminetetraacetic acid [EDTA], 0.1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 10 μg/mL pepstatin A, 20 μg/L leupeptin, 10 μg/mL aprotinin, and 2 mM dithiothreitol [DTT]). A total of 50 μL of reaction buffer (100 mM HEPES, 20% v/v glycerol, 0.5 mM EDTA, and 5 mM DTT) containing 200 μM DEVD-*p*NA substrate was then added to each well containing cell lysates and incubated in a 5% CO_2 incubator at 37 °C. After 3 h of incubation, levels of released pNA were measured at 405 nm with a reference wavelength of 450 nm on a UV-max microplate reader. Data were expressed as the percentage of active caspase-3 activity with respect to the DMSO control.

Phytochemical Analyses. Individual anthocyanin glycosides present in açai were quantified according to the HPLC conditions of Del Pozo-Insfran et al. (*9*) using a cyanidin standard (Polyphenols Laboratories AS, Sandnes, Norway). Anthocyanins were characterized based on PDA spectral interpretation from 200 to 600 nm, comparison to authentic standards (Polyphenols Laboratories AS, Sandnes, Norway), and identification, which was additionally confirmed following acid hydrolysis into their respective aglycones with 2 N HCl in 50% v/v methanol for 60 min at 90 °C. Major flavonoids and phenolic acids present in açai were separated by HPLC using the chromatographic conditions of Del Pozo-Insfran et al. (*9*). Polyphenolics were identified by spectroscopic interpretation, retention time, and comparison to authentic standards (Sigma Chemical Co., St. Louis, MO). Polyphenolic derivatives were quantified using equivalents of their respective free form.

The six isolates (fractions I−VI) obtained from the extraction of açai pulp and the three hydrolyzed isolates (fractions VII−IX) containing anthocyanin and/or polyphenolic aglycones were evaluated for antioxidant capacity using the oxygen radical absorbance capacity assay against a standard of Trolox as described by Talcott et al. (*10*). Each isolate was appropriately diluted in pH 7.0 phosphate buffer prior to pipetting into a 96-well microplate with corrections made for background interference due to phosphate buffer and/or extraction solvents. Total soluble phenolics of each hydrolyzed or non-hydrolyzed fraction were quantified as gallic acid equivalents using the Folin−Ciocalteu assay (*10*).

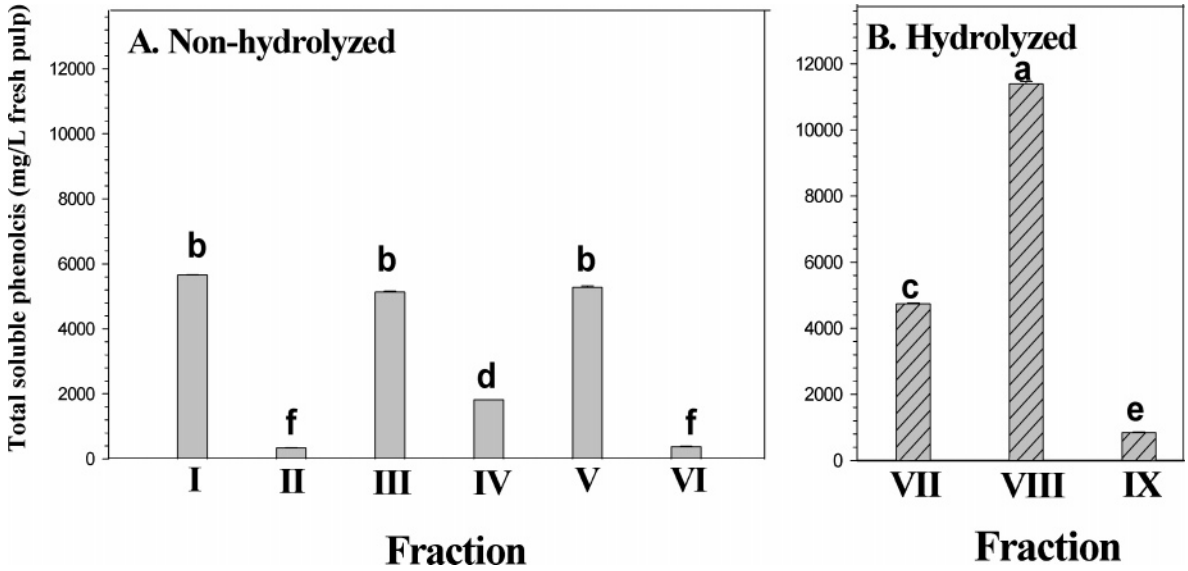

Figure 2. Total soluble phenolic content of (**A**) non-hydrolyzed and (**B**) hydrolyzed (2 N HCl in 50% v/v methanol for 60 min at 90 °C) phytochemical fractions of açai (*E. oleracea* Mart.). Bars represent standard error of the mean ($n = 6$). Polyphenolic compounds present in fractions III–V were acid hydrolyzed into their respective aglycones (fractions VII, IX, and VIII, respectively). Total soluble phenolics were quantified using gallic acid equivalents. Bars with different letters across all fractions are significantly different (LSD test, $P < 0.05$).

