Açaí (Euterpe oleracea Mart.) Modulates Oxidative Stress Resistance in Caenorhabditis elegans by Direct and Indirect Mechanisms

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Abstract

Açaí (Euterpe oleracea Mart.) has recently emerged as a promising source of natural antioxidants. Despite its claimed pharmacological and nutraceutical value, studies regarding the effects of açaí in vivo are limited. In this study, we use the Caenorhabditis elegans model to evaluate the in vivo antioxidant properties of açaí on an organismal level and to examine its mechanism of action. Supplementation with açaí aqueous extract (AAE) increased both oxidative and osmotic stress resistance independently of any effect on reproduction and development. AAE suppressed bacterial growth, but this antimicrobial property did not influence stress resistance. AAE-increased stress resistance was correlated with reduced ROS production, the prevention of sulfhydryl (SH) level reduction and antimicrobial property did not influence stress resistance. AAE increased polyglutamine protein aggregation and decreased proteasome activity. Our findings suggest that natural compounds available in AAE can improve the antioxidant status of a whole organism under certain conditions by direct and indirect mechanisms.

Introduction

Açaí (Euterpe oleracea Mart.) is an exotic fruit originally native to Central and South America that grows in the floodplains of the Amazon region [1]. Traditionally, açaí is used as a medicinal plant and as a staple food in many parts of Brazil. In recent years, açaí pulp has gained international attention as a functional food due to its nutritional benefits and therapeutic promise. Composition analysis shows that açaí pulp contains approximately 13% protein, 48% lipids, 1.5% total sugar and several other nutrients such as lignans, dietary fiber and polyphenols [2]. The main polyphenols found in açaí are anthocyanins, proanthocyanidins (specifically cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside) and other flavonoids [2,3]. The overall pharmacological properties of açaí are related to its antiproliferative, anti-inflammatory, antioxidant and cardioprotective effects [4].

Açaí pulp and its polyphenolic fractions show high antioxidant activity based on various in vitro assays, predominantly against DPPH, superoxide and hydroxyl radicals and hypochlorous acid [5–11]. In vitro cell-based assays have also demonstrated that the pulp can reduce ROS production in human erythrocytes and polymorphonuclear (PMN) cells exposed to oxidative stress [8,9]. Brain tissue cells pretreated with açaí decreased H2O2-induced damage to lipids and proteins and reduced the activities of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) to basal levels [12].

Despite its claimed pharmacological and nutraceutical value, studies regarding the effects of açaí in vivo are limited [4]. Dietary açaí in rats reduces ROS production by neutrophils, increases GSH levels, reduces protein damage and activates the gene expression of glutathione peroxidase (GPx) and γ-glutamylcysteine synthetase (γ-GCS) in the liver [13]. In humans, intervention studies of volunteers ingesting an açaí juice blend showed a
significant increase in serum antioxidant capacity and a reduction of lipid peroxidation [6,14]. In another study, açaí puree improved select markers of metabolic disease risk in overweight adults [15]. The high superoxide and peroxyl radical scavenging capacity of açaí pulp suggests that açaí has anti-aging properties. In *Drosophila melanogaster*, dietary açaí supplementation increased the lifespan of female flies fed a high fat diet and also under oxidative stress induced by *sod1* RNAi [16]. In another fruit fly, *Anastrepha ludens*, açaí supplementation promoted survival in flies on diets with high fat and high sugar while decreasing lifetime reproductive output [17].

In addition to flies, genetic and pharmacological modifications of stress resistance and lifespan mechanisms have been well elucidated in the nematode *Caenorhabditis elegans*. A number of genes and pathways have been identified to modulate lifespan in *C. elegans*. These highly conserved pathways include the insulin/IGF-1 receptor-like signaling pathway, which depends on the transcription factor DAF-16/FOXO. Reduced insulin/IGF-1 signaling activates DAF-16 nuclear localization, which in turn induces the expression of genes that increase lifespan and promote resistance to various stresses [18]. SKN-1 is another transcription factor regulated by the insulin/IGF-1 signaling pathway [19]. SKN-1 contributes to the increased stress tolerance and longevity resulting from reduced insulin/IGF-1 signaling independently of DAF-16 [19]. Using this model, several studies have demonstrated the beneficial effects of natural products in promoting stress resistance and longevity, including extracts from *Ginkgo biloba* [20], blueberry [21], *Cinnamomum cassia* bark [22] and cranberry [23].

In the present study, we use the *C. elegans* model to evaluate the *in vivo* antioxidant properties of açaí on an organismal level and to unveil its mechanism of action. Our results indicate that açaí has anti-aging properties. In *Drosophila melanogaster*, dietary açaí supplementation increased the lifespan of female flies fed a high fat diet and also under oxidative stress induced by *sod1* RNAi [16]. In another fruit fly, *Anastrepha ludens*, açaí supplementation promoted survival in flies on diets with high fat and high sugar while decreasing lifetime reproductive output [17]. The high superoxide and peroxyl radical scavenging capacity of açaí pulp suggests that açaí has anti-aging properties. In *Drosophila melanogaster*, dietary açaí supplementation increased the lifespan of female flies fed a high fat diet and also under oxidative stress induced by *sod1* RNAi [16]. In another fruit fly, *Anastrepha ludens*, açaí supplementation promoted survival in flies on diets with high fat and high sugar while decreasing lifetime reproductive output [17].

