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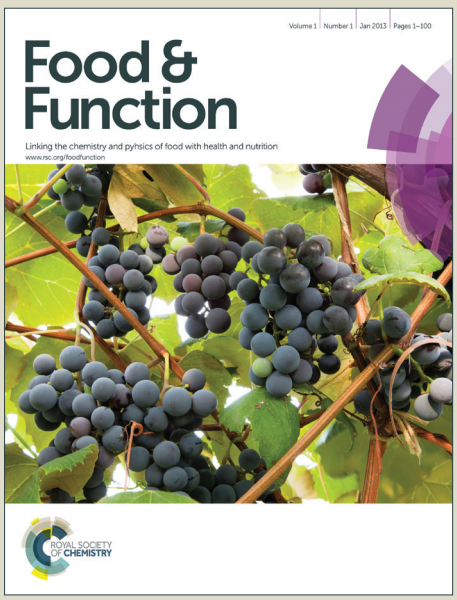

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ANTI-INFLAMMATORY ACTIVITY OF POLYPHENOLICS FROM AÇAI (*Euterpe oleracea* Martius) IN INTESTINAL MYOFIBROBLASTS CCD-18Co CELLS

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ABSTRACT

The demand for tropical fruits high in polyphenolics including açai (*Euterpe oleracea* Mart.) has been increasing based on ascribed health benefits and antioxidant properties. This study evaluated the anti-inflammatory activities of açai polyphenolics in human colon myofibroblastic CCD-18Co cells to investigate the suppression of reactive oxygen species (ROS), and mRNA and protein expression of inflammatory proteins. Non-cytotoxic concentrations of açai extract, 1-5 mg gallic acid equivalent/L, were selected. The generation of ROS was induced by lipopolysaccharide (LPS) and açai extract partially reversed this effect to 0.53-fold of the LPS-control. Açai extract (5 mg GAE·L^{-1}) down-regulated LPS-induced mRNA-expression of tumor necrosis factor alpha, TNF-α (to 0.42-fold), cyclooxygenase 2, COX-2 (to 0.61-fold), toll-like receptor-4, TLR-4 (to 0.52-fold), TNF receptor-associated factor 6, TRAF-6 (to 0.64-fold), nuclear factor kappa-B, NF-κB (to 0.76-fold), vascular cell adhesion molecule 1, VCAM-1 (to 0.71-fold) and intercellular adhesion molecule 1, ICAM-1 (to 0.68-fold). The protein levels of COX-2, TLR-4, p-NF-κB and ICAM-1 were induced by LPS and the açai extract partially reversed this effect in a dose-dependent manner. These results suggest the anti-inflammatory effect of açai polyphenolic extract in intestinal cells are at least in part mediated through the inhibition of ROS and the expression of TLR-4 and NF-κB. Results indicate the potential for açai polyphenolics in the prevention of intestinal inflammation.

Keywords: *açai, polyphenolics, intestinal, colon, inflammation.*

INTRODUCTION

The incidence of inflammatory bowel disease has been increasing in the U.S. and worldwide over the last two decades [1]. Chronic intestinal inflammation primarily implies a dysfunction of the intestinal mucosa, and may indicate a decreased function of the tight junction barriers as well as an overproduction of pro-inflammatory mediators that include cytokines, chemokines and adhesion molecules and are associated with a dysregulation of the immune system. Chronic intestinal inflammation may lead to several disorders involving inflammatory bowel diseases (IBDs; Crohn's disease and ulcerative colitis), food allergies, and autoimmune diseases, including celiac disease and type 1 diabetes that have been associated with increased cancer risk [2-4].

The molecular mechanism of inflammatory injury can be at least partially attributed to the generation and release of reactive oxygen species from activated neutrophils and macrophages. This over-production of ROS may cause tissue injury by damaging membrane structures through lipid peroxidation [5, 6]. In addition, ROS propagate inflammation by stimulating the release of cytokines that stimulate the recruitment of additional neutrophils and macrophages. Thus, free radicals are key-mediators that initiate or promote inflammation and consequently, their neutralization by dietary compounds may help to attenuate inflammation [7, 8].

There is increasing evidence that myofibroblasts influence the integrity of the intestinal mucosa through secretion of cytokines and chemokines, when stimulated with lipopolysaccharide (LPS) [9-11]. LPS is a major integral component of the outer membrane of gram-negative bacteria and is one of the most potent stimuli of inflammation, the first mechanism of defense by the host against infective agents [12]. LPS is recognized by the

Toll-like receptor (TLR)-4, a proinflammatory cell surface receptor that is expressed on cells of the innate immune system as well as epithelial cells. Upon activation, TLR-4 induces NF-κB[13], which in turn, transcriptionally upregulates the expression of many proinflammatory cytokines (TNF-α and IL-6), adhesion molecules (intracellular adhesion molecule - 1 (ICAM-1) and vascular cell adhesion molecule – 1 (VCAM-1), and also activates the expression of cyclo-oxygenase-2 (COX-2), an enzyme required in the synthesis of prostaglandin-2 (PGE-2) as proinflammatory mediator [14-17].