Statistical Analysis. Antiproliferative and pro-apoptotic effects were evaluated as a 9 × 8 full factorial that included nine açai phytochemical fractions evaluated at eight different total soluble phenolic concentrations. Data for bioactive assays represent the mean of two independent experiments each containing three replicates. Data for phytochemical evaluations represent the mean of three replicates. Multiple linear regression, analysis of variance, and Pearson correlations were conducted using JMP software (SAS, Cary, NC) and mean separation using the LSD test ($P < 0.05$).

RESULTS AND DISCUSSION

Phytochemical Content. Predominant polyphenolics in açai included cyanidin 3-glucoside, ferulic acid, epicatechin, *p*-hydroxy benzoic acid (1, 120, 250, 112, 104 mg/L pulp, respectively) in accordance to our previous phytochemical characterizations (*9*). (+)-Catechin, pelargonidin 3-glucoside, gallic acid, protocatechuic, and free ellagic acid were also present at ca. 60 mg/L, while vanillic and *p*-coumaric acid were found at lower concentrations (32.7 and 17.8 mg/L, respectively). Other polyphenolic compounds that shared spectroscopic characteristics with gallic and ellagic acid were also detected and were tentatively identified either as gallotannins (32.7 and 17.8 mg of gallic acid equivalents/L, respectively) or as an ellagic acid glycoside (17.8 mg of ellagic acid equivalents/L), respectively.

Single strength açai fruit pulp contained 1173 mg/L total anthocyanins, 960 mg/mL phenolic acids and flavonoids (quantified by HPLC), and 5660 mg/L total soluble phenolics (in gallic acid equivalents; **Figure 2**). Polyphenolic levels chosen for cell culture experiments were based on absorption studies with cyanidin 3-glucoside, the predominant polyphenolic present in açai. According to reported pharmacokinetic parameters (*1*, *11*−*13*), a maximum plasma concentration of 45.4 μg/L can be reached after 60 min of oral consumption of this anthocyanin at 580 mg. Therefore, cyanidin 3-glucoside levels from 0 to 50 μg/L that were evaluated as these concentrations could be potentially achieved in human plasma after consumption of 0−500 mL of whole açai pulp (0−567 mg/L anthocyanins). Cyanidin concentrations from 50 to 400 μg/L were also evaluated since these levels might be achieved in plasma after the consumption of a concentrated nutraceutical product made from açai. The bioactive properties of the different açai phytochemical fractions were reported in total soluble phenolic levels (0−10.7 μM), following the molar relationship between cyanidin 3-glucoside and soluble phenolics (1:13.7) present in the whole pulp. Fractions were evaluated at equivalent phenolic acid, flavonoid, and/or anthocyanin levels (quantified by HPLC) as compared to those contained in the whole pulp, to accurately compare the bioactive properties of the whole pulp with each of açai phytochemical fractions.

Antioxidant and Metal Reducing Activity of Açai Phytochemical Fractions. Fractionation of açai phytochemicals based on solubility and affinity characteristics was conducted to determine the distribution of compounds with antioxidant and metal ion reducing activity (evaluated using the Folin−Ciocalteu assay) among the isolates. Results showed that the antioxidant and total soluble phenolic content of each fraction presented nonadditive contributions to that of the whole pulp (**Figures 2** and **3**), presumably due to physical and/or chemical interactions that occurred among phytochemical constituents. Acid hydrolysis affected in a different manner the soluble phenolic and antioxidant levels of each fraction. At equimolar concentrations, the glycosidic forms of phenolic acids and flavonoids induced a higher magnitude of change in cell parameters (proliferation and apoptosis) than their respective aglycone forms, while the opposite trend was observed for anthocyanin aglycones.

Similar concentrations of total soluble phenolics and antioxidant capacity were observed for all non-hydrolyzed anthocyanin-containing fractions (I, III, IV, and V), while the remaining fractions had appreciably lower values (**Figures 2A** and **3A**). Results also demonstrated that nonadditive antioxidant and metal reducing capacity contributions were observed from ethyl acetate-soluble phenolics (fraction IV) and isolated anthocyanins (fraction V) when compared to that isolate containing both types of polyphenolics (C_{18} bound polyphenolic fraction; fraction III). The latter effect indicated that the radical scavenging and metal reducing capacity of the latter isolate (fraction III) was lower than expected from the sum of each individual isolate (fractions IV and V). Individual anthocyanin and

