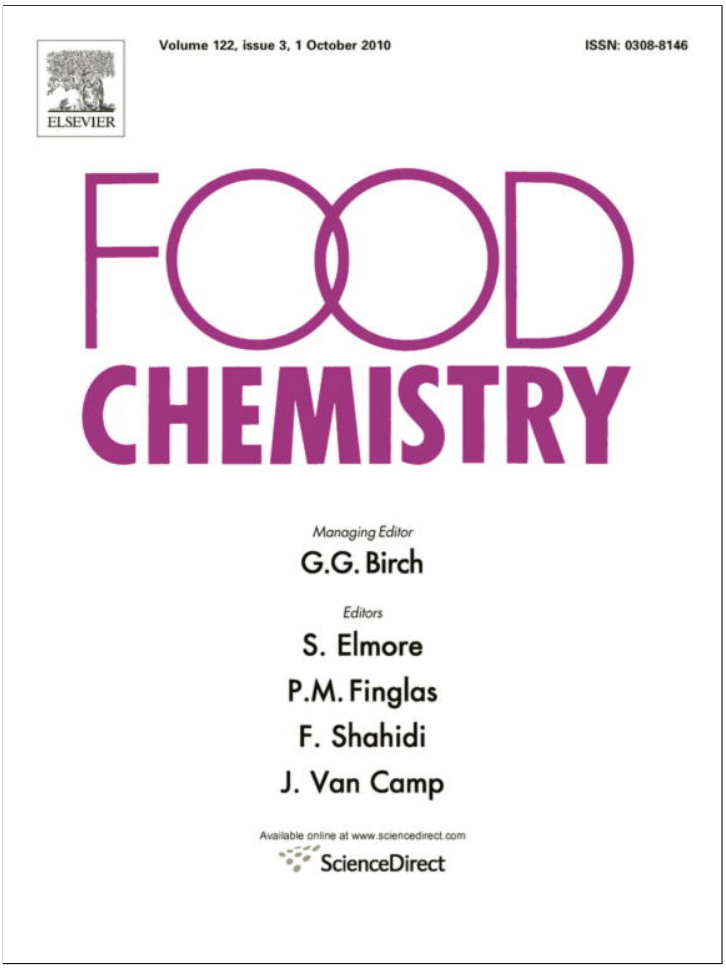

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Anti-oxidant capacities of flavonoid compounds isolated from acai pulp (*Euterpe oleracea* Mart.)

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ABSTRACT

Acai fruit (*Euterpe oleracea* Mart.) has been demonstrated to exhibit extremely high anti-oxidant capacity. Seven major flavonoids were isolated from freeze-dried acai pulp by various chromatographic methods. Their structures were elucidated as orientin (**1**), homoorientin (**2**), vitexin (**3**), luteolin (**4**), chrysoeriol (**5**), quercetin (**6**), and dihydrokaempferol (**7**) by NMR, MS and compared with the reported literature. Compounds **3** and **6** were reported from acai pulp for the first time. Anti-oxidant capacities of these flavonoids were evaluated by oxygen radical absorbance capacity (ORAC) assay, cell-based anti-oxidant protection (CAP-e) assay and reactive oxygen species (ROS) formation in polymorphonuclear (PMN) cells (ROS PMN assay). ORAC values varied distinctly (1420–14,800 μmol TE/g) among the seven compounds based on numbers and positions of hydroxyl groups and/or other substitute groups. The ORAC values of aglycones are generally higher than that of glycosides. CAP-e results indicated that only three compounds (**4**, **6** and **7**) could enter the cytosol and contribute to the reduction of oxidative damage within the cell. The ROS PMN assay showed that five compounds (**2**–**3** and **5**–**7**) demonstrated exceptional effects by reducing ROS formation in PMN cells, which produced high amounts of ROS under oxidative stress. In evaluating the anti-oxidant capacity of natural products, combining both chemical and cell-based assays will provide more comprehensive understanding of anti-oxidant effects and potential biological relevance.

1. Introduction

Acai (*Euterpe oleracea*) belongs to the family Arecaceae (palm tree). It is indigenous to South America. The palm trees grow at a density of 2500–3500 trees per hectare, serving as the predominant canopy plant inside the Amazon River delta in Para and Amapa states, Brazil, covering a range of over three million hectares (Brondizio, Safar, & Siqueira, 2002). The nutrient-dense and polyphenol-rich pulp of acai fruit is consumed as a fruit juice (Rodriques et al., 2006). The single seed, which accounts for 87% of the fruit, is expelled, while the skin is removed during pulping. A popular juice in Brazil prepared from the pulp of acai is consumed in a variety of beverages and food preparations (Schauss, 2010; Schauss et al., 2006a). Acai pulp has received much attention in recent years due to its extremely high anti-oxidant capacity and its role as a "functional food" or food ingredient. Freeze-dried acai pulp had dramatically high anti-oxidant value measured by ORAC (1027 μmol TE/g) (Schauss et al., 2006a), which is indeed higher than that of any fruit or vegetable that have been analysed and reported by USDA (Wu et al., 2004). Anti-oxidant capacities and other bioactivities of acai were studied in human, animal and cell culture models (Del, Percival, & Talcott, 2006; Jensen, Schauss, Beaman, & Ager, 2009; Jensen et al., 2008; Spada et al., 2009).