**Materials and Methods**

**Chemicals and Reagents**

M199, Fungizone, penicillin, streptomycin and L-glutamine were purchased from Gibco by Life Technologies (Saint Aubin, France). Methanol, HPLC-grade acetonitrile, HPLC-grade water, formic acid, DPPH (2,2-diphenyl-1-picryl-hydrazyl), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and cyanidin 3-O-glucoside were purchased from Aldrich (St. Louis, MO, USA). Cyanidin 3-O-glucoside was obtained from Litiécnica Alimentos L.T.D.A (San José, PR, Brazil). The total anthocyanin content of açaí powder. A molar absorptivity of 26,900 M⁻¹ cm⁻¹ and a molecular mass of 449.2 g/mol were used for cyanidin-3-glucoside.

**Caenorhabditis elegans Strains and Maintenance**

The following strains were used in this study: Bristol N2 (wild-type; WT); LD1171, I003 (ges-1;GFP); CL2166, dvl-1;[pAF15:gsc-1::GFP::NL5]; CF1553, mubs84[pAD76; cod-3::GFP]; VP198, kbs5 [gck-1::GFP + rol-6(sa1006)]; TJ 356, zls356[pGP30;daf-16::GFP::pRF4::rol-6]; BA17, fem-1(ha17); EU1, skh-1([af67] IV/nT1; CF1036, dys-16(mab60); AU3, ncy-1(2g3); KU4, sek-1(km4); VC6, jk-l(2g3); AM1, aos-1(2m1); and MT2805, unc-43(a498n1186). All *C. elegans* strains were maintained at 20°C on solid nematode growth medium (NGM) seeded with *E. coli* (OP50) as a food source according to Brenner [24].

**Human Umbilical Vein Endothelial Cells (HUVECs) Culture**

HUVECs isolated previously by Boulanger et al. [25] were collected from the veins of umbilical cords obtained from Jeanne de Flandres Maternity (Lille University Hospital, France). Cells were cultured in M199 (2.5 μg/mL Fungizone, 100 μg/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 150 mM Hepes) supplemented with 20% FBS. HUVECs were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. HUVECs were treated with 10 mM Kanamycin (KAN).

**Anthocyanin Quantification**

Total anthocyanin was quantified by the pH differential method as described by Giusti et al. [26]. Diluted samples were added to 0.025 M chloride buffer (pH 1.0) and 4.0 M sodium acetate buffer (pH 4.5). After incubation in the dark for 30 min at room temperature, absorbances were determined simultaneously as absorption maxima for the visible light spectrum and at 700 nm (SP-220, Biospectro, PR, Brazil). The total anthocyanin content was expressed in mg of cyanidin-3-glucoside equivalent per 100 g of açaí powder. A molar absorptivity of 26,900 M⁻¹ cm⁻¹ and a molecular mass of 449.2 g/mol were used for cyanidin-3-glucoside.

The quantification of cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside was performed in duplicate using a modified method from Gordon et al. [27]. Briefly, 0.25 g of sample was diluted in 3 mL of methanol/water/acetic acid (50:49.5:0.5, v/v/v). After shaking for 5 min, the sample was sonicated for 20 sec and centrifuged at 8,000 rpm for 10 min at 10°C. The supernatant was collected and the pellet was re-extracted twice. The three supernatants were pooled, transferred into a 10 mL volumetric flask and diluted with methanol/water/acetic acid mix. After filtration through a 0.45 μm filter, each sample was analyzed by LC-MS/MS. Mass spectrometry analyses were carried out on a Thermo Scientific TSQ Quantum Discovery MAX triple-stage quadrupole mass spectrometer (Thermo Fisher Scientific, Courtaboeuf, France) with an electrospray ionization (ESI) probe coupled to an Accela HPLC system (Thermo Fisher Scientific, Courtaboeuf, France). The analytical separation was performed on a Symmetry Shield RP_18 column, 150×2.1 mm, 3.5 μm (Waters, Guyancourt, France), at 20°C. The mobile phase was composed of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B) and was eluted at a flow rate of 0.2 mL/min. The HPLC gradient started with 5% B for 20 min, reaching 30% B at 40 min and 95% B at 45 min. Mass spectra for the determination of anthocyanins were obtained by using positive ionization mode.
Monitoring (SRM) mode. The specific transitions m/z 449.0 → m/z 287.1 and m/z 595.0 → m/z 287.1 were used for the detection and quantification of cyanidin 3-O-gluco side and cyanidin 3-O-rutinoside, respectively. As no stable isotopically labeled internal standard for anthocyanins is commercially available, the quantification was performed using the standard addition method and expressed as mg/100 g of dry matter.

**DPPH Radical Scavenging Assay**

The DPPH Radical Scavenging Activity of AAE was determined as described by Brand-Williams et al. [28]. In short, 100 μL of açai extract (1, 10 and 100 mg/mL) was added to 3.9 mL of methanol (Trolox) in the concentration range 200–800 μM DPPH dissolved in 80% methanol. The mixture was homogenized and kept in the dark for 30 min at room temperature, after which the absorbance at 515 nm was determined (SP-220, Biospectro, PR, Brazil). A calibration curve was prepared using 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) in the concentration range 200–800 μM. The percentage of inhibition was determined according to the following equation:

\[
\% \text{ Scavenging activity} = \left(1 - \frac{\text{Abs Sample 515}}{\text{Abs Control 515}}\right) \times 100
\]

**Body Length and Brood Size Assays**

To measure body length, first-larval-stage animals (L1) were treated with control solution (S basal) or 100 mg/mL AAE until the third larval stage (L3) and then transferred to NGM plates with *E. coli* OP50 until the next day. Images were captured (Axio Imager Z2, Zeiss, NY, USA) of one-day-old animals, and body length was measured along the animal axis using NIH Image J software.