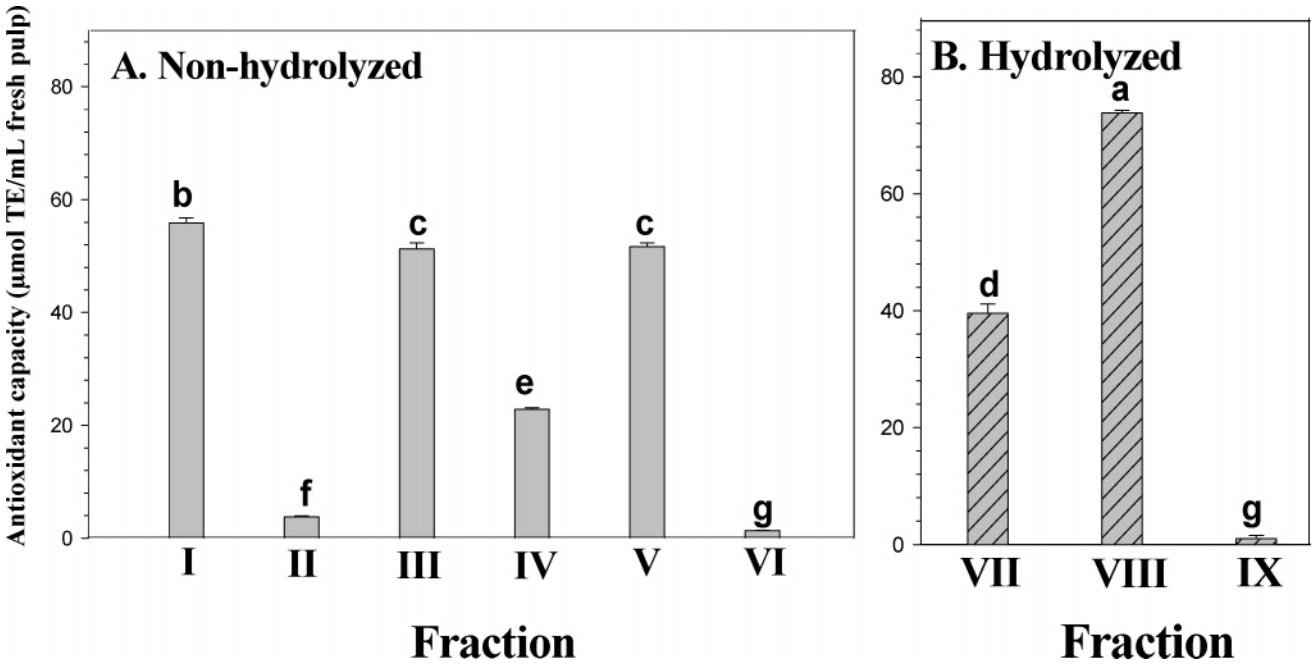

Figure 3. Antioxidant capacity of (**A**) non-hydrolyzed and (**B**) hydrolyzed (2 N HCl in 50% v/v methanol for 60 min at 90 °C) phytochemical fractions of açai (*E. oleracea* Mart.). Polyphenolic compounds present in fractions III–V were acid hydrolyzed into their respective aglycones (fractions VII, IX, and VIII, respectively). Antioxidant capacity quantified using Trolox equivalents (TE). Bars with different letters across all fractions are significantly different (LSD test, $P < 0.05$).

polyphenolic fractions were recombined again for analysis, and their activity did not differ from that of the C_{18} bound polyphenolics.

Acid hydrolysis caused marked increases in total soluble phenolics (2.2-fold; **Figure 2B**) and antioxidant capacity (1.4-fold; **Figure 3B**) in the fraction containing isolated anthocyanins (fraction VII) when compared to its non-hydrolyzed counterpart (fractions V) in accordance to previous investigations with anthocyanins (*14*). However, acid hydrolysis decreased levels for fractions containing phenolic acids and flavonoids (fractions VII and IX). Between fractions III and VII, the antioxidant capacity and total soluble phenolics decreased by 7 and 27%, respectively, as compared to 56 and 96% between fractions IV and IX. These results suggested that the glycosidic form of açai non-anthocyanin polyphenolics was either destroyed during hydrolysis or that the resultant aglycones were less effective in radical scavenging or metal reduction potential. Such an effect was also observed by Anselmi et al. (*15*) and Garcia-Conesa et al. (*16*), which showed that at equal molar proportions, the free ferulic acid form presented <50% antioxidant levels than its dehydro dimers. Interactions among the aglycone forms could also have occurred and increased when compared to their glycoside forms. Other studies (*17*−*19*) also have demonstrated antagonistic interactions between individual polyphenolics and have concluded that the diversity of antioxidant polyphenolics present in food create a complex matrix from which detailed evaluations are difficult to make. Moreover, the effectiveness of an antioxidant compound is dependent on the polarity of the testing system, the nature of the radical, and the type of substrate protected by the antioxidant (*9*, *17*−*19*).