Açai (*Euterpe oleracea* Martius), a palm-fruit native to the Brazilian Amazon, is a rich source of polyphenols, particularly in anthocyanins, proanthocyanidins and other flavonoids [18]. Among the anthocyanins, the cyanidin-3-glucoside and the cyanidin-3-rutinoside are predominantly expressed in acai (*Euterpe oleracea* Martius) within a concentration-range of 947.0 to 1256.0 mg/kg of acai pulp, while isoorientin and orientin is predominantly expressed within the non-anthocyanin compounds at much lower concentrations of 34.8 to 53.1 mg/kg [19]. Açai has gained popularity in a variety of beverages and food preparations [20, 21] and its trade has increased internationally due to ascribed antioxidant [22-26], anti-cancer [27-29] and anti-inflammatory properties that may be helpful in the reduction of inflammatory chronic diseases [20, 30-33]. Additionally, some reports indicate the cytotoxic activities of acai polyphenols *in vitro* [34] and *in vivo* [35, 36] .

While several studies have demonstrated the anti-inflammatory activities of polyphenolics from different plant-based foods, limited information is available regarding the effects of açai polyphenolics on cellular signaling pathways involved in intestinal inflammation. Thus, the purpose of this study was to evaluate the effect of açai

polyphenolic extract on pro-inflammatory biomarkers and involved signaling pathways in LPS-stimulated CCD-18Co normal colon myofibroblast cells.

MATERIAL AND METHODS

Chemicals, antibodies, and reagents

The following reagents and materials were used in the described study: Folin-Ciocalteu reagent and 2'7'-dichlorofluorescein diacetate (DCFH-DA) (Fisher Scientific, Pittsburgh, PA); Lipopolysaccharide (LPS), dimethyl sulfoxide (DMSO) (Sigma, St Louis, MO); HPLC grade solvents, hydrogen peroxide (Across) (VWR International, Bristol, CT). Bradford reagent (BioRad, Hercules, CA); antibodies against NF-κB p65, phospho-NF-κB p65, ICAM-1, COX-2 (Cell Signaling Technology, Beverly, MA); antibody against β-actin (Sigma-Aldrich, St Louis, MO); antibodies for VCAM-1 and TLR-4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); Primers for real time-PCR (Integrated DNA Technologies, Inc., San Diego, CA); ELISA kit for ICAM-1 (Invitrogen, Grand Island, NY).

Polyphenols Extraction

Frozen, açai pulp (*Euterpe oleracea* Martius) was imported from Belém, PA, Brazil, which was processed from the edible pericarp of the fruit, with the inedible seed discarded. Açai pulp was clarified into single strength juice and concentrated 10-fold by Yakima Fruit Works, Inc (Moxee, WA) in a proprietary process. The concentrated was kindly provided to the Department of Nutrition and Food Science at Texas A&M

University. The açai juice concentrate was stored at -20°C upon arrival. Polyphenolics were concentrated using a C18 Sep-Pak Vac 20 cm3 column (Waters Corporation, Milford, MA) under vacuum using acidified (0.1% HCl) methanol and water. The methanol was evaporated in a rotavapor (Buchi Laborthechnik AG, Flawil, Switzerland) at <40°C, redissolved in water and dimethyl sulfoxide (DMSO) (60:40 v/v) and stored at -80 °C for further analyses and quality control. The extract was normalized to a maximum concentration of 0.1% DMSO in cell culture.

Chemical Analyses

Total soluble phenolics were quantified using the Folin-Ciocalteu assay according to a modified methodology described by Singleton and Rossi [37], quantified as gallic acid equivalents (GAE). The antioxidant capacity was determined with the oxygen radical absorbance capacity (ORAC) assay, modified from Ou, et al. [38], with fluorescein as fluorescent probe, 2,2-azobis(2-amidinopropane) dihydrochloride as generator of peroxyl radicals. Reduction of fluorescence was monitored using a FLUOstar Omega microplate reader (BMG Labtech Inc., Durhan, NC) at 485 nm excitation and 520 nm emission. Results were quantified in μmol of Trolox equivalents per mL of extract.

The anthocyanin profile of the acai juice concentrate was analyzed by revered phase HPLC using analytical methods previously described[29]. Identification and quantitation was based on their spectral characteristics and retention time, as compared to authentic standards (Sigma Chemical Co., St. Louis, MO).

Cell Culture

The non-cancer colon myofibroblast CCD-18Co cells were obtained from ATCC (Manassas, VA) and cultured using DMEM medium supplemented with 20% of fetal bovine serum, 1% sodium pyruvate (100mM), 1% non-essential amino acids (10mM) and 1% penicillin/streptomycin solution (Invitrogen, Carlsbad, CA). Cells were maintained at 37 ºC at 5% CO_2 atmosphere. Polyphenolics were diluted to a known concentration of total polyphenolics and normalized to contain a maximum concentration of 0.1% DMSO (water: DMSO, 60:40) in the culture medium that did not show any cytotoxic activities when compared to untreated control cells.