To determine total progeny production, ten fourth-larval-stage (L4) worms, previously treated with control solution (S Basal) or 100 mg/mL AAE since L1, were placed onto the *E. coli* OP50 lawn on individual NGM plates in the presence or absence of 100 mg/mL AAE. During the egg-laying period, nematodes were transferred onto new plates every 24 h for five days until the end of the reproductive period. The F1 progeny from each individual worm was counted after approximately two days. The total progeny numbers for each plate were calculated and divided by the number of animals [29]. Both experiments were conducted three times.

**Longevity**

The longevity assay was performed with the adult sterile strain *unc-43(n498n1186)*, treated with or without 2.5 mg/mL AAE for 16 h, followed by incubation for 30 min with 10 μM H2DCFDA. Cells were washed twice and incubated in 0.5 mM PBS containing 30 mM H2DCFDA for 1 h. Thirty animals in experimental triplicates of each group were then transferred into the wells of a 96-well microtiter plate containing 200 μL PBS. The fluorescence quantification was carried out on a multi-label microplate Reader VICTOR X3 (Perkin Elmer, Massachusetts, USA) using excitation at 485 nm and emission at 535 nm.

**Bacterial Growth Curve**

*E. coli* OP50 growth was evaluated over 4 h in the presence of 100 mg/mL AAE. All OD readings at 600 nm were normalized to the OD of the control group at time zero. Bacterial growth was measured in three individual experiments.

**In vivo Measurement of ROS**

ROS production was measured in *C. elegans* and HUVECs using the fluorescent probe 2′, 7′-dichlorofluorescein diacetate (H2DCFDA). ROS production in *C. elegans* was performed as described by Shi et al. [31]. Synchronized L1 animals were treated with control solution (S basal) or 100 mg/mL AAE for 48 h. L4 worms were incubated in 1 mL PBS containing 1 mM H2O2 for 2 h. Subsequently, the worms were washed twice and incubated in 0.5 mM PBS containing 30 mM H2DCFDA for 1 h. Thirty animals in experimental triplicates of each group were then transferred into the wells of a 96-well microtiter plate containing 200 μL PBS. The fluorescence quantification was determined using a flow cytometer with excitation at 488 nm and emission at 525 nm. In both *C. elegans* and HUVECs, the fluorescence of the control group was used to normalize the values from all other groups. The experiments were repeated at least four times.

**Stress Resistance Assays**

To evaluate oxidative stress resistance, N2 wild-type animals and *skn-1(zu67), daf-16(mu86), ayu-1(ag3), sek-1(km4), juk-1(gk7), osr-1(tm174)* and *unc-43(n498n1186)* mutants were treated with control solution (S basal) or 100 mg/mL AAE from L1 until L4 and then with 7.5 mM tert-buty hydrogen peroxide (t-BOOH) in M9. To perform the oxidative stress resistance assay in dead bacteria, NGM plates seeded with *E. coli* OP50, with or without 100 mg/mL AAE, were treated with 10 mM KAN. Survival was measured at 3, 6, 9 and 12 h. We analyzed five wells, each with approximately ten worms, for each experimental group. Worms were prodded with a platinum wire and scored as dead if they displayed no pharyngeal pumping or movement [30].

Thermotolerance assays were performed with hermaphrodites on adult day 5, after the majority of egg-laying had ceased. Animals treated at 20°C with control solution (S basal) or 100 mg/mL AAE from L1 until adult day 5 were transferred onto 3-cm NGM agar plates supplemented as indicated above and then incubated at 35°C for 12 h. Survival was monitored at 6, 9 and 12 h and scored as the number of animals responsive to gentle touch as a fraction of the original number of animals on the plate. Animals that had died from desiccation on the sides of the plate were excluded [21]. To quantify the percent of motile worms under acute osmotic stress, animals were treated with control solution (S basal) or 100 mg/mL AAE for 68 h from L1 and then transferred to new plates containing 500 mM NaCl. The percentage of worms that moved outside a 7-mm circle was monitored at 15, 30 and 60 min. All experiments measuring oxidative stress resistance were conducted at least twice. The heat stress and motility under acute osmotic stress assays were each performed three times.
between the total and free sulfhydryl groups. The experiment was performed as described by Sedlak and Lindsay [34]. The total and free serum sulfhydryl groups were estimated using Ellman’s reagent.

Analysis of gene expression by qPCR

Transgenic worms containing reporter genes were treated with control solution (S basal) or 100 mg/mL AAE for 48 h at L1, followed by the presence or absence of oxidative stress. The total and free serum sulfhydryl groups were determined as the difference between the total and free sulfhydryl groups. The experiment was repeated twice.

Table 1. Determination of anthocyanins present in açaí aqueous extract (AAE).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (mg/100 g)</th>
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<tbody>
<tr>
<td>Total monomeric anthocyanins</td>
<td>31.0±2.4</td>
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<tr>
<td>Cyanidin 3-O glucosideb</td>
<td>8.8±0.9</td>
</tr>
<tr>
<td>Cyanidin 3-O rutinosideb</td>
<td>8.7±0.6</td>
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The results are expressed as the mean ± SEM (standard error of the mean).

Table 2. In vitro antioxidant activity of açaí aqueous extract (AAE) measured by DPPH assay.

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Table 2. In vitro antioxidant activity of açaí aqueous extract (AAE) measured by DPPH assay.

<table>
<thead>
<tr>
<th>% inhibition (Mean ± SEM)</th>
<th>AAE (mg/mL)</th>
<th>AAE + OP50</th>
<th>AAE + OP50 + KAN</th>
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</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.07±0.75*</td>
<td>4.38±0.98*</td>
<td>−0.71±0.28b</td>
<td>0.10±0.16b</td>
</tr>
<tr>
<td>10</td>
<td>53.30±5.61a</td>
<td>49.53±4.07a</td>
<td>18.55±2.18b</td>
<td>25.68±2.22b</td>
</tr>
<tr>
<td>100</td>
<td>79.61±3.33a</td>
<td>77.30±3.49a</td>
<td>73.46±4.54*</td>
<td>78.35±1.78a</td>
</tr>
</tbody>
</table>

DPPH, 2,2-diphenyl-1-picrylhydrazyl; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; OP50, E. coli strain, KAN, Kanamycin.