Antiproliferative Effects of Açai Phytochemical Fractions. The antiproliferative and pro-apoptotic activities of açai whole pulp were then evaluated in a cell culture model using HL-60 cells and were compared to açai individual phytochemical fractions to evaluate how these fractions contributed to the overall bioactive properties of the whole pulp. The observed cytostatic activities were compared to quercetin aglycone, a polyphenolic compound with strong antiproliferative activity in various cancer cell lines (*7*).

When HL-60 cells were exposed to açai phytochemical fractions for 24 h, a dose-dependent decrease in cell viability was observed for all fractions (**Figure 4**) with the exception of the lipophilic and C_{18} nonretained fractions (fractions II and VI, respectively), which independently of their concentration reduced cell viability by <5% (data not shown). The non-hydrolyzed, anthocyanin-containing fractions (fractions I, III, and V; **Figure 4A**) strongly suppressed the proliferation of HL-60 cells, with apoptosis induction, in a dose-dependent manner. A strong suppression of HL-60 cell growth by anthocyanin-rich extracts was also observed in previous investigations (*3*, *8*, *20*). Chang et al. (*3*) showed a 75% decrease in cell viability after 24 h exposure to a 3 mg/mL anthocyanin-rich extract isolated from hibiscus flowers (4% delphinidin 3-glucoside), while Fimognari et al. (*20*) showed that the addition of cyanidin 3-glucoside at 0.2 mg/L resulted in a 37% reduction in viable HL-60 cells.

Similar to observations for antioxidant capacity and total soluble phenolics, ethyl acetate soluble phenolics (fraction IV) and anthocyanins (fraction V) were nonadditive in their contribution to the cell anti-proliferation activity of the C_{18} bound phenolics (fraction III) and the whole fruit pulp (fraction I) fraction. These results again indicated that antagonistic interactions among açai phytochemicals unfavorably impacted their individual antiproliferative properties. Katsube et al. (*8*) demonstrated that although the anthocyanin composition of various berry extracts was similar, their antiproliferative activity significantly differed. These authors hypothesized that the bioactive properties of each extract were influenced by various factors including concentrations and forms of individual phytochemicals, interactions between fruit constituents, and the properties of the food matrix. Other investigations also have reported that important antagonistic, synergistic, or additive effects occurred when individual mixtures of polyphenolics were present together in biological systems and that possibly the