Cell Proliferation

Cells were seeded (2 x 10^4 onto a 24-well plate) and incubated for 24h to allow cell attachment. The number of cells from the pre-treatment wells (0-time) was quantified and the growth medium was replaced with 500 µL medium containing the açai polyphenolics (from 1 to 5 mg GAE·L^{-1}). The concentration range was chosen based on the acai concentration that had anti-inflammatory effect without reducing CCD-18 cell proliferation. Cytotoxic concentrations were not considered for this study, since non-cancer cells are studied. Following incubation for 48h, cell numbers were determined using an electronic particle counter (Z2™ Series, Beckman Coulter, Inc, Fullerton, CA). Net growth was calculated as the difference in number of cells between final incubation time (48h) and 0-time.

Generation of Reactive Oxygen Species (ROS)

CCD-18Co ($5x10^3$/mL) cells were seeded in a 96-well plate and incubated for 24h. Thereafter, CCD-18Co cells were pre-treated for 24h with different extract concentrations (1-10 mg GAE·L^{-1}) and stimulated with LPS (2 µg·mL^{-1}) for 4h after 48h incubation. After washing with phosphate buffer pH 7.0 (PBS) cells were incubated with 10 µM DCFH-DA for 30 min at 37°C. Fluorescence intensity was determined after 30 min using a fluorescent microplate reader (BMG Labtech Inc., Durham, NC) at 485 nm excitation and 520 nm emission. Following, Janus green staining was used to determine relative cell number in each well and results are expressed as relative ROS intensity/relative cell number as previously described[39].

mRNA Analysis by real-time PCR

Attached cells were pre-treated with varying concentrations of polyphenolics for 24h and stimulated with LPS (2 µg·mL^{-1}) for 4h before mRNA extraction and analysis. Total RNA was isolated according to the manufacturer's protocol using the mirVana™ extraction kit (Applied Biosystems, Foster City, CA) and samples were evaluated for nucleic acid quality and quantity using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Enriched mRNA was used to synthesize cDNA using a Reverse Transcription Kit (Invitrogen Corp., Grand Island, NY) according to the manufacturer's protocol. qRT-PCR was carried out with the SYBR Green PCR Master Mix from Applied Biosystems (Foster City, Ca) on an ABI Prism 7900 Sequence Detection System (Applied Biosystems Inc, Foster City, CA). Primers were designed using the Primer Express software (Applied Biosystems, Foster City, CA) (Table 1). Each primer was

homology-searched by NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Product specificity was examined by dissociation curve analysis.

Western-blotting and Enzyme-linked Immunosorbent Assay (ELISA)

Cells were seeded (2 x 10^5 cells/well) in 6-well plate and incubated for 24h to allow cell attachment. Cells were pre-treated with polyphenolics for 24h and subsequently stimulated with LPS (2 $\mu g \cdot mL^{-1}$). Cell lysates were obtained after 24 h and analyzed by Western blots. Cell culture supernatants were analyzed by ELISA assays. For Western blot analysis, cells were washed with PBS and lysated with RIPA (Radio-Immunoprecipitation Assay) buffer (1.0% Igepal CA-630 (NP-40), 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1 mM EDTA) and 1% proteinase inhibitor cocktail (Sigma,-Aldrich) for 30 min in ice. Solid cellular debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was collected and stored at -80°C. Protein content was determined using the Bradford reagent (Bio-Rad, Hercules, CA) following the manufacturer's protocol. For each lane 60 µg of protein was diluted with Laemmli's loading buffer, boiled for 5 min, loaded on an acrylamide gel (10%) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 100 V for 2h. Proteins were transferred by wet blotting onto 0.2 µm PVDF membrane (Bio-Rad, Hercules, CA). Membranes were blocked using 5% milk in 0.1% PBS-Tween (PBS-T) for 1h and incubated with primary antibodies (1:1000) in 3% bovine serum albumin in PBS-T overnight at 4°C with gentle shaking, followed by incubation with the secondary antibody (1:2000) in 5% milk PBS-T for 2h. Reactive bands were visualized with a luminal reagent (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) as previously performed [40].

The CCD-18Co supernatant was assessed by ELISA using an Invitrogen kit (Invitrogen Corp. Grand Island, NY) for ICAM-1 according to the manufacturer's protocol. The final content of ICAM-1 (ng/mg protein) was calculated after quantifying the protein concentration by Bradford. Data were normalized using untreated control cells.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 15.0 (SPSS Inc., Chicago, IL). Data represent mean values with their standard deviations (SD) or standard error of mean (SE) as indicated in the figure legends, corresponding to four or more replicates. Duncan pairwise comparisons were used for establishing statistically significant differences at the 5% level of probability.