Polyglutamine (PolyQ) Aggregation Quantification

Transgenic worms carrying the reporter gene vha-6::Q44::YFP were treated with control solution (S basal) or 100 mg/mL AAE since L1. Photographs of one-, four-, eight- and twelve-day-old animals were taken with a fluorescence microscope (Axio Imager Z2, Zeiss, NY, USA), and the numbers of aggregates were counted. The experiment was repeated three times.

Proteasome Activity Quantification

In vitro 26S proteasome activity assays were performed as described by Kisselev and Goldberg [35]. Approximately 5,000 N2 wild-type animals were treated with control solution (S basal) or 100 mg/mL AAE for 48 h at L1. L4 worms were then harvested and sonicated. The lysates were centrifuged at 20,000 x g for 20 min at 4°C. Protein extract was quantified using the QuantiPro BCA Assay Kit (Sigma Aldrich, St. Louis, MO, USA). The proteasome activity was calculated as the difference between the total activity and the activity remaining in the presence of 20 μM MG-132.

Data Analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (San Diego, CA). The results were plotted as the mean ± SEM (standard error of the mean) of at least two individual experiments. Data were subjected to the Kolmogorov-Smirnov test for normality. For data with a normal distribution, Student’s t test was used to compare pairs of groups, whereas a one-way ANOVA followed by Tukey’s post-test was used to compare three or more groups. Nonparametric data were analyzed using the Mann-Whitney test when comparing two groups and the Kruskal-Wallis test followed by Dunn’s post-test for comparing three or more groups. All survival curves were plotted as the mean ± SEM. The statistical significance was determined as p<0.05.
Results

AAE phytochemical composition and antioxidant capacity in vitro and in vivo

We first characterized the composition of anthocyanins and the in vitro antioxidant capacity of our extract. AAE presents 31.0±2.4 mg/100 g of the total anthocyanins measured by the pH differential method. Cyanidin 3-O glucoside and cyanidin 3-O rutinoside are the two major anthocyanins in açai pulp; AAE presents 8.8±0.9 mg/100 g of cyanidin 3-O glucoside and 8.7±0.9 mg/100 g of cyanidin 3-O rutinoside, as determined by LC-MS/MS analysis (Table 1). Açai’s polyphenolic compounds have already been associated with its in vitro antioxidant capacity [3,36,37]. We next determined the antioxidant capacity of 1, 10 and 100 mg/mL AAE by the DPPH radical scavenging activity method. We observed that AAE displays increasing in vitro antioxidant capacity in a dose-dependent manner (Table 2). At 100 mg/mL AAE, the highest concentration tested, the DPPH inhibition was 79.61%, which is equivalent to 800 μM Trolox, the reference standard, which shows 80.90% inhibition. Moreover, the percentage of DPPH inhibition for any concentration tested was not significantly altered when the extracts were mixed with E. coli OP50. Thus, we used AAE at 100 mg/mL with live E. coli OP50 for the subsequent experiments.

Figure 1. Effect of açai aqueous extract (AAE) on wild-type C. elegans body length and progeny. A). L1 animals were treated with control solution (S basal) or 100 mg/mL AAE until L3 and then transferred onto NGM plates with OP50 until the next day. Images were captured of one-day-old animals, and body length was measured along the animal axis using NIH Image J software. There was no significant difference between groups, as determined by two-tailed Student’s t-test. B, C) Progeny profiles were measured in animals treated with control solution (S basal) or 100 mg/mL AAE. Animals were transferred individually to NGM plates and moved daily until the end of the reproductive period. The results were plotted as the mean ± SEM for each day (B) and total final progeny (C). There was no significant difference between groups by a two-tailed Student’s t-test.

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AAE does not alter C. elegans development and progeny

As toxic compounds usually delay C. elegans development and progeny [38], we characterized the effect of AAE treatment on these two biological parameters. Body length was determined in one-day-old adults treated with or without 100 mg/mL AAE from L1 until L3 and then transferred to NGM plates with E. coli OP50. We did not observe a significant difference in body length between control (1020.0 ± 11.6 μm) and AAE-treated animals (994.3 ± 10.5 μm) (Figure 1A). To measure the total number of progeny, animals were treated with control solution (S basal) or 100 mg/mL AAE beginning at L1. After five days at 20°C, the animals were incubated at 35°C and survival was monitored at 6, 9 and 12 h. There was no significant difference between curves by the Log-rank (Mantel-Cox) test. These results suggest that 100 mg/mL AAE is not toxic to C. elegans, as it does not interfere with development and progeny (Figure 1A, 1B and 1C).