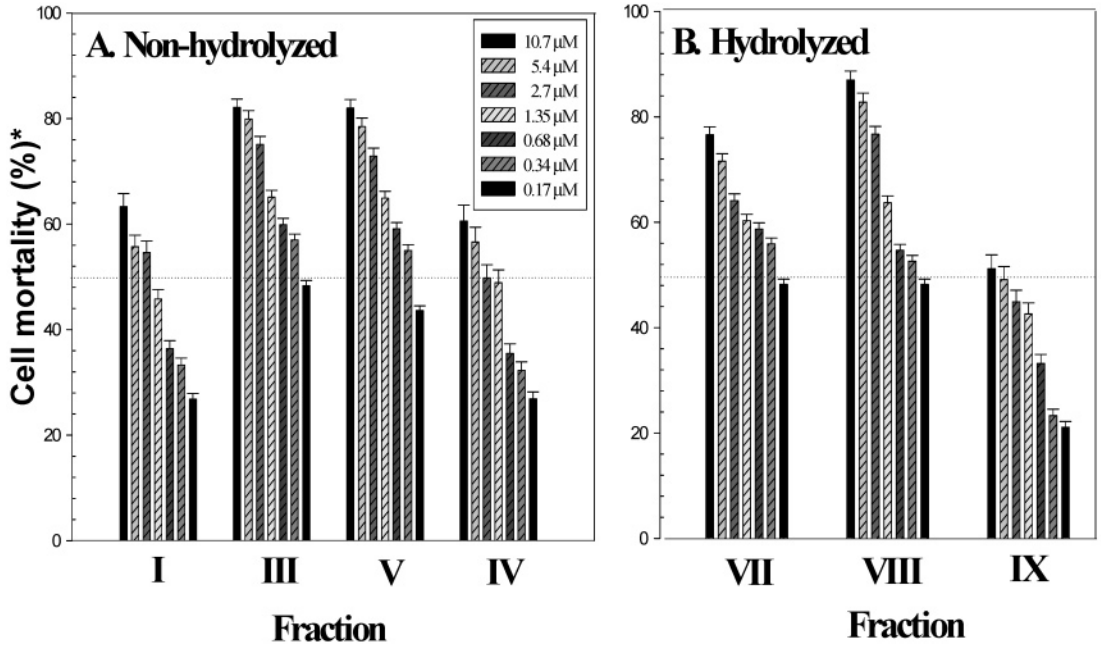

Figure 4. HL-60 cell mortality induced by different (**A**) non-hydrolyzed and (**B**) hydrolyzed (2 N HCl in 50% v/v methanol for 60 min at 90 °C) açai phytochemical fractions after 24 h exposure of whole pulp soluble phenolic concentrations of 0–10.7 μM. Fractions III–IX were tested at equivalent polyphenolic and/or anthocyanin content (quantified by HPLC) as fraction I. Each value is the mean ± SE of two replicate studies (each $n = 3$). Measurement of cell viability was based on the mitochondrial function of a control treatment containing DMSO at 2 mL/L. Tested fractions included whole pulp (fraction I), lipophilic fraction (fraction II), C_{18} bound phenolics and anthocyanins (fraction III), ethyl-acetate soluble polyphenolics (fraction IV), anthocyanins (fraction V), C_{18} nonretained (fraction VI), hydrolyzed C_{18} bound phenolics and anthocyanins (fraction VII), hydrolyzed anthocyanins (fraction VIII), and hydrolyzed ethyl-acetate soluble polyphenolics (fraction IX).

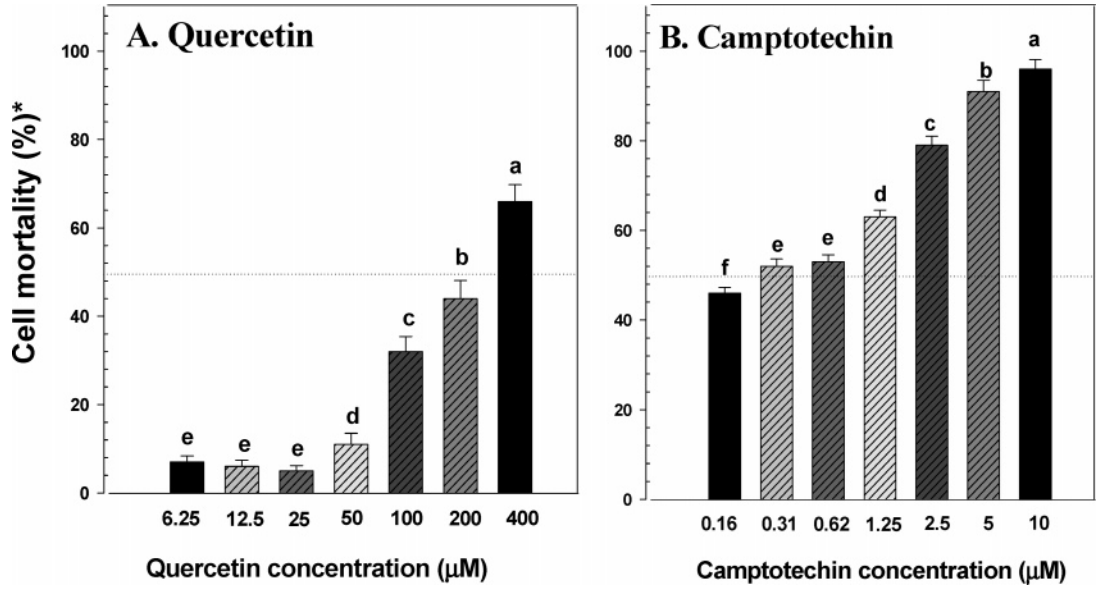

Figure 5. HL-60 cell mortality induced after 24 h exposure of (**A**) quercetin aglycone (0–400 μM) and (**B**) camptotechin (0–10 μM). Each value is the mean ± SE of two replicate studies (each $n = 3$). Measurement of cell viability was based on mitochondrial function of a control treatment containing DMSO at 2 mL/L.

mixture of different phytochemicals in whole fruit and vegetable extracts, not single compounds, may be responsible for the observed health benefits reported in epidemiological and clinical studies (*3*, *5*, *6*, *8*).

At equivalent individual polyphenolic and anthocyanin concentrations, fractions containing ethyl acetate-soluble phenolics (fractions I and IV) presented lower cell mortality than the other fractions containing anthocyanins (III and V). Both fractions III and V at 10.7 μM reduced cell viability to 18%, while at equivalent phytochemical concentrations, fractions I and IV reduced proliferation to 38%. When evaluated at their lowest concentration (0.17 μM), fractions III and V reduced cell viability by 50% (IC_{50} value), while a comparable growth inhibition was obtained by fractions I and IV when tested at 1.35 μM. Quercetin at ~200 μM (**Figure 5A**) and camptotechin at 0.31 μM (**Figure 5B**) also reduced the HL-60 cell population by 50%. Only a slight growth inhibition was observed by quercetin at ≤50 μM, while exposure at 400 μM resulted in a 66% growth inhibition; the latter being similar to that observed for fractions I and IV at 10.7 μM.

Acid hydrolysis affected the antiproliferative activity of each fraction in a different manner for each of the tested fractions.