Since its high anti-oxidant capacity was revealed, numerous research investigations have focused on analyses and assessments associated with the anti-oxidant capacities of the acai pulp, juice or extracts (Honzel et al., 2008; Rufino, Fernandes, Alves, & Brito, 2009; Schauss, Jensen, Wu, & Scherwitz, 2009; Schauss et al., 2006a; Wu et al., 2004). Polyphenols have been associated with the anti-oxidant activity in fruits and vegetables. Major polyphenolic components found in acai include anthocyanins, proanthocyanidins, other flavonoids and lignans, etc. (Gallori, Bilia, Bergonzi, Barbosa, & Vincieri, 2004; Schauss et al., 2006b). Anthocyanins and proanthocyanidins are considered major anti-oxidants in fruits, but their concentrations are relatively low in acai (Schauss et al., 2006b). The contributions of the anthocyanins to the overall anti-oxidant capacities of acai were estimated to be only approximately 10% (Lichtenthäler et al., 2005). A recent paper also suggested that the components other than anthocyanins in acai contributed to antiproliferative activity against C-6 rat brain glioma cells (Hogan et al., 2010). The flavonoids were found to be

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the major polyphenols in acai (Gallori et al., 2004; Schauss et al., 2006b). Many studies have demonstrated that flavonoids have strong anti-oxidant activities and anti-inflammatory properties (Beara et al., 2009; Leong et al., 2010; Li et al., 2009). However, very few studies have determined the anti-oxidant capacity of individual polyphenol compounds found in acai pulp. There is only one study that has primarily studied lignans (Chin, Chai, Keller, & Kinghorn, 2008).

On the other hand, due to given limitations in analytical procedures, flavonoids have only been tentatively identified by HPLC or mass spectrometric methods, resulting in many flavonoids in the pulp remaining unidentified, much less determined for possible bioactivity. Therefore, the first objective of this study was to identify the major flavonoid compounds in acai pulp. Systemic isolation and fractionation in freeze-dried acai pulp is being reported in this paper. Seven flavones and their C-glycosides were obtained from freeze-dried acai pulp and their structures were elucidated by NMR, MS and the results compared to the literature. Among them, two compounds were identified in acai pulp for the first time.

The vast majority of studies that assess anti-oxidant capacities of acai and other anti-oxidant rich fruits and berries have utilised chemical-based assays. Unfortunately, such assays do not reflect or correlate with the test item's cellular response. The second objective of this study was to evaluate the anti-oxidant capacities of these flavonoids with one chemical-based assay and two cell-based assays: ORAC, CAP-e assay and ROS PMN assay. By doing these additional assays, we hope to understand not only their *in vitro* anti-oxidant capacities, but also their behaviours in living cells to reduce oxidative stress.

2. Materials and methods

2.1. Plant material

Freeze-dried acai (*Euterpe oleracea*) fruit pulp was obtained (Earth Fruits LLC, Belem, Brazil). The fruit was collected in Para state, Brazil, and processed within hours of harvesting to pure pulp and stored at −20 °C until transferred for freeze drying.

2.2. Chemicals and reagents

2.2.1. Extraction and isolation

Ninety five percentage EtOH, MeOH, petroleum ether and $CHCl_3$ were purchased from Shanghai Zhengxing Chemical Plant (Shanghai, China). EtOAc and acetone were obtained from Sinopharm Chemical Reagent Corporation (Shanghai, China). Silica gel (100–200 mesh) and Sephadex LH-20 were supplied by the Branch of Qingdao Marine Chemical Corporation (Qingdao, China) and Shanghai Juyuan Biotechology Corporation (Shanghai, China), respectively. Diatomite was obtained from Sinopharm Chemical Reagent Corporation (Shanghai, China).

2.2.2. ORAC-based assay

2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH) was purchased from Wako Chemicals, USA (Richmond, VA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and fluorescein (sodium salt) (FL) were obtained from Sigma–Aldrich (Milwaukee, WI). Potassium phosphate dibasic (K_2HPO_4) and potassium phosphate monobasic (KH_2PO_4) were obtained from VWR (West Chester, PA). Microplates (48-well, Falcon 3230) were purchased from VWR (West Chester, PA).

2.2.3. CAP-e assay and inhibition of ROS formation by PMN cells

The following buffers and reagents were obtained from Sigma–Aldrich (St. Louis, MO): phosphate-buffered saline (PBS), RPMI-1640 culture medium, hydrogen peroxide 30% solution (H_2O_2), dimethyl sulfoxide (DMSO), Histopaque 1077, and Histopaque 1119. Dichloroflurescein diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, OR), a subdivision of Invitrogen (Carlsbad, CA).

2.3. Instrumentation

Electrospray ionisation mass spectrometry (ESIMS) data were measured on Micromass Q-TOF spectrometer (Milford, MA). ^1H NMR spectra were recorded on Varian Inova 400 or 500 MHz NMR spectrometer (Palo Alto, CA) using tetramethylsilane (TMS) as an internal standard in dimethyl sulfoxide-d_6 (DMSO-d_6). ORAC analyses were carried out on a FLUOstar Galaxy plate reader (BMG Labtech, Duraham, NC). A fluorescence filter with an excitation wavelength of 485 nm and an emission wavelength of 520 nm was used.