AAE increases resistance to oxidative and hyperosmotic stress conditions

As AAE shows in vitro antioxidant activity and is not toxic to C. elegans, we next evaluated whether AAE treatment has a protective effect under normal and oxidative, heat and hyperosmotic stress conditions. To monitor longevity under normal conditions, fem-1(hc17) mutants grown at 25°C were treated with control solution (S basal) or 100 mg/mL AAE beginning at L1. AAE treatment had no effect on the C. elegans aging profile (p = 0.2997) (Figure 2A). Oxidative stress resistance assays were performed in wild-type animals treated with control solution (S basal) or 100 mg/mL AAE from L1 until L4 and then with 7.5 mM t-BOOH in M9. AAE showed increased oxidative stress resistance when compared to the control (p = 0.0002) (Figure 2B). The effect of AAE on heat tolerance was analyzed at 35°C in five-day-old wild-type animals with or without 100 mg/mL AAE treatment from L1. AAE did not protect C. elegans against heat stress when compared to the control group (p = 0.7388) (Figure 2C). Finally, we analyzed motility under acute osmotic stress at 500 mM NaCl. For the
dead bacteria. We observed that wild-type animals treated with oxidative stress resistance assay in animals treated with AAE on mL AAE exerted an antimicrobial effect, we repeated the all times analyzed (Figure 3A). Because we observed that 100 mg/100 mg/mL AAE. AAE treatment decreased bacteria growth at growth, measuring the OD over 4 h in the presence or absence of promoted by AAE could be a secondary response to a possible [39,40], we investigated whether the increased oxidative stress independence of its antimicrobial effect.

The protective effect of AAE against oxidative stress is independent of its antibacterial effect. ***p<0.001 related to the respective control by the Log-rank (Mantel-Cox) test.

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motility assay. L1 wild-type animals were treated with 100 mg/mL AAE for 68 h and then transferred to NGM plates with 500 mM NaCl. AAE treatment significantly increased motility under the osmotic stress condition (Figure 1D). After 1 h, 48.31% of the animals treated with 100 mg/mL AAE showed motility compared to 27.70% from the control group (p<0.05). We conclude that 100 mg/mL AAE protects against oxidative and hyperosmotic stress conditions but not under normal and heat stress conditions.

The protective effect of AAE against oxidative stress is independent of its antimicrobial effect.

As bacteria play a role in C. elegans mortality and stress resistance [39,40], we investigated whether the increased oxidative stress promoted by AAE could be a secondary response to a possible AAE antimicrobial property. First, we evaluated E. coli OP50 growth, measuring the OD over 4 h in the presence or absence of 100 mg/mL AAE. AAE treatment decreased bacteria growth at all times analyzed (Figure 3A). Because we observed that 100 mg/mL AAE exerted an antimicrobial effect, we repeated the oxidative stress resistance assay in animals treated with AAE on dead bacteria. We observed that wild-type animals treated with 100 mg/mL AAE presented increased survival on 7.5 mM t-BOOH compared to controls (p<0.0001) (Figure 3B). These results show that AAE treatment does not increase C. elegans oxidative stress resistance through an antimicrobial property.

AAE improves redox status in C. elegans and HUVECs under oxidative stress conditions.

Potential antioxidants can act directly to neutralize reactive oxygen species (ROS) and thus prevent the cellular response to increased oxidative stress to combat and remove ROS. To evaluate the capacity of AAE to neutralize ROS in vivo, we assessed ROS production in two experimental models, C. elegans and HUVECs, using the H2DCFDA fluorescent probe. We did not detect an effect of AAE treatment on basal ROS levels in either C. elegans or HUVECs (data not shown). We observed a significant increase in fluorescence intensity in worms (Figure 4A) and cells (Figure 4B) treated with their respective stress conditions. However, AAE treatment significantly reduced intracellular ROS accumulation in C. elegans under stress conditions (p<0.01) (Figure 4A). In HUVECs, the stress condition increased ROS production by 16-fold relative to the control, and AAE diminished this increase by up to 6-fold (p<0.001) (Figure 4B).

As AAE exhibited an in vivo antioxidant capacity, we next evaluated whether AAE would improve redox status in wild-type C. elegans. We assessed the levels of total sulphydryl (SH) groups in animals treated with control solution (S basal) or 100 mg/mL AAE from L1 until L4. The treatment of C. elegans with 100 mg/ml of AAE for 48 h did not increase the levels of total protein sulphydryl groups (515.97±2.73 vs. 512.2±52.75 μmol/mg of protein). The stress condition significantly decreased the levels of SH groups (515.97±32.73 vs. 279.73±5.51 μmol/mg of protein) (p<0.05). However, when the worms were first exposed to AAE and then submitted to the stress condition with t-BOOH, this pretreatment was able to prevent the reduction of protein sulphydryl group levels induced by t-BOOH (601.18±58.25 vs. 279.73±5.51 μmol/mg of protein) (p<0.05) (Figure 4C).

To correlate the in vivo antioxidant effect of AAE treatment with a molecular response in C. elegans, we analyzed the gene expression of γ-glutamyl cysteine synthetase-1 (gcs-1) and glutathione-s-transferase-4 (gst-4). GCS-1 is the limiting enzyme of glutathione (GSH) synthesis and is expressed at high levels in the intestine in response to oxidative stress but at low levels under normal conditions in an SKN-1-dependent manner [41]. gst-4::GFP is expressed primarily in the muscles and the hypodermis under normal conditions, and is increased in response to a variety of oxidative stress treatments [42,43]. Transgenic worms containing reporter genes were treated with control solution (S basal) or 100 mg/mL AAE for 48 h beginning at L1 and either in the presence or absence of oxidative stress. The fluorescence signals of gcs-1::GFP and gst-4::GFP animals treated with 100 mg/mL of AAE for 48 h were not significantly increased compared to the control group (Figure 4D–E). Exposure to 7.5 mM t-BOOH for 1 h increased gcs-1::GFP and gst-4::GFP expression, and AAE significantly prevented the upregulation of gcs-1::GFP (Figure 4D) but not gst-4::GFP (Figure 4E). Taken together, these results suggest that AAE functions as a direct antioxidant through the removal of ROS, preventing the decrease in SH groups mediated by the stress condition.

Increased oxidative stress resistance induced by AAE treatment depends on DAF-16 and OSR-1/UNC-43/SEK-1.