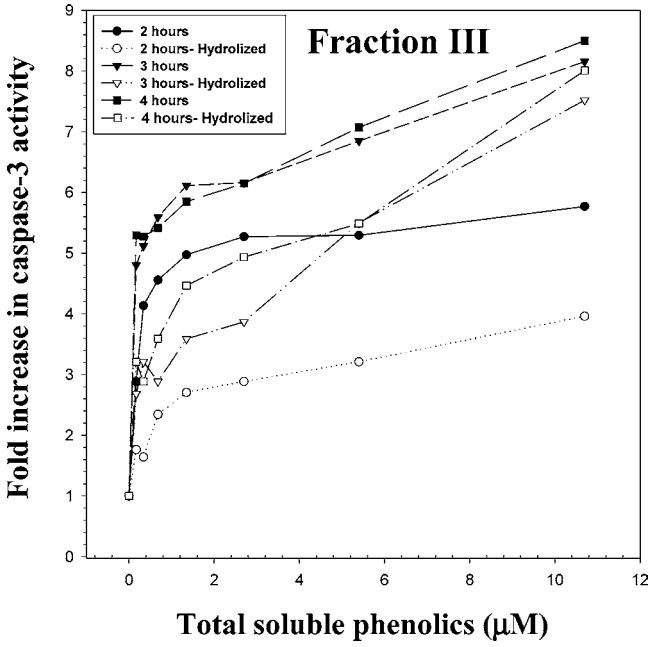

Figure 6. Caspase-3 activation in HL-60 cells after different exposure times (2–4 h) of açai C_{18} bound phenolics (fraction III) in both their glycosidic (closed symbols) and aglycone (open symbols) forms. The C_{18} bound phenolic fraction was tested at equivalent polyphenolic and anthocyanin contents (quantified by HPLC) as the whole pulp fraction (0–10.7 μM). Fold-increase in caspase-3 activity (relative fluorescence units) was compared to a control treatment containing DMSO at 2 mL/ L. Each value is the mean ± SE of two replicate studies (each $n = 3$).

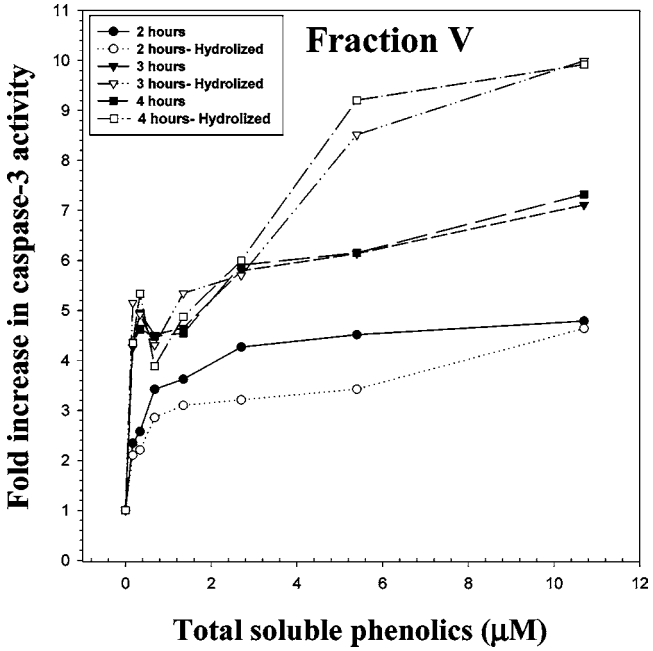

Figure 7. Caspase-3 activation in HL-60 cells after different exposure times (2–4 h) of açai anthocyanins (fraction V) in both their glycosidic (closed symbols) and aglycone (open symbols) forms. The anthocyanin fraction was tested at equivalent anthocyanin content (quantified by HPLC) as the whole pulp fraction (0–10.7 μM). Fold-increase in caspase-3 activity (relative fluorescence units) was compared to a control treatment containing DMSO at 2 mL/L. Each value is the mean ± SE of two replicate studies (each $n = 3$).

The non-hydrolyzed C_{18} bound phenolic fraction (fraction IV) induced a higher magnitude of change in vital cell parameters (proliferation and apoptosis) than their respective aglycone forms (fraction IX; **Figure 4B**) at concentrations ≥1.35 μM. However, the differences were not as prominent as those observed for antioxidant and metal reducing capacity. While acid hydrolysis of the anthocyanin glycosides (fraction VIII) slightly increased their antiproliferative effects (<9%), hydrolysis significantly decreased the cytostatic effect of the phenolic acids and flavonoids (fraction IX). At concentrations >0.68 μM, anthocyanin aglycones (fraction VIII) presented similar antiproliferative effects than the whole pulp fraction (fraction I) yet at significantly lower concentrations (4-fold). The IC_{50} value for both hydrolyzed anthocyanin-containing fractions was 0.17 μM, while isolated phenolics (fraction IX) presented a similar effect but at a higher concentration (5.4 μM).