2.4. Extraction and isolation

The freeze-dried acai pulp powder (1800 g) was mixed with diatomite, percolated with 95% alcohol for 2 weeks. After evaporation of solvents under the vacuum, the residue (244 g) was then mixed with diatomite, extracted with petroleum ether, chloroform, ethyl acetate, acetone, and methanol, successively. The $CHCl_3$ and EtOAc fractions were then chromatographed over silica gel and Sephadex LH-20 columns and purified by preparative thin layer chromatography (PTLC) (Fig. 1).

$CHCl_3$ extract (9 g) was loaded into an open silica gel column ($CHCl_3$:MeOH = 90:1). Fraction 27–34 was subjected to Sephadex LH-20 column, eluted with MeOH. Sub-fraction 11–18 was then purified by PTLC (petroleum ether:EtOAc = 1:3) to get compound **5** (R_f = 0.34, 8 mg).

EtOAC extract (19 g) was loaded into an open silica gel column, using a gradient of increasing polarity with $CHCl_3$ and MeOH mixture as solvent.

Fraction 71–86 ($CHCl_3$:MeOH = 50:1) was subjected to Sephadex LH-20 column, eluted with MeOH. Sub-fraction 16–24 was then purified using PTLC ($CHCl_3$:MeOH = 5:1) to yield compound **7** (R_f = 0.61, 7 mg).

Fraction 87–99 ($CHCl_3$:MeOH = 30:1) was subjected to Sephadex LH-20 column, eluted with MeOH. Compound **4** (3 mg) came from sub-fraction 21–23 by recrystallisation in MeOH.

Fraction 100–112 ($CHCl_3$:MeOH = 10:1) was subjected to Sephadex LH-20 column, eluted with MeOH. Sub-fraction 33–40 was then purified by PTLC ($CHCl_3$:MeOH = 5:1) to yield compound **6** (R_f = 0.29, 9 mg).

Fraction 118–136 ($CHCl_3$:MeOH = 10:1) was subjected to Sephadex LH-20 column, eluted with MeOH. Compound **3** (2 mg) was obtained from sub-fraction 11–12 by recrystallisation in MeOH.

Fraction 137–159 ($CHCl_3$:MeOH = 10:1) was subjected to Sephadex LH-20 column, eluted with MeOH. Sub-fraction 14–26 was then purified using PTLC (EtOAc:MeOH:H_2O = 8:2:1) to afford compounds **1** (R_f = 0.55, 21 mg) and **2** (R_f = 0.49, 11 mg).

2.5. Oxygen radical absorbance capacity (ORAC) assay

Seven pure flavonoids (>95%) were weighed exactly, dissolved in MeOH and diluted properly with phosphate buffer (0.75 M, pH 7.0). The dilution factors were in the range of 50–800 folds depending on the compounds. The ORAC assay was conducted based on the method reported by our group (Wu et al., 2004). Briefly, the assay was carried out on a FLUOstar Galaxy plate reader used with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The temperature of the incubator was set to 37 °C. The micro plate loaded with samples and

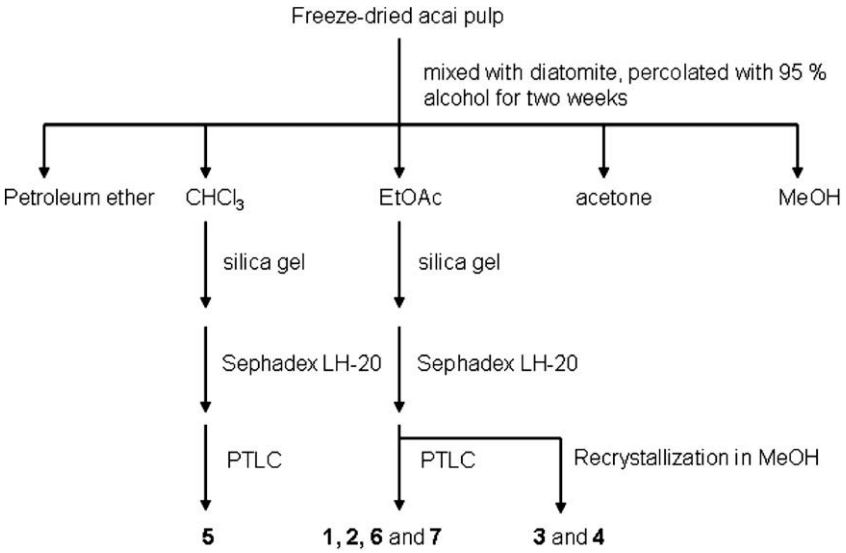


Fig. 1. The process of extraction, isolation and fractionation of flavonoids from acai.

standards were incubated for 5 min prior to run. Fluorescein was used as fluorescence probe; AAPH was used as peroxyl generator; Trolox as standard. The results were expressed as μmol Trolox equivalent (TE) per gramme.

2.6. Purification of red blood cells (RBC) and polymorphonuclear (PMN) cells

Healthy human volunteers between the ages of 20 and 50 years served as blood donors after informed consent, as approved by the Sky Lakes Medical Center Institutional Review Board (Klamath Falls, OR). Peripheral venous blood samples were drawn into sodium heparin and layered onto a double-gradient of Histopaque 1119 and 1077. The vials were centrifuged at 2400 rpm for 25 min. The PMN-rich lower interface was harvested using sterile transfer pipettes. PMN cells and the RBC fraction were washed twice in PBS without calcium or magnesium at 2400 rpm for 10 min. Then the core of the packed RBC was transferred into new vials and again washed twice in PBS without calcium or magnesium at 2400 rpm for 10 min. RBC aliquots were stored at 4 °C until use in the CAP-e assay. PMN cells were used immediately for the ROS assay.