Certain flavonoids protect against oxidative stress either directly, by radical scavenging, or indirectly, by inducting antioxidant enzymes and thus increasing the stress resistance of
the organism. It has been shown in *C. elegans* that phytochemicals and plant extracts activate several stress response pathways [21,23,44]. To verify whether, in addition to its direct role, AAE protection under oxidative stress could also require these pathways, we performed the oxidative stress resistance assay in wild-type and mutant animals treated with 100 mg/mL AAE or control solution (S basal).

DAF-16/FOXO, the downstream target of insulin-like signaling in *C. elegans*, is a transcription factor required for both lifespan regulation and stress resistance [45,46]. The nuclear translocation of DAF-16 is positively regulated by c-Jun N-terminal kinase (JNK-1) in parallel with insulin-like signaling [46]. We therefore investigated whether oxidative stress resistance induced by AAE treatment depends on JNK-1/DAF-16 signaling. AAE treatment did not increase *daf-16(mu86)* mean survival (*p* = 0.1782), whereas it increased the mean survival of *jnk-1(gk7)* (*p* < 0.0001) (Figure 5A; Table 3). These results suggest that AAE may increase oxidative stress resistance via DAF-16 independently of JNK-1.

SKN-1/Nrf is a transcription factor that promotes the expression of antioxidant or detoxification enzymes, thus increasing stress resistance and longevity in *C. elegans* [19,30]. Under oxidative stress, SKN-1 functions depend on p38 MAPK signaling through the NSY-1/SEK-1/PMK-1 pathway [47]. We measured the survival of *nsy-1(ag3)*, *sek-1(km4)* and *skn-1(zu67)* mutants treated with AAE. AAE treatment prolonged the mean survival of *nsy-1(ag3)* (*p* = 0.0005) and *skn-1(zu67)* (*p* = 0.0002) but not *sek-1(km4)* (*p* = 0.7483) (Figure 5B; Table 3). These results suggest that the AAE antioxidant effect does not depend on SKN-1, and may act via the p38 pathway, but only through SEK-1.

In *C. elegans*, survival under hyperosmotic stress requires the OSR-1/UNC-43/SEK-1 stress response pathway [48]. We also tested whether the protective effect of AAE treatment under

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**Figure 4. Effect of açai aqueous extract (AAE) on redox status in wild-type *C. elegans* and HUVECs.**

A) *C. elegans* was treated with control solution (S basal) or 100 mg/mL AAE for 48 h and then submitted to the presence or absence of 1 mM *H₂O₂* for 2 h. The results are expressed as *H₂DCFDA* fluorescence relative to the untreated control. B) HUVECs were treated with or without 2.5 mg/mL AAE for 16 h and then incubated in 0.25 mM *H₂O₂* for 1 h. The fluorescence was measured by flow cytometry. The results are expressed as *H₂DCFDA* fluorescence relative to the untreated control. Different letters indicate significant differences by one-way ANOVA followed by Tukey’s post-test. C) To measure the levels of total SH groups, animals were treated with control solution (S basal) or 100 mg/mL AAE from L1 until L4 and then incubated with or without 5 mM t-BOOH for 1 h. *p* values were determined by a two-tailed Student’s *t*-test, and groups were significantly different when *p* < 0.05 in *C. elegans*. Transgenic worms containing reporter genes were treated with control solution (S basal) or 100 mg/mL AAE for 48 h beginning at L1 and then with or without the oxidative stress condition. After a 1-h recovery period, photographs were taken on a fluorescence microscope. For (D) *gcs-1::GFP* and (E) *gst-4::GFP* animals, GFP fluorescence signals were measured using NIH Image J software. Different letters correspond to significant differences by the Kruskal-Wallis test followed by Dunn’s post-test.

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oxidative stress conditions depends on OSR-1 and UNC-43. AAE treatment did not extend the mean survival of *unc-43(n498n1186) (p = 0.7320) (Figure 5C, Table 3). In *osr-1(rm1) mutants, AAE treatment reduced the mean survival under oxidative stress (p = 0.0009) (Figure 5C, Table 3). These findings suggest that AAE modulates oxidative stress resistance through OSR-1 and two of its downstream effectors: UNC-43 and SEK-1.

AAE treatment induces *ctl-1 and *gst-7 expression in a DAF-16-dependent manner

As AAE treatment increased oxidative stress resistance in *C. elegans through DAF-16, we investigated whether AAE treatment activates DAF-16 nuclear translocation under normal and stress conditions. The results showed that under normal growth conditions, AAE treatment did not induce DAF-16::GFP nuclear translocation (Figure 6A–B). Moreover, the AAE treatment did not alter *daf-16 mRNA levels in wild-type animals (Figure 6C). The stress condition used in this study (7.5 mM t-BOOH for 1 h) also did not increase the fraction of animals showing DAF-16::GFP nuclear localization (Figure 6B). However, under stress conditions, AAE treatment significantly reduced DAF-16::GFP nuclear localization (Figure 6B). One possible explanation for this finding is that AAE modifies DAF-16 to increase its activity but not its concentration. To further test this hypothesis, we measured the
expression of the DAF-16 stress target genes after AAE treatment in wild-type animals and daf-16 mutants (Figure 6D). The results showed that the transcripts levels of ctl-1 and gis-7 were upregulated in wild-type animals treated with AAE in a manner that was dependent upon daf-16 (Figure 6D). However, AAE treatment did not induce neither sod-3 mRNA (Figure 6D) nor sod-3::GFP expression (Figure S1). Taken together, these results suggest that AAE treatment increases oxidative stress resistance by activating antioxidant genes via DAF-16.