RESULTS AND DISCUSSION

Total phenolics, anthocyanins and antioxidant capacity

The açai extract contained 30,000 mg GAE·L^{-1} of total phenolics and 6,000 mg·L^{-1} of total anthocyanins as determined with the Folin-Ciocalteu assay[37] and differential pH spectrophotometric method[41], respectively. HPLC analysis indicated the total anthocyanin content to be 2386.9 mg·L^{-1}, predominantly including cyanidin-rutinoside (1395.3 mg·L^{-1}) and cyanidin-3-O-glucoside (451.5 mg·L^{-1}) as reported in our previous study[42]. Similar results regarding the anthocyanins content in *Euterpe oleracea* M. fruits was also found by Pacheco-Palencia et al. (2009).

The antioxidant capacity of açai polyphenolic 10-fold concentrate was determined by the ORAC assay to be 788.16 µmol TE·mL^{-1}, and this value was comparable to the

amount found by Pacheco-Palencia, et al. [19] (87.4 ± 4.4 μmol TE·g^{-1}). This level of *in vitro* antioxidant activity is comparable to other fruits with similar polyphenolic composition (50−100 μmol TE·g^{-1} fresh weight) such as Chardonnay grapes, pink grapefruits, Bing cherries, raspberries, black plums, Elliot blueberries, and blackberries [43] attributable to their high concentrations of anthocyanins, flavonols and procyanidins.

Cell Proliferation and Generation of Reactive Oxygen Species (ROS)

The açai polyphenolic extract (1-5 mg GAE·L^{-1}) did not inhibit ($p>0.05$) net cell growth of human colon myofibroblast CCD-18Co cells after 48h of incubation within the selected concentration range (Figure 1). Furthermore, the açai polyphenolic extract (1-5 mg GAE·L^{-1}) had a protective effect on the production of ROS in CCD-18 cells with and without LPS-challenge (Figure 2). LPS (2 μg·mL^{-1}) challenge induced ROS up to 1.2-fold and the açai polyphenolic extract at 5 mg GAE·L^{-1} prevented this effect and down-regulated ROS levels to 0.84-fold compared to LPS-challenged cells ($p<0.05$) (Figure 2B).

The reduction of ROS by açai polyphenolics in CCD-18Co cells are in concordance with previous research performed by this research group. Açai polyphenolics (5-20 mg GAE·L^{-1}) decreased high glucose-induced ROS generation to 0.40-fold compared to untreated controls cells in HUVEC cells [31]; red wine polyphenolics (25-100 mg GAE·L^{-1}) reduced the LPS-induced generation of ROS to 0.58-fold compared to untreated controls in CCD-18 cells [44]; black and red cowpea varieties (2-20 mg GAE/L) significantly inhibited the generation of ROS stimulated by LPS in CCD-18Co cells [45]. Flavonol-rich fractions of yaupon holly leaves (*Ilex vomitoria*, Aquifoliaceae) protected CCD-18Co cells against reactive oxidative species (ROS) [46]. The reduction of ROS by açai polyphenolics was also

observed by Guerra, et al. [47] *in vivo*, where the addition of açai pulp to the diet for non-diabetic animals caused an approximately 2.6-fold reduction of ROS production compared to the control group. Thus, dietary polyphenols from açai, can play an important role in the improvement of antioxidant status as they are able to reduce the generation of ROS in intestinal cells. This antioxidant effect may be important since overproduction of ROS may result in oxidative stress and this can cause significant damage to cellular proteins, lipids and DNA [48]. ROS may induce NF-κB activity by oxidative modification of its cysteine residues, IκB degradation and oxidative enhancement of upstream signal cascades [49]. Moreover, *in vitro* and *in vivo* studies have previously demonstrated that ROS and other reactive free radicals are involved in inflammatory responses [50, 51], that may trigger other chronic diseases.

TLR-4, TRAF-6, TNF-α and COX-2 pro-inflammatory cytokines

The Toll-like Receptor-4 (TLR-4) pathway has been reported to be activated within innate immune responses of intestinal myofibroblasts [52]. TLR-4, when activated by LPS, stimulates signals transmitted through the recruitment of more than a dozen different signaling proteins, including TRAF-6, an intracellular distal signaling mediator to lipopolysaccharide (LPS)/toll-like receptor (TLR)-4 signaling, that plays a role in cytokine production and inflammation through activating transcription factors, including nuclear factor-κB (NF-κB) [13, 53]. NF-κB also mediates the synthesis of the cytokine TNF-α and the expression of cyclooxygenase 2 (COX-2)[54].

In this study, açai polyphenolics reduced the expression of TLR-4 within a concentration range of 1-10 mg GAE·L^{-1}. mRNAs expression of TLR-4 (Toll-like receptor 4) and TRAF-6 (TNF receptor-associated factor 6) was induced by LPS to 1.5-fold and 1.3-fold compared to the untreated control, respectively. The açai polyphenolic extract reduced the expression of TLR-4 and TRAF-6 to 0.52-fold and 0.64-fold, respectively, at 5 mg GAE·L^{-1} in cells stimulated by LPS (Figure 3A).