2.7. Cell-based anti-oxidant protection of erythrocytes (CAP-e) assay

CAP-e assay was conducted following the method published by Honzel et al. (2008), but using an accelerated and more sensitive microplate-based protocol.

RBC cell suspension was prepared for the CAP-e assay by adding packed RBC (0.1 ml) into PBS (10 ml). The cell suspension was distributed in a V-bottom 96-well microtiter plate. Twelve wells were not treated with any source of anti-oxidants, and served as negative controls (six wells) and positive controls (six wells) for minimum versus maximum oxidative damage. Twelve wells were treated with a standard source of a known anti-oxidant (gallic acid) across six different serial dilutions, where each dilution was tested in duplicate. The remaining wells were treated with the isolated compounds from acai, where each compound was tested at 6 serial dilutions, and each dilution was tested in duplicate. RBC were incubated with anti-oxidants for 20 min, which was chosen based on testing of various incubation times on RBC anti-oxidant uptake of standard anti-oxidant compounds. Anti-oxidants not absorbed by the cells were removed by washing twice in PBS at 2400 rpm for 2.5 min. The cells were lysed and the precursor dye was added to the wells. Incubation was performed at room temperature for 15 min, followed by two washes. Oxidation was carried out using the peroxyl free radical generator AAPH for 1 h. The green fluorescence intensity, as a measure of oxidative damage, was measured at 488 nm using a Tecan Spectrafluor plate reader (40 flashes, optimal gain). The inhibition of oxidative damage was calculated as the reduced fluorescence intensity of product-treated cells, compared to cells treated only with the oxidising agent. The CAP-e value reflects the IC_{50} dose of the test product. This is then compared to the IC_{50} dose of the known anti-oxidant gallic acid. The CAP-e value was expressed as gallic acid equivalent (GAE) per gramme.

2.8. Inhibition of reactive oxygen species (ROS) formation by polymorphonuclear (PMN) cells

Evaluation of inhibition by seven flavonoids of ROS production was performed by using primary human polymorphonuclear (PMN) cells (ROS PMN assay) as previously described (Honzel et al., 2008). Freshly purified human PMN were exposed to the test products. During the incubation with a test product, any anti-oxidant compounds able to cross the cell membrane can enter the interior of the PMN cells. Compounds capable of transducing a signal across the cell membrane can do so. Then the cells were washed, loaded with the DCF-DA dye, which turns fluorescent upon exposure to reactive oxygen species. Formation of ROS was triggered by addition of H_2O_2. The fluorescence intensity of the PMN cells was evaluated by flow cytometry. The low fluorescence intensity of untreated control cells served as a baseline and PMN cells treated with H_2O_2 alone served as a positive control. If the fluorescence intensity of PMN cells exposed to an extract, and subsequently exposed to H_2O_2, was reduced compared to H_2O_2 alone, this indicates that a test product has anti-oxidant and/or anti-inflammatory effects. In contrast, if the fluorescence intensity of PMN cells exposed to test product was increased compared to H_2O_2 alone, this indicates that a test product has pro-inflammatory effects.

2.9. Statistical analysis

Statistical analysis was performed using Sigma Stat 3.5 for Windows (San Jose, CA). Statistical significance was tested using

Student's *t*-test with a *P* value of less than 0.05 indicating a significant difference between data sets.

3. Results and discussion

3.1. Characterisation of compounds

According to the initial screening, flavonoid compounds were mainly present in $CHCl_3$ and EtOAc fractions (Fig. 1). Therefore, we separated and purified compounds from these two fractions. After repeated separation by various chromatographic methods, seven known compounds were obtained (Fig. 2). Their structures were elucidated by ESIMS, ^{1}H NMR spectra and compared with literature.

Orientin (**1**), yellowish amorphous powder; ESIMS: m/z = 447 $[M-H]^-$; ^{1}H NMR (DMSO-d_6) ppm: δ 13.12 (1H, s, 5-OH), 7.32 (1H, d, J = 8.4 Hz, H-6′), 7.23 (1H, s, H-2′), 6.58 (1H, d, J = 8.4 Hz, H-5′), 6.19 (1H, s, H-3), 5.71 (1H, s, H-6), 4.72 (1H, d, J = 9.6 Hz, glc-H-1″), 3.92–3.26 (5H, m, glc-H-2″-6″). The data were consistent with the known compound (Leong et al., 2010).

Homoorientin (**2**), yellowish amorphous powder; ESIMS: m/z = 447 $[M-H]^-$; ^{1}H NMR (DMSO-d_6) ppm: δ 13.62 (1H, s, 5-OH), 7.19 (1H, d, J = 8.4 Hz, H-6′), 7.15 (1H, s, H-2′), 6.59 (1H, d, J = 8.4 Hz, H-5′), 6.19 (1H, s, H-3), 5.92 (1H, s, H-8), 4.56 (1H, d, J = 9.6 Hz, glc-H-1″), 4.16–3.15 (5H, m, glc-H-2″-6″). The data were consistent with the known compound (Leong et al., 2010).