AAE increases polyglutamine protein aggregation and decreases proteasome activity

OSR-1 is a secreted protein that couples with SEK-1/MAPKK through UNC-43/CaMKII to promote resistance to osmotic stress [48]. A loss of OSR-1 function constitutively activates gdh-1 expression and glycerol accumulation [49]. Glycerol replaces inorganic ions in the cytoplasm and functions as a chemical chaperone that aids in the refolding of misfolded proteins [50].

It is possible that AAE increases both oxidative and osmotic stress resistance by directly blocking OSR-1 activity and inducing gdh-1 expression. To test this hypothesis, we measured osr-1 mRNA levels and gdh-1::GFP expression in animals treated or not with 100 mg/mL AAE for 48 h. The AAE treatment did not modify osr-1 gene expression under normal conditions (Figure 7A) and reduced gdh-1::GFP expression (Figure 7B). Nevertheless, AAE treatment could interfere in OSR-1 activity which is coupled with the pathway UNC-43/SEK-1 in order to promote oxidative stress resistance.

Alternatively, AAE may function as a mild stressor, increasing hypertonic stress and/or protein homeostasis. High ionc stress is a well-known disruptor of protein secondary structure that can increase protein aggregation [51]. To test the hypothesis that AAE could increase hypertonic stress, we quantified aging-induced polyglutamine protein aggregation in vha-6::Q44::YFP transgenic worms. Q44 animals exhibit a diffuse fluorescence throughout the intestine until they reach adulthood, when focal fluorescent aggregates gradually increase. At one-day-old, Q44::YFP aggregation was not different between the control and 100 mg/mL AAE-treated groups (Figure 7C), whereas four-day-old Q44::YPF adult animals treated with 100 mg/mL AAE had approximately twice as many aggregates as control animals (p<0.05) (Figure 7C). Although the average number of aggregates increased with age, the AAE treatment had no effect on Q44::YPF aggregation in adult worms at eight- and twelve-day-old (Figure 7C). This result suggests that AAE treatment accelerates the aggregation formation at the young adult stage. To evaluate whether AAE treatment impairs protein homeostasis, we measured proteasome activity in animals treated with control solution (S basal) from L1 until L4. Proteasome chymotrypsin-like activity was monitored by SLLVV-MCA digestion in L4 worm extracts containing equal amounts of total protein. AAE decreased proteasome degradation activity by 2.5-fold relative to the controls (p<0.05) (Figure 7D). These results suggest that AAE may increase oxidative and osmotic stress resistance via increased ionic strength and/or impairment of protein homeostasis.

Discussion

Açai (Euterpe oleracea Mart.) has been highly praised in recent few years for having a wide range of health-promoting and therapeutic benefits due to its nutritional value and high levels of polyphenolic compounds, especially anthocyanin and proanthocyanidin. Although a great number of studies have reported that açai treatment in vivo provides antiproliferative, anti-inflammatory, antioxidant and cardioprotective effects [4], in vivo studies are still lacking. Here, we employed an in vivo approach to investigate the antioxidant properties of açai and its underlying mode of action by using the model organism C. elegans. Our results reveal that açai protects against oxidative stress through both direct and indirect mechanisms. AAE increases resistance to oxidative and osmotic stress independently of any effect on reproduction, development and bacterial growth. AAE treatment directly reduces ROS production and prevents SH level reduction and gcs-1 activation under oxidative stress conditions. Oxidative stress resistance is also indirectly mediated by AAE through DAF-16 and the OSR-1/UNC-43/SEK-1 osmotic stress pathway.

Many dietary polyphenols have antioxidant activity, and this activity is generally attributed to their ability to directly neutralize reactive oxygen and nitrogen species. The findings presented here support a direct mechanism of action for AAE through the reduction of ROS production induced by hydrogen peroxide (H2O2) in C. elegans and HUVECs. Many authors have already demonstrated that dietary supplementation with polyphenols and phytochemicals significantly decreases ROS production under stress conditions in C. elegans. For example, the flavonoids epicatechin, quercetin, rutin and Ginkgo biloba extract EGb761 reduce the ROS accumulation induced by thermal stress [52–54]. Similarly, treatment with myricetin, a flavonoid commonly used as
Figure 6. Effect of açai aqueous extract (AAE) on DAF-16::GFP nuclear localization and the expression of its stress-inducible targets. Transgenic worms containing reporter genes were treated with control solution (S basal) or 100 mg/mL AAE for 48 h from L1 and then with or without the oxidative stress condition. A, B) DAF-16::GFP, worms were classified as cytosolic, intermediate or nuclear according to their subcellular distribution of DAF-16. C) mRNA level of daf-16 in wild-type animals treated or not with 100 mg/mL AAE for 48 h. D) mRNA levels of DAF-16 stress-inducible genes sod-3, ctl-1 and gst-7 in wild-type and daf-16 animals treated or not with 100 mg/mL AAE. #p = 0.07, *p < 0.05, **p < 0.01 by a two-tailed Student’s t-test.

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Figure 7. Effect of açai aqueous extract (AAE) on protein homeostasis. A) mRNA level of osr-1 in wild-type animals treated or not with 100 mg/mL AAE for 48 h. B) Transgenic worms carrying the reporter gene gpdh-1::GFP were treated or not with 100 mg/mL AAE for 48 h and then photographs were taken on a fluorescent microscope. GFP fluorescence signals were measured using NIH Image J software. ***p < 0.001 by a two-tailed Student’s t-test. C) Transgenic worms carrying the reporter gene vha-6::Q44::YFP were treated with control solution (S basal) or 100 mg/mL AAE starting at L1. Photographs of one-, four-, eight- and twelve-day-old animals were taken on a fluorescence microscope, and the numbers of aggregates were counted. *p < 0.05 by a one-tailed Student’s t-test. D) Animals were treated with 100 mg/mL AAE or control solution (S basal) from L1 until L4. Proteasome chymotrypsin-like activity was monitored by SLLVY-MCA digestion in L4 worm extracts containing equal amounts of total protein. ***p < 0.001 by a two-tailed Student’s t-test.