Pro-apoptotic Effects of Açai Phytochemicals. The pro-apoptotic activity of each açai phytochemical fraction was assessed by measuring caspase-3 activity following treatment of HL-60 cells to investigate if its activation was responsible for the observed anti-proliferative effects of HL-60 cells by açai phytochemicals. A time-course response of caspase-3 activation was also evaluated to determine if the pro-apoptotic response induced by açai phytochemicals differed due to their composition or form (glycosides vs aglycones).

Phytochemical compounds present in açai were shown to induce HL-60 cell mortality through caspase-3 activation in a dose- and time-dependent manner. Maximum caspase activity was reached after 3 h of phytochemical exposure for the non-hydrolyzed anthocyanin containing fractions (fractions III and V; **Figures 6** and **7**), while maximum activity was reached at 4 h for the fraction containing phenolic acids and the flavonoid fraction (fraction IV; **Figure 8**). Caspase-3 activity did not significantly change after 4 h up and to 6 h of phytochemical

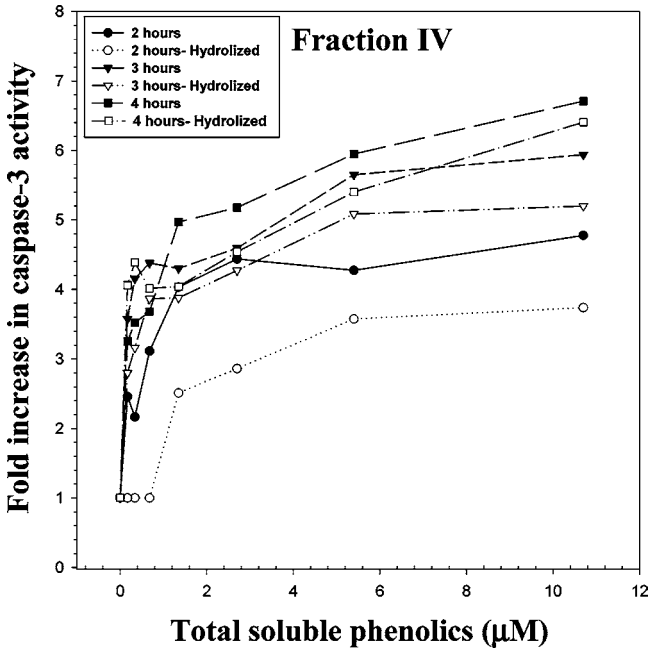

Figure 8. Caspase-3 activation in HL-60 cells after different exposure times (2–4 h) of açai ethyl acetate soluble phenolics (fraction IV) in both their glycosidic (closed symbols) and aglycone (open symbols) forms. The polyphenolic fraction was tested at equivalent polyphenolic content (quantified by HPLC) as the whole pulp fraction (0–10.7 μM). Fold-increase in caspase-3 activity (relative fluorescence units) was compared to a control treatment containing DMSO at 2 mL/L. Each value is the mean ± SE of two replicate studies (each $n = 3$).

exposure for all tested fractions (data not shown). Results also indicated that greater rates of caspase-3 activation were observed at lower phenolic concentrations (0.17−2.7 μM). Increases in