Additionally, our findings also revealed that LPS-induced protein expression of COX-2 (cyclooxygenase-2) was down-regulated by treatment with the açai polyphenolic extract at concentrations of 1-10 mg GAE·L^{-1}. LPS induced the gene expression of TNF-α (tumor necrosis factor alpha) and COX-2 after 4h to 2.7-fold and 3.7-fold, respectively, compared to untreated control cells. The açai extract partially reversed this effect and reduced the expression of TNF-α and COX-2 in LPS-stimulated cells to 0.47-fold and 0.61-fold, respectively at 5 mg GAE·L^{-1} (Figure 3B). Thus, the down-regulation of these pro-inflammatory biomarkers by açai polyphenolic extract possibly may reduce the risk of intestinal inflammation.

The down-regulation of biomarkers such as TLR-4, TRAF-6, TNF-α and COX-2 by açai polyphenolics is central to the reduction of inflammation. TNF-α is produced during an initial inflammatory response; it initiates and propagates the production of cytokines, chemokines, and endothelial adhesion molecules [55]. COX-2 is one of the inducible enzymes in excessive inflammatory responses that can regulate the production of prostaglandins [56]. COX-2 is frequently overexpressed in colonic adenoma and carcinoma [57].

Similarly, previous reports demonstrated the anti-inflammatory effects of different polyphenols involving these inflammatory biomarkers. Noratto, et al. [46] showed that flavonol-rich fractions of yaupon holly leaves (*Ilex vomitoria*, Aquifoliaceae) (10-40 mg GAE·L^{-1}) down-regulated the gene expression of NF-κB, TLR-4, TRAF-6 and COX-2 in CCD-18Co cells. Cianciulli, et al. [12] showed that resveratrol (10-50 μM) concentration-dependently inhibited the expression of COX-2 mRNA and inhibited the translocation of NF-κB p65 subunits from the cytosol to the nucleus in the LPS-treated Caco-2 human intestinal cells. Xie, et al. [30] showed that velutin, a flavone isolated from the pulp of açai fruit (*Euterpe oleracea* Mart.) (2.5-5 μM) inhibited the expression of proinflammatory cytokines TNF-α and IL-6 by inhibiting NF-κB activation in RAW 264.7 peripheral macrophages.

NF-κB and Adhesion Molecules

NF-κB is a complex transcription factor that controls the expression of several proteins important for cellular adhesion and also the expression of many genes involved in immunoregulation, growth regulation, inflammation and cell survival [58, 59].

Results from this study show that the mRNA of nuclear factor-κB (NF-κB) was up-regulated by LPS up to 1.34-fold compared to the untreated control and açai polyphenolics prevented this effect maintaining NF-κB mRNA levels down to 0.76-fold at 5 mg GAE·L^{-1}. The mRNAs of VCAM-1 and ICAM-1 adhesion molecules were up-regulated by LPS to 2.6-fold and 2.5-fold of the untreated control, respectively. The açai polyphenolic extract at 5 mg GAE·L^{-1} prevented this effect and down-regulated the expression of VCAM-1 and

ICAM-1 in LPS-stimulated cells to 0.71-fold and 0.68-fold, respectively (Figure 4A). The protein expression of ICAM-1 and p-NF-κB was also reduced by açai polyphenolics (Figure 4B). Overall, açai polyphenolics significantly reduced the expression of different target genes under LPS stimulation.

These results are in agreement with previous studies from our laboratory that showed that açai polyphenolics inhibited LPS-induced inflammation in human vascular endothelial cells (HUVEC), by inhibition of adhesion molecules and NF-κB activation at gene and protein expression levels [31]. Moreover, red wine polyphenolics decreased the expression of mRNA of LPS-induced mediators of inflammation including NF-κB, ICAM-1, VCAM-1 in CCD-18Co cells [44]. Furthermore, other studies demonstrated the effects of different flavonoids in the reduction of VCAM-1 and ICAM-1 stimulated by LPS [60] and by ox-LDL [61] in HUVEC cells. Kim, et al. [62] also showed the inhibition of ICAM-1 stimulated by high glucose by flavonoids in human vein endothelial cells. In addition, Yi, et al. [63] demonstrated that different flavonoids, including anthocyanins, flavonols, flavones and isoflavones also inhibited NF-κB activation as well as the expression of VCAM-1 and ICAM-1 induced by ox-LDL in human vascular endothelial cell line EA.hy926. Active NF-κB participates in the control of transcription of over 150 target genes, including the expression of various inflammatory cytokines, chemokines, immunoreceptors, and cell adhesion molecules [64]. Thus, the down-regulation of NF-κB gene expression by açai extract also decreases the expression of the adhesion molecules VCAM-1 and ICAM-1, and consequently leukocyte adhesion and transmigration may be reduced [65].