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Açai’s Antioxidant Effect

In Vivo

A natural chemopreventive, also reduces ROS accumulation in wild-type animals exposed to H₂O₂ [55]. Conversely, Guha et al. [23] demonstrated that cranberry extract (CBE) supplementation was not effective in reducing ROS levels in worms exposed to paraquat or in protecting worms exposed to oxidativo stress.

Many antioxidants can also increase oxidative stress resistance by inducing the transcription of cytoprotective proteins. Flavonoids and phytochemicals have been shown to increase the expression of protective genes in C. elegans such as gst-4 [42], gcs-1 [56] and sod-3 [31]. In our work, AAE treatment did not alter the expression of gst-4, gcs-1 or sod-3 under normal conditions, but it prevented gcs-1 activation under oxidative stress conditions. Moreover, AAE treatment prevented SH level reduction under oxidative stress conditions. Similarly, GSH levels in epicatechin-treated worms under thermal stress were restored to normal levels [52]. These findings support a direct mode of action for AAE.
These results can be partially explained by the high levels of polyphenols present in acai. In this scenario, the radical-scavenging properties of AAE's polyphenols, demonstrated both in vitro and in vivo, directly neutralize ROS produced under stress condition. The depletion of sulfhydryl groups, the most abundant and important nonenzymatic defense molecules, especially GSH, reflects intracellular oxidation. Thus, a reduction of the cellular stress environment promoted by AAE is able to avert the oxidation of sulfhydryl groups and the activation of gcs-1, the limiting enzyme of glutathione (GSH) synthesis (Figure 8).

We have demonstrated that AAE treatment also increases acute osmotic stress resistance in C. elegans. This activity can be explained not only by the radical-scavenging properties of AAE but also by an indirect activity through the activation of protective signaling pathways. Our genetic analysis indicates that stress resistance mediated by AAE is dependent on the DAF-16 and OSR-1/UNC-43/SEK-1 osmotic stress pathways.

The DAF-16/FOXO transcription factor is regulated by the insulin signaling pathway and is considered a key regulator of many important biological processes, including lifespan, metabolism and stress responses [57]. The nuclear translocation of DAF-16 and sod-3 expression were reported for different extracts and polyphenols such as cranberry extract [23] and Monascus-fermented Dioscorea [31], quercetin [38], myricetin and kaempferol [59]. Our results showed that AAE treatment does not increase oxidative stress resistance in daf-16(mu86) mutant animals, suggesting that AAE treatment protects against oxidative stress in a DAF-16-dependent manner. Nevertheless, AAE treatment does not increase DAF-16 nuclear localization or sod-3 expression under normal conditions. One possible explanation for the requirement of DAF-16 on AAE-induced oxidative stress resistance without nuclear localization is that AAE might modify DAF-16 to increase its activity but not its concentration. Furthermore, DAF-16 activation by AAE in the nucleus might upregulate specific subsets of genes other than sod-3. In fact, AAE treatment increased the gene expression of ctl-1 and gst-7 in a DAF-16-dependent manner.

OSR-1 is a master regulator of C. elegans survival in hyperosmotic environments [48]; OSR-1 couples with SEK-1/ MAPKK through UNC-43/CaMKII to promote resistance to chronic osmotic stress. The requirement of UNC-43 has been reported for the extended lifespan induced by blueberry [21], cranberry [23] and quercetin [44]. However, the prolongevity effect induced by these phytochemicals depends differently on OSR-1 and SEK-1. For example, SEK-1, but not OSR-1, is required for quercetin-induced longevity [44], whereas OSR-1, but not SEK-1, is required for cranberry-induced longevity [23]. Our genetic study indicates that OSR-1 is required for AAE-induced oxidative stress resistance as well as UNC-43 and SEK-1.

Wilson et al. [21] found that blueberry polyphenol-induced longevity is dependent on the OSR-1/UNC-43/SEK-1 pathway. These results suggest that the OSR-1/UNC-43/SEK-1 pathway is a key target for anthocyanins, the predominant polyphenols present in acai and blueberry.

Notably, prolongevity and thermotolerance are common features observed in C. elegans exposed to phytochemical interventions [21,23,44,54]. The ability of these phytochemicals to increase resistance to heat stress may be due to their similar polyphenol content. Despite this similarity, neither of these effects were observed in the animals treated with AAE. Although blueberry and acai extracts have similar polyphenol content, other bioactive metabolites are found in acai extract. These compounds include peonidin 3-rutinoside and peonidin 3-glucoside, cyanidin 3-arabinoxylarabinoside, cyanidin 3-arabinoside and cyanidin 3-acetyl hexose [2,60]. As a number of experiments have shown that different phytochemicals may interact and synergize to exert their biological functions, it is not unexpected that AAE treatment does not increase thermotolerance in C. elegans.

Guha et al. [23] observed that CBE treatment decreases osmotic stress resistance in C. elegans. The authors explained this susceptibility through a depression of CaMKII/p38 MAPK signaling by CBE, based on the observation that osr-1 mRNA is upregulated by CBE treatment. osr-1 upregulation by flavonoids was also demonstrated by Xue et al. [61]. In our study, we
demonstrated that AAE treatment increases acute osmotic stress resistance in \textit{C. elegans}. However, the AAE treatment did not modify \textit{os-1} gene expression under normal conditions and reduced \textit{gdh-1::GFP} expression. These results