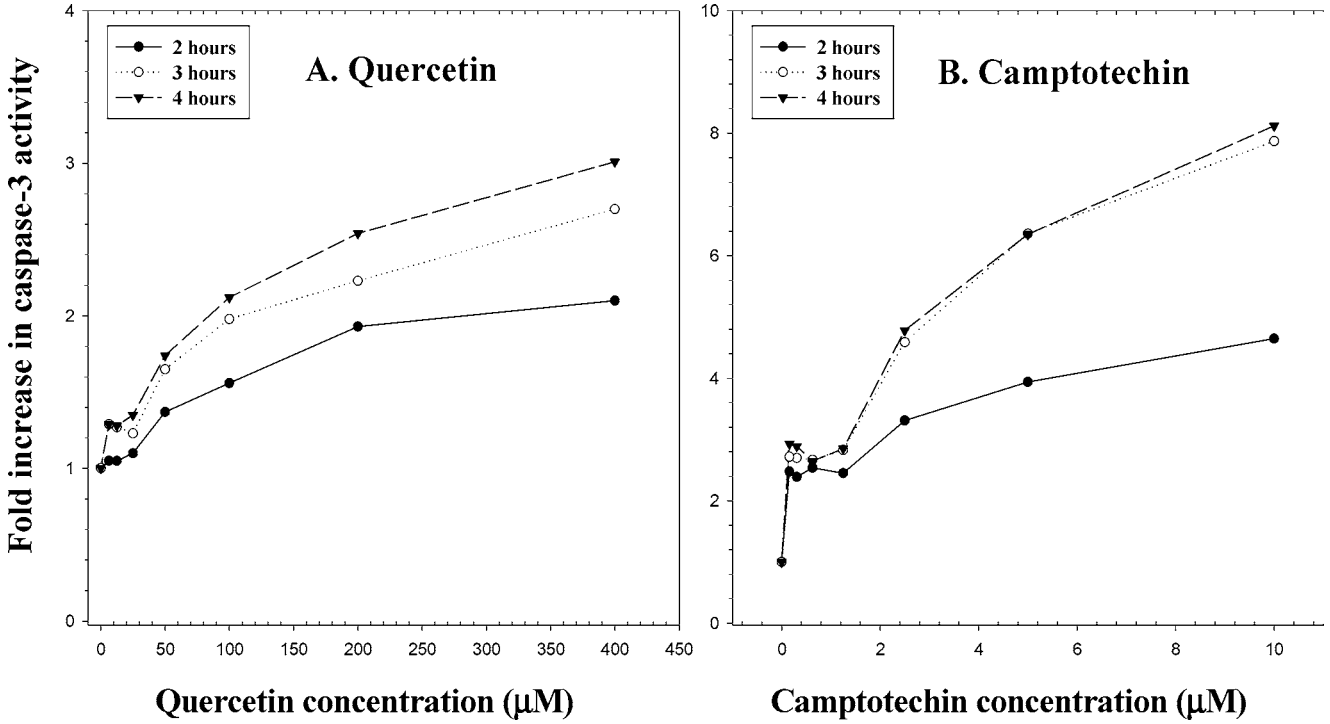

Figure 9. Caspase-3 activation in HL-60 cells after different exposure times (2–4 h) of (**A**) quercetin (0–400 μM) and (**B**) camptotechin (0–10.7 μM). Fold-increase in caspase-3 activity (relative fluorescence units) was compared to a control treatment containing DMSO at 2 mL/L. Each value is the mean ± SE of two replicate studies (each $n = 3$).

caspase-3 activity where inversely correlated ($r = 0.82$) to cell viability for all fractions. Generally, a 5-fold increase in caspase-3 activity correlated to a 50% cell mortality for each açai fraction, including camptotechin and quercetin (**Figure 9**). Furthermore, the maximum cell growth inhibition (ca. 82%) observed for the anthocyanin-containing fractions (fractions I, III, and V) was associated with an 8.2-fold increase in caspase-3 activity, which was similar to the antiproliferative effect of camptotechin at 2.5 μM (**Figure 9B**). These results suggested that apoptosis induced by açai phytochemicals involves a caspase-3 mediated mechanism; however, questions remain as to how caspase-3 is activated by them. Chang et al. (*3*) showed that apoptosis induced by an anthocyanin-rich extract from Hibiscus was through the activation of p38 and c-Jun kinases and that caspase-8 mediated the induction of caspase-3 activity. Fimognari et al. (*20*) observed that 30 h exposure of cyanidin 3-glucoside at 0.2 μg/mL (cyanidin levels corresponding to 5.4 μM açai soluble phenolics) resulted in a 36% increase in the fraction of apoptotic cells and that the induction of apoptosis did not require a functional p53 pathway.

Aglycone forms showed a slow onset of caspase-3 activation when compared to their corresponding glycosidic forms (**Figures 6−8)**, yet hydrolyzed fractions induced similar caspase-3 activation as their respective glycoside forms after 4 h of phytochemical exposure. Results also showed that polyphenolic compounds with low antioxidant and metal reducing (fractions VII and IX) properties could exert strong cytostatic properties and induce apoptosis. This effect also was observed by Tokalov et al. (*6*), who additionally demonstrated that the structure and concentration of a particular phytochemical determines which cell cycle phase is preferentially affected within a specific cancer cell type. These authors showed that flavonoids from *Parafavella denticulata* at concentrations ≤20 μM induced a block in the G_2/M phase of HL-60 cells, while at higher concentrations, a larger fraction of cells were present in the S phase. The longer time required for aglycones to induce the same magnitude of caspase-3 activation than their respective glycosides could have occurred due to the fact that these compounds might be interacting differently with a particular intracellular signal transduction pathway and/or that compounds stabilize each other (intermolecular copigmentation) so that their stability is enhanced, and therefore, the signaling pathways are stimulated for a longer period of time (*6*, *7*).

The present study demonstrated that polyphenolics present in açai reduced the proliferation of HL-60 leukemia cells through caspase-3 activation in a dose- and time-dependent manner. Results also indicated that antagonistic interactions among açai phytochemicals unfavorably impacted their individual pro-apoptotic and antiproliferative properties and thus reaffirmed the importance of investigating real food systems when evaluating the potential health benefits of these compounds.

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