Vitexin (**3**), yellowish amorphous powder; ESIMS: m/z = 431 $[M-H]^-$; ^{1}H NMR (DMSO-d_6) ppm: δ 10.27 (1H, s, 5-OH), 8.01 (2H, d, J = 8.4 Hz, H-2′, 6′), 6.92 (2H, d, J = 8.4 Hz, H-3′, 5′), 6.77 (1H, s, H-3), 6.28 (1H, s, H-6), 4.92 (1H, d, J = 9.6 Hz, glc-H-1″), 4.88–3.76 (5H, m, glc-H-2″-6″). The data were consistent with the known compound (Leong et al., 2010).

Luteolin (**4**), yellowish amorphous powder; ESIMS: m/z = 285 $[M-H]^-$; ^{1}H NMR (DMSO-d_6) ppm: δ 12.94 (1H, s, 5-OH), 7.40 (1H, d, J = 8.4 Hz, H-6′), 7.40 (1H, s, H-2′), 6.89 (1H, d, J = 8.4 Hz, H-5′), 6.63 (1H, s, H-3), 6.44 (1H, s, H-8), 6.18 (1H, s, H-6). The data were consistent with the known compound (Hartwig, Maxwell, Joseph, & Phillips, 1990).

Chrysoeriol (**5**), yellowish amorphous powder; ESIMS: m/z = 299 $[M-H]^-$; ^{1}H NMR (DMSO-d_6) ppm: δ 12.94 (1H, s, 5-OH), 7.36 (1H, d, J = 8.4 Hz, H-6′), 7.32 (1H, s, H-2′), 6.74 (1H, d, J = 8.4 Hz, H-5′), 6.34 (1H, s, H-3), 5.78 (1H, s, H-8), 5.54 (1H, s, H-6), 3.71 (3 H, s, OCH_3-3′). The data were consistent with the known compound (Awaad, Maitland, & Soliman, 2006).

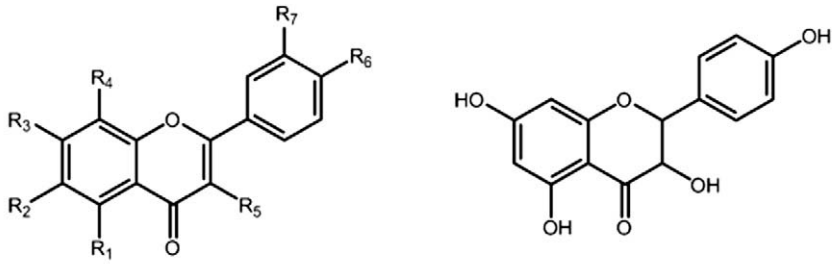
Quercetin (**6**), yellowish amorphous powder; ESIMS: m/z = 301 $[M-H]^-$; ^{1}H NMR (DMSO-d_6) ppm: δ 12.42 (1H, s, 5-OH), 9.26 (1H, s, 3-OH), 7.66 (1H, d, J = 2.0 Hz, H-2′), 7.54 (1H, dd, J = 8.0, 2.0 Hz, H-6′), 6.88 (1H, d, J = 8.0 Hz, H-5′), 6.40 (1H, d, J = 2.0 Hz, H-6), 6.18 (1H, d, J = 2.0 Hz, H-8). The data were consistent with the known compound (Awaad et al., 2006).

Dihydrokaempferol (**7**), yellowish amorphous powder; ESIMS: m/z = 287 $[M-H]^-$; ^{1}H NMR (DMSO-d_6) ppm: δ 11.96 (1H, s, 5-OH), 7.30 (2H, d, J = 8.4 Hz, H-2′, 6′), 6.78 (2H, d, J = 8.4 Hz, H-3′, 5′), 5.79 (1H, d, J = 1.6 Hz, H-8), 5.74 (1H, d, J = 1.6 Hz, H-6), 4.99 (1H, d, J = 11.2 Hz, H-2), 4.50 (1H, d, J = 11.2 Hz, H-3). The data were consistent with the known compound (Nafady et al., 2003).

After a literature search, orientin (**1**), homoorientin (**2**), vitexin (**3**), luteolin (**4**) and quercetin (**6**) were isolated and identified as pure compounds from acai for the first time. Two of them, vitexin and quercetin (**3** and **6**), were reported from acai for the first time. Though some previous studies identified some flavonoids from acai, only a few of them (Chin et al., 2008) were separated and obtained. Most flavonoids were tentatively identified by HPLC-UV or HPLC-MS analysis (Gallori et al., 2004; Pacheco-Palencia, Duncan, & Talcote, 2009), therefore the bioactivities of pure compounds from acai could not be studied. It is very important to obtain the pure compounds from acai for studying responsible compounds having anti-oxidant capacities in acai.

