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Flavonoids from acai (*Euterpe oleracea* Mart.) pulp and their antioxidant and anti-inflammatory activities ☆

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ABSTRACT

Five flavonoids, (2S,3S)-dihyrokaempferol 3-*O*-β-D-glucoside (**1**) and its isomer (2R,3R)-dihydrokaempferol 3-*O*-β-D-glucoside (**2**) , isovitexin (**3**), velutin (**4**) and 5,4′-dihydroxy-7,3′,5′-trimethoxyflavone (**5**), were isolated from acai (*Euterpe oleracea* Mart.) pulp. The structures of these compounds were elucidated based upon spectroscopic and chemical analyses. To our knowledge, compounds **1**, **2**, **4** and **5** were identified from acai pulp for the first time. The *in vitro* antioxidant activities of these compounds were evaluated by the oxygen radial absorbance capacity (ORAC) assay. The ORAC values varied distinctly (4458.0–22404.5 μmol Trolox equivalent (TE)/g) from 5,4′-dihydroxy-7,3′,5′-trimethoxyflavone (**5**) to isovitexin (**3**) and were affected by the numbers/positions of hydroxyl groups, substitute groups, as well as stereo configuration. The anti-inflammatory effects of these compounds were screened by the secreted embryonic alkaline phosphatase (SEAP) reporter assay, which is designed to measure NF-κB activation. Velutin (**4**) was found to dose-dependently inhibit SEAP secretion in RAW-blue cells induced by LPS, with an IC_{50} value of 2.0 μM. Velutin (**4**) also inhibited SEAP secretion induced by oxidised LDL, indicating potential athero-protective effects.

1. Introduction

Acai fruits gained popularity in North America and in the European countries lately as a new "super fruit" largely due to its extremely high antioxidant capacity and potential anti-inflammatory activities (Schauss, Wu, Prior, Ou, Huang, et al., 2006). Antioxidant and anti-inflammatory activities of acai pulp or acai juice have been studied in human, animal and cell culture models (Del Pozo-Insfran, Percival, & Talcott, 2006; Jensen et al., 2008; Spada et al., 2009). In a recent study of the effects of acai pulp supplementation on the lifespan of oxidative stressed female flies, molecular analysis revealed that supplementation with acai pulp restored the transcript level of lethal (2) essential for life (l(2)efl), a downstream event of the oxidative stress response pathway Jun-*N*-terminal kinase (JNK), in loss-of-function and reduction-of-function sod1mutants. This resulted in a significant life span extension, suggesting that the compounds in acai may play a role in cell signalling and changes in gene expression (Sun et al., 2010).

Major polyphenolic components in acai pulp include anthocyanins, proanthocyanidins, other flavonoids and lignans (Chin, Chai, Keller, & Kinghorn, 2008; Gallori, Bilia, Bergonzi, Barbosa, & Vincieri, 2004; Schauss, Wu, Prior, Ou, Patel, et al., 2006). Among them, the flavonoids were found to be the major polyphenols. Flavonoids are ubiquitously present in fruits and vegetables. As a group, flavonoids have been shown to exhibit strong antioxidant capacities. The mechanism of antioxidant activity of flavonoids involves the direct scavenging or quenching of oxygen free radicals or excited oxygen species, as well as the inhibition of oxidative enzymes that generate these reactive oxygen species (Pietta, 2000; Terao, 2009). Flavonoids have also shown anti-inflammatory activity in both the proliferative and exudative phases of inflammation (Rathee et al., 2009).

In our previous paper (Kang et al., 2010), seven known flavonoids were isolated and their antioxidant activities were evaluated using three assays. The first objective of this study was to isolate and identify additional flavonoids from acai pulp. Furthermore, antioxidant and anti-inflammatory activities were evaluated by the oxygen radial absorbance capacity (ORAC) assay and the secreted embryonic alkaline phosphatase (SEAP) reporter assay.

☆ Mentioning of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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The SEAP reporter assay was designed to measure the nuclear factor-kappa B (NF-κB) activation (Berger, Hauber, Hauber, Geiger, & Cullen, 1988; Moon, Hahn, Lee, & Kim, 2001). As a major transcription factor, NF-κB plays a key role in regulating the genes responsible for innate and adaptive immune responses (Brasier, 2006; Hoffmann, Natoli, & Ghosh, 2006). The activation of NF-κB has been shown to mediate inflammation by increasing the expression of pro-inflammatory cytokines, chemokines and enzymes (Pahl, 1999). In the SEAP reporter assay, lipopolysaccharide (LPS) or oxidised LDL (oxLDL) was used as stimuli; the later one may particularly implicate potential protective actions of test compounds against atherosclerosis.

2. Materials and methods

2.1. Instrumentation

Optical rotations were measured on a Perkin-Elmer 341 digital polarimeter (Waltham, USA). The UV spectra were obtained on a Shimadzu-2500PC spectrophotometer (Kyoto, Japan). The circular dichroism (CD) spectra were recorded in MeOH using a JASCO J-810 spectropolarimeter (Easton, USA). The infrared (IR) spectra were obtained with a NEXUS 670-FTIR spectrophotometer (San Jose, USA). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Inova 500 MHz NMR spectrometer (Palo Alto, USA). Electrospray ionisation mass spectrometry (ESIMS) was carried out using Micromass Q-Tof mass spectrometer (Milford, USA). High performance liquid chromatography (HPLC) was performed in a Agilent 1100 HPLC equipped with a UV detector (Palo Alto, USA); a C-18 Dikma Diamonsil analytical column (250 × 4.6 mm, 5 μm) (Scarborough, Canada) and a C-18 Waters uBondapak column (3000 × 7.8 mm, 10 μm) (Milford, USA) were employed for analysis and preparation respectively.