CONCLUSION

In summary, polyphenols from açai reduced basal levels of ROS and prevented LPS-induced generation of ROS in colon myofibroblasts within a concentration-range that did not show any cytotoxicity for these non-cancer cells. Moreover, the açai extract down-regulated the expression of pro-inflammatory genes and proteins in LPS-stimulated CCD-18Co cells, that are involved in inflammation of the gastrointestinal tract. These results suggest that the anti-inflammatory activities of açai polyphenolics in intestinal cells involve the inhibition of toll-like receptor-4 (TLR-4) and nuclear factor kappa-B (NF-κB). Future *in vivo* studies should be performed in order to verify the potential of polyphenols from açai as candidates for dietary intervention in the prevention of intestinal inflammation and associated diseases.

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AUTHORS CONTRIBUTION

All authors participated in the design, interpretation of the studies and statistical analysis of the data and writing and final review of the manuscript. MD, HM and AR conducted the experiments.

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Tab.1 Human Primers Sequences

Gene	Primers Sequences	
	Forward	**Reverse**
GAPDH	5'-CCTCCCGCTTCGCTCTCT -3'	5 ' -TGGCGACGCAAAAGAAGA -3'
NF-κB	5'-TGGGAATGGTGAGGTCACTCT-3'	5'- TCCTGAACTCCAGCACTCTCTTC-3'
ICAM-1	5'-TGGCCCTCCATAGACATGTGT-3'	5'- TGGCATCCGTCAGGAAGTG-3'
VCAM-1	5'-ACAGAAGAAGTGGCCCTCCAT-3'	5'-TGGCATCCGTCAGGAAGTG-3'
TNF-α	5'- TGTGTGGCTGCAGGAAGAAC-3'	5'- GCAATTGAAGCACTGGAAAAGG-3'
TLR-4	5'- TGGTGTCCCAGCACTTCATC-3'	5'- GCCAGGTCTGAGCAATCTCATA-3'
TRAF-6	5′-AGAGTTTGCCGTCCAAGCA-3′	5′-TGGTAGAGGACGGACACAGACA-3′
COX-2	5′-AGGGTTGCTGGTGGTAGGAA-3′	5′-GGTCAATGGAAGCCTGTGATACT-3′

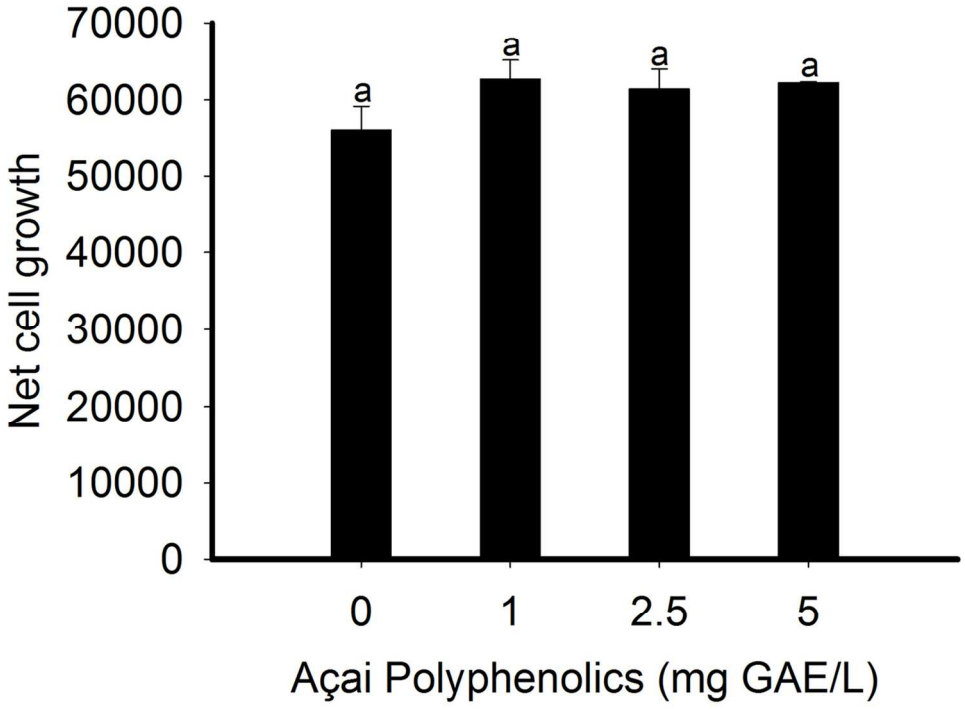

Fig.1 Açai polyphenolic extract (1-5 mg GAE·L^{-1}) do not inhibit ($p>0.05$) CCD-18Co net growth.
127x97mm (300 x 300 DPI)