3.2. Anti-oxidant capacity from ORAC

The ORAC values obtained for the seven flavonoids from ORAC assay is shown in Table 1. ORAC values varied distinctly among these seven compounds (1420–14800 μmol TE/g) based on the numbers and positions of hydroxyl groups and other substitute groups. A previous study reported that with compounds having the same basic chemical structure, the ORAC value of flavonoid was proportional to the number of hydroxyl substitutions on the structure (Cao, Sofic, & Prior, 1997). However, according to our study, the ORAC value of each flavonoid was not only determined by the number of hydroxyl groups, but also depended on the positions of hydroxyl groups in the structures. The structure of vitexin (**3**) is very similar to that of orientin (**1**). The difference is a lack of one hydroxyl group at C-3′ of vitexin (**3**). ORAC values of these two



1 $R_1 = R_3 = R_6 = R_7 = OH$, $R_4 = glc$, $R_2 = R_5 = H$

2 $R_1 = R_3 = R_6 = R_7 = OH$, $R_2 = glc$, $R_4 = R_5 = H$

3 $R_1 = R_3 = R_6 = OH$, $R_4 = glc$, $R_2 = R_5 = R_7 = H$

4 $R_1 = R_3 = R_6 = R_7 = OH$, $R_2 = R_4 = R_5 = H$

5 $R_1 = R_3 = R_6 = OH$, $R_7 = OCH_3$, $R_2 = R_4 = R_5 = H$

6 $R_1 = R_3 = R_5 = R_6 = R_7 = OH$, $R_2 = R_4 = H$

Fig. 2. The structures of seven flavonoid compounds (1–7) isolated from acai. The corresponding compound names are shown in Table 1.

Table 1
Antioxidant capacities from ORAC assay and CAP-e assay[a].

No.	Compound name	ORAC (μmol TE/g)	CAP-e (GAE/g)
1	Orientin	1700 ± 78.9	N/A[b]
2	Homoorientin	1420 ± 63.3	N/A
3	Vitexin	14,800 ± 451	N/A
4	Luteolin	7870 ± 350	5040 ± 260
5	Chrysoeriol	4400 ± 189	N/A
6	Quercetin	12,300 ± 1070	5510 ± 443
7	Dihydrokaempferol	8390 ± 93.8	3980 ± 126

[a] Data was expressed as mean ± SD ($n = 3$).
[b] No value was obtained.

compounds were very different. Vitexin (**3**) has the highest ORAC (14800) among the seven compounds, whereas ORAC of orientin (**1**) was only 1700. The results also showed that ORAC values of aglycones are generally higher than that of C-glycosides. For instance, the ORAC value of the aglycone luteolin (**4**) was four times higher than that of glycosides, orientin and homoorientin (**1** and **2**). The *O*-methylation of the hydroxyl groups reduced the ORAC values (about two times), such as chrysoeriol (**5**) vs. luteolin (**4**). Likely the conjugation between 4′-OH and 4-C=O distinctly affects the anti-oxidant activity (e.g. vitexin and dihydrokaempferol, **3** and **7**), while mono-OH substitution at 4′ (vitexin, **3**) has stronger anti-oxidant activity (approximate 8–10 times) than di-OH substitution at 3′ and 4′ (e.g. orientin and homoorientin, **1** and **2**).

3.3. CAP-e anti-oxidant capacity

Anti-oxidant capacities of the seven flavonoids were also measured by the recently developed cell-based anti-oxidant protection (CAP-e) assay. Data from the CAP-e assay reflects whether anti-oxidants can enter into and protect live cells from oxidative damage. The results of CAP-e values of seven flavonoids are shown in Table 1. Remarkably, orientin, homoorientin, vitexin and chrysoeriol (**1**–**3** and **5**) did not have any CAP-e values at any tested concentrations (Fig. 3), which meant that they were not able to penetrate into live cells and provided protection against oxidative stress. As these compounds were not able to enter into and protect RBC in the CAP-e assay, no inhibition of oxidative damage was seen, and no IC_{50} were calculated. The other three compounds, luteolin, quercetin, and dihydrokaempferol (**4**, **6** and **7**) could enter into the live cells with quercetin (**6**) displaying the highest CAP-e value. The IC_{50} for compounds **4**, **6**, and **7** were 0.00635 ± 0.00033, 0.00581 ± 0.00047, and 0.00803 ± 0.00025 mg/ml, respectively.

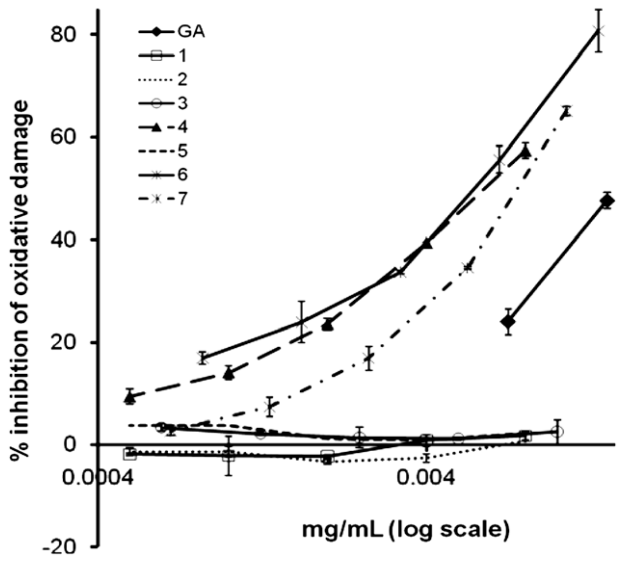


Fig. 3. CAP-e values of seven flavonoid compounds. The corresponding compound names are shown in Table 1. GA represents gallic acid, which was used as standard.

The results showed that the compounds (**4**, **6** and **7**) being able to get into living cells were all aglycones, and their C-glycosides (**1**–**3**) cannot penetrate into living cells. Surprisingly, chrysoeriol (**5**), as an aglycone, was not able to enter living cells at all. It is the only aglycone that bears a methoxy group. So we speculate that this structural property may increase its hydrophobicity thus prevent this compound from entering living cells.