2.2. Plant material

The fruits of *Euterpe oleracea* Mart. were harvested in Para state, Brazil, in September, 2009. A voucher specimen was deposited at Embrapa Amazônia Oriental (Belém, Brazil). The fruits were processed within hours of harvesting to pure pulp and stored at −20 °C until transferred for freeze drying. The frozen pulp was lyophilised at Liotecnica Tecnologia em Alimentos (Sao Paolo, Brazil) and the freeze-dried pulps were transported into the US and supplied by Earth Fruits (South Jordan, USA).

2.3. Chemicals and reagents

Methanol (MeOH), 95% ethanol (EtOH), *n*-butyl alcohol (n-BuOH), petrol ether, and chloroform ($CHCl_3$) were purchased from Shanghai Zhengxing Chemical (Shanghai, China). Ethyl acetate (EtOAc) and acetone were obtained from Sinopharm Chemical Reagent (Shanghai, China). Silica gel (100–200 mesh) and Sephadex LH-20 were supplied by the Branch of Qingdao Marine Chemical (Qingdao, China) and Shanghai Juyuan Biotechnology (Shanghai, China), respectively. Diatomite was obtained from Sinopharm Chemical Reagent (Shanghai, China). 2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and fluorescein (sodium salt) (FL) were obtained from Aldrich (Milwaukee, USA). Potassium phosphate dibasic (K_2HPO_4), potassium phosphate monobasic (KH_2PO_4) and Microplates (48-well, Falcon 3230) were obtained from VWR (West Chester, USA).

2.4. Extraction and isolation procedure

The freeze-dried acai pulp powder (3000 g) was mixed with diatomite, percolated with 95% alcohol for two weeks. After evaporation of the solvents under the vacuum, the residue (500 g) was then extracted with petroleum ether, $CHCl_3$, EtOAC, and *n*-BuOH, in succession. The EtOAc and *n*-BuOH fractions were further separated by open column with silica gel, Sephadex LH-20, preparative thin layer chromatography (PTLC) and preparative high performance liquid chromatography (HPLC) respectively. The EtOAC extract (50 g) were loaded into an open silica gel column and eluted with $CHCl_3$–MeOH mixtures with increasing polarity. Fraction 27–34 ($CHCl_3$:MeOH = 90:1) was subjected to a Sephadex LH-20 column, eluted with MeOH. Sub-fraction 12–13 was then purified by PTLC (petroleum ether:EtOAc = 1:3) to get compounds **4** (R_f = 0.75, 4 mg) and **5** (R_f = 0.81, 3 mg).

Fraction 137–159 ($CHCl_3$:MeOH = 10:1) was submitted to a Sephadex LH-20 column and eluted with MeOH. Sub-fraction 12–13 was further purified using HPLC with a Waters uBondapak column. The mobile phase was CH_3CN–H_2O (containing 0.1% formic acid) (20:80, v/v), at a flow rate of 1 ml/min to yield compound **3** (3 mg). The chromatographic profile was detected at 270 nm.*n*-BuOH extract (80 g) was separated with a Sephadex LH-20 column eluted with MeOH, followed by further purification using HPLC on a Waters uBondapak column (flow rate, 1 ml/min; detection wave length, 270 nm), with CH_3CN–H_2O (containing 0.1% formic acid) (30:70, v/v) as the mobile phase, to yield compounds **1** (15 mg) and **2** (7 mg).

2.5. ORAC assay

The ORAC assay was conducted based on the method reported previously (Wu et al., 2004). Briefly, the assay was carried out on a FLUOstar Galaxy plate reader (BMG Labtech, Durham, USA) 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. Fluorescein was used as fluorescence probe; 2,2'-azobis (2-methylpropionamide) dihydrochloride (AAPH) was used as peroxyl generator; Trolox was used as standard. The results were expressed as μmol TE per gram.

2.6. SEAP reporter assay

RAW-Blue cells (Invitrogen, San Diego, USA) are derived from RAW264.7 macrophages with chromosomal integration of a SEAP reporter construct inducible by NF-κB and AP-1. RAW-Blue mouse macrophage cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, USA) and zeocineosin (200 μg/ml). All cell culture reagents were purchased from Invitrogen (San Diego, USA).

RAW-Blue cells (1×10^5 cells/well) were pretreated with compounds for 3 h, stimulated by LPS (100 ng/ml, Invitrogen, San Diego, USA) or oxLDL (100 ng/ml, Academy Biomedical, Houston, USA) for 18 h. Luteolin was isolated from the acai pulp described in our previous study (Kang et al., 2010). The supernatants were collected for the SEAP secretion assay. The QUANTI-Blue™ powder was dissolved in endotoxin-free water and sterile filtered (0.22 μm) (QuantiQuanta-blue substrate). RAW-Blue cell supernatant (40 μL/well) was added to QuantiQuanta-blue substrate (160 μL/well) and incubated at 37 °C for 0.5–1 h. The absorbance was measured at 620 nm in a Polarstar microplate reader (BMG Labtech, Durham, USA).