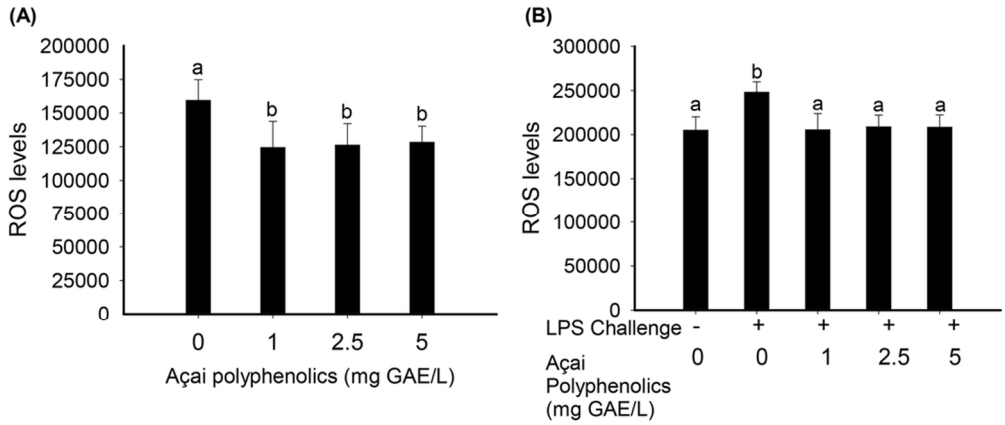

Fig.2 Açai polyphenolics inhibit reactive oxygen species (ROS) generation in CCD-18Co colon myofibroblasts cells (A) without and (B) with LPS challenge. Values are mean ± SE (n=6). Different letters indicate a significant difference, by Duncan Test, compared to the DMSO-treated control ($p \leq 0.05$).
97x40mm (300 x 300 DPI)

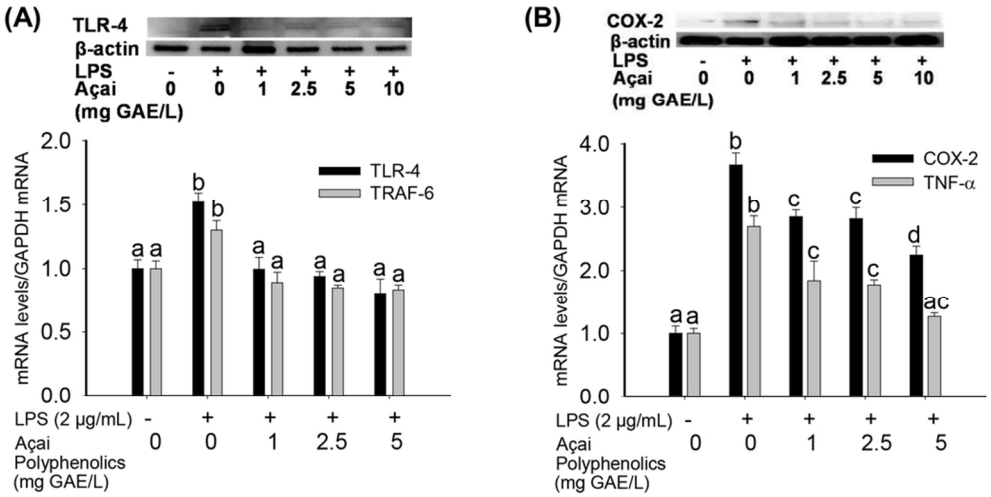

Fig.3 Effects of açai polyphenolics on mRNA and protein expression of TLR-4, TRAF-6, TNF-α and COX-2 pro-inflammatory cytokines induced with LPS in CCD-18Co cells (A, B). Cells were treated with DMSO (control vehicle) or with different concentrations of açai polyphenolic extract dissolved in DMSO (1–10 mg GAE·L^{-1}) for 24h and were stimulated with LPS for 4 hours. Values are mean ± SE (n≥3). Different letters indicate a significant difference, by Duncan test, compared to the untreated control ($p \leq 0.05$). Data of real time RT-PCR were analyzed as a ratio to the mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
89x43mm (300 x 300 DPI)