3.4. Inhibition of ROS formation by ROS PMN assay

Inhibition effects of the seven flavonoids against ROS formation were evaluated by the ROS PMN assay are shown in Fig. 4. The ROS PMN assay monitors the combined effect of a test product on an inflammatory cell type, and the data reflects a combination of at least three different mechanisms: (1) anti-oxidants penetrate into the cell and neutralise free radicals, similar to the CAP-e assay; (2) anti-inflammatory compounds mediate cell signalling at the cell surface, reprogramming the PMN cell to a less inflammatory behaviour, resulting in a reduction in formation of ROS; and (3) pro-inflammatory compounds capable of supporting innate immune functions mediate a signal at the cell surface, resulting in an increase in the PMN cell function, thus increasing the production of ROS (Honzel et al., 2008). All compounds tested were shown to reduce ROS formation at various doses. However, their behaviours were very different. Orientin (**1**) and luteolin (**4**) showed dose responses in which only higher doses displayed inhibition effects (Fig. 4). Four compounds, homoorientin, vitexin, quercetin, and dihydrokaempferol (**2**, **3**, **6** and **7**), were shown to reduce ROS formation best at the lowest concentrated dilution of 8×10^{-7} mg/ml (Fig. 4). Interestingly, chrysoeriol (**5**) exhibited inhibition effects indistinguishably at doses varied from 8×10^{-7} to 8×10^{-3} mg/ml (Fig. 4). One apparent reason for these dramatic behavioural differences is the chemical structure. Chrysoerial (**5**), for example, is the only one among these seven compounds that bears a methoxy group. This structural characteristic might be responsible for the unique inhibitory effects of this compound, which inhibited ROS formation at both low and high doses ranging from 8×10^{-7} to 8×10^{-3} mg/ml (Fig. 4). But exactly how the chemical structure affects ROS production and the potent anti-inflammatory effect seen at the lowest dose tested for these compounds warrant further investigation.

3.5. Results of anti-oxidant capacities from different assays

In this study, anti-oxidant capacities of seven flavonoids isolated from acai were investigated by three assays. Of these three assays, ORAC is one of most widely used chemical-based anti-oxidant assays, which measures anti-oxidant inhibition of peroxyl radical induced oxidation (Prior, Wu, & Schaich, 2005). However, a chemical-based assay is solely based on chemical reaction and do not reflect the cellular physiological conditions, including cellular uptake versus signal transduction. Mechanisms of anti-oxidants going beyond direct scavenging of free radicals may be involved in disease prevention and health promotion. Therefore, there is need for cell-based anti-oxidant assays (Liu & Finley, 2005). The CAP-e assay is a newly developed cell-based assay using erythrocytes to address the question of whether anti-oxidants in complex natural products enter the cytosol and contribute to the reduction of oxidative damage within the cell. The assay allows for semiquantification specifically of those anti-oxidants that are capable of penetrating into live cells (Honzel et al., 2008). Based on the CAP-e data, another cell-based as-

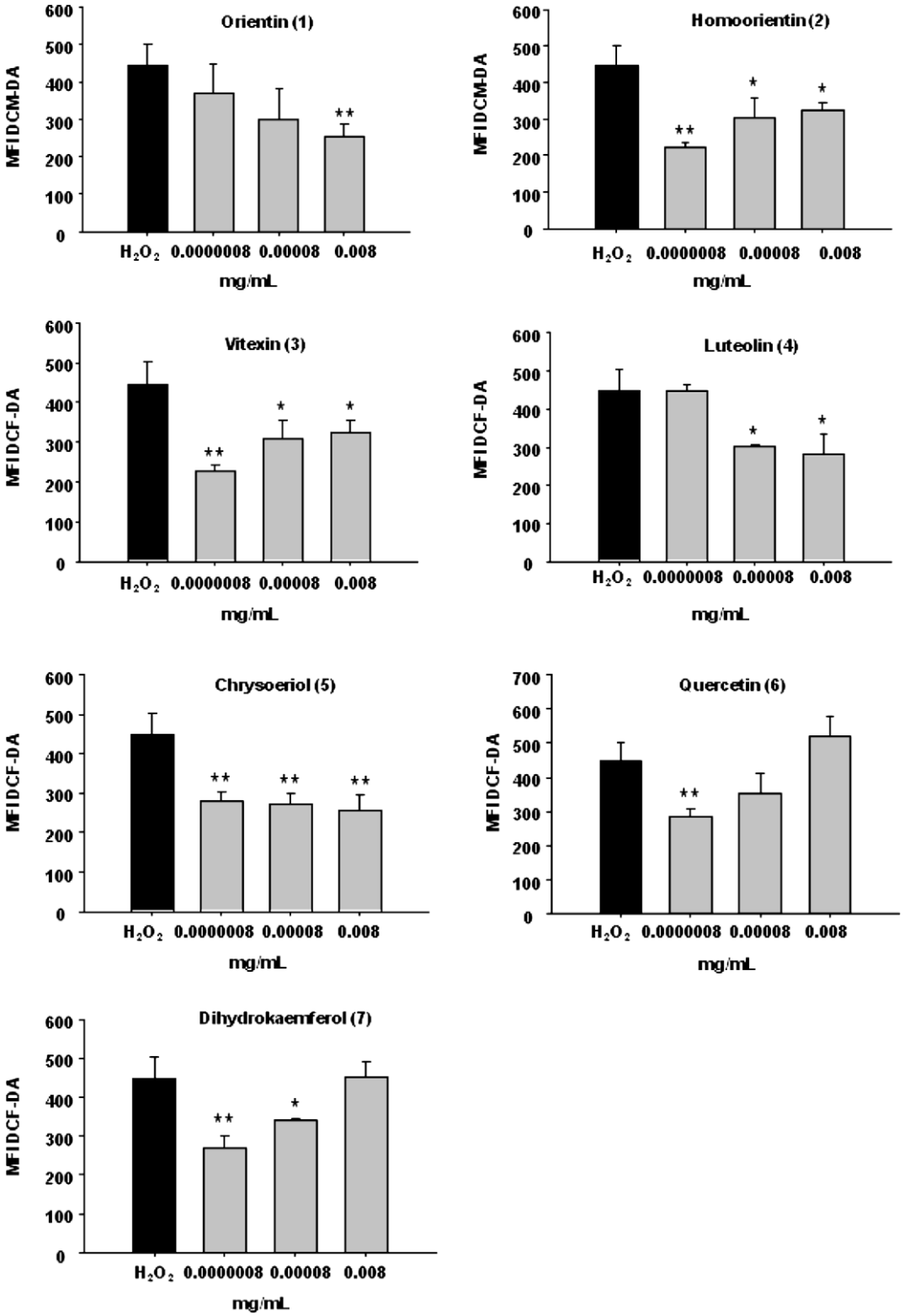


Fig. 4. Results from the ROS PMN assay for seven flavonoid compounds. The mean fluorescence intensity (MFI) for the reporter dye DCF-DA is plotted. It is proportional to ROS formation under each assay condition. Black bars indicate H_2O_2 treatment and grey bars indicate different doses of each flavonoid ($^{*}P < 0.05$ and $^{**}P < 0.01$).

say using primary pro-inflammatory PMN cell was performed, which allows for a more complex assessment of the properties of natural products *in vitro*, were conducted. PMN cells are an important part of our innate immune defense and are capable of rapid production of ROS in response to both oxidative damage and pro-inflammatory stimuli such as microbial invaders. The PMN cell can respond to compounds in natural products extracts in the three aforementioned mechanisms. Thus, data obtained from a PMN-based assay may be interpreted better in the context of data from the CAP-e assay (Honzel et al., 2008).

The combination of ORAC to CAP-e data and ROS PMN data give a good foundation for making further decisions and fully

understand the scope of acai compounds' anti-oxidant capacities. For instance, vitexin (**3**) responsible for the highest ORAC value may not match the three compounds, luteolin, quercetin and dihydrokaempferol (**4**, **6** and **7**) that performed best in the CAP-e assay. The four compounds, orientin, homoorientin, vitexin and chrysoeriol (**1**–**3** and **5**) that showed no bioactivity in the CAP-e bioassay, may be responsible for the signalling at the cell membrane level, where entry into the cell is not necessary for the biological activity involved in the ROS PMN assay. Basically, the seven compounds were divided into four groups according to the results from three assays. Group 1, orientin (**1**), showed relatively low ORAC, and does not penetrate into live cells. Group 2, homoorientin, vitexin and chrysoeriol (**2**, **3**, **5**), had exceptional anti-inflammatory effect and compound **3** had the highest ORAC, but neither of these compounds were able to penetrate into live cells. Group 3, luteolin (**4**), had some anti-inflammatory effect, relatively high ORAC, and can penetrate into live cells. Group 4, quercetin and dihydrokaempferol (**6**–**7**), both of them have demonstrated exceptional anti-inflammatory effects and the ability to penetrate into live cells. Remarkably, quercetin (**6**) in group 4 displayed the best anti-oxidant capacities in all three assays, which included high ORAC and CAP-e value at the lowest dose of 8×10^{-7} mg/ml inhibiting ROS formation.

4. Conclusions

In this study, seven flavonoids (flavones and their C-glycosides) were isolated and structurally identified from freeze-dried acai pulp. Vitexin and quercetin (**3** and **6**) were reported from acai pulp for the first time. Our data confirmed that flavonoids are major polyphenols in acai pulp. In order to determine the major anti-oxidants in acai pulp, the anti-oxidant capacities of these flavonoids were evaluated with one chemical-based assay and two cell-based assays: ORAC, CAP-e assay and ROS PMN assay. However, anti-oxidant capacities of these compounds varied significantly based on their chemical structures and the assays used. In general, flavonoid aglycones showed not only higher anti-oxidant capacities than their C-glycosides, but also the ability to penetrate into living cells. Except for sugar moieties, the methoxy group appears to be another important structural factor that could lead to dramatic changes of flavonoids in terms of their anti-oxidant capacity and the ability to penetrate into living cells.

Due to the complexity of the anti-oxidant defense system in the body, a single anti-oxidant assay cannot reflect all aspects of activities of a given compound. In evaluating the anti-oxidant capacities of natural products, combining both chemical and cell-based assays will provide a useful approach towards understanding the anti-oxidant effects of natural compounds and their biological relevance to any health benefits observed *in vivo*. Such combined use of assays may prevent premature conclusions being made about which compounds are responsible for a foods biological effects, based on reliance of chemical assays.

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