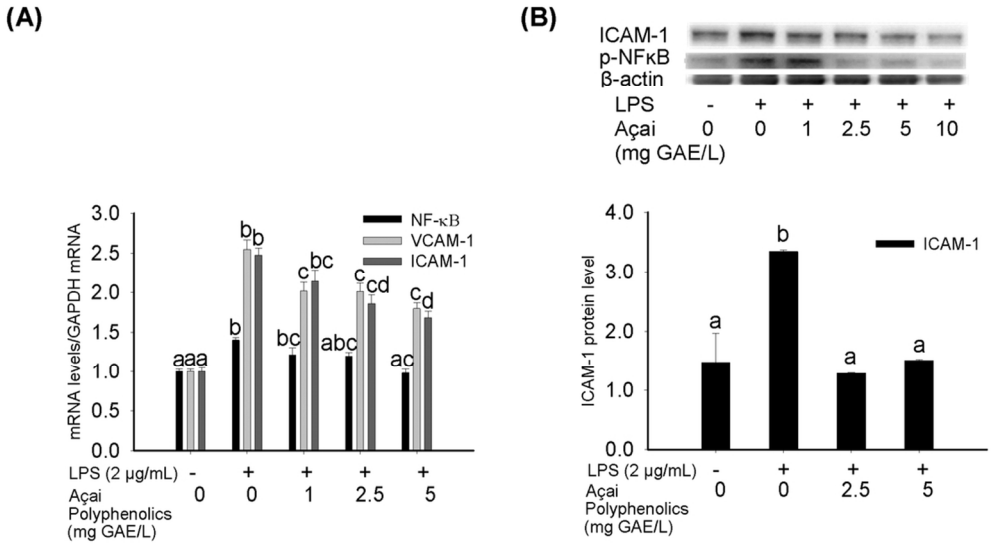

Fig.4 Effects of açai polyphenolics on mRNA (A) and protein (B) expression of NF-κB, p-NF-κB, ICAM-1 and VCAM-1 adhesion molecules induced with LPS in CCD-18Co cells. (B) Protein expression using ELISA kit and Western Blot technique. Cells were treated with DMSO (control vehicle) or with different concentrations of açai polyphenolic extract dissolved in DMSO (1–10 mg GAE·L-1) for 24h and were stimulated with LPS for 4 hours. Values are mean ± SE (n≥3). Different letters indicate a significant difference, by Duncan test, compared to the untreated control (p≤0.05). Data of real time RT-PCR were analyzed as a ratio to the mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
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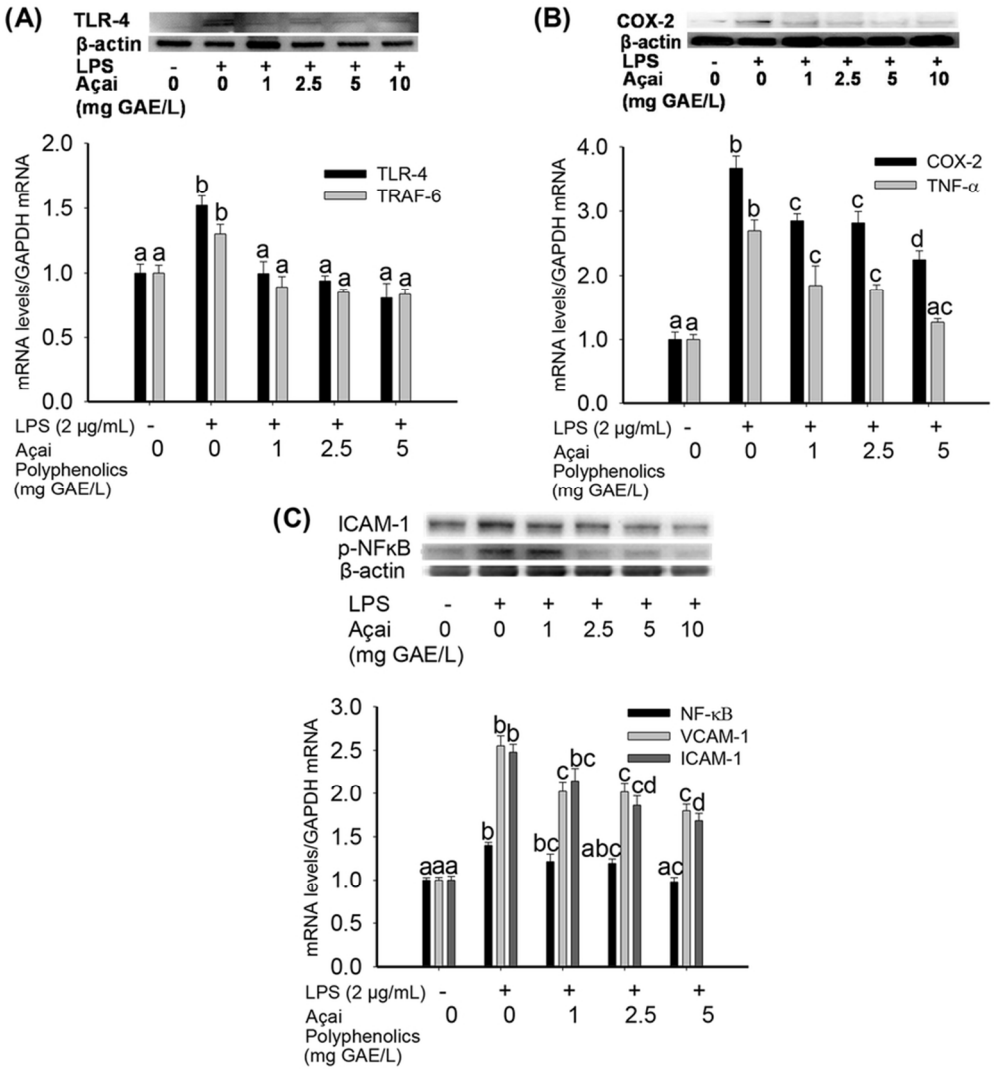

Açai polyphenolics reduce mRNA and protein expression of pro-inflammatory cytokines induced with LPS in CCD-18Co cells.
80x87mm (300 x 300 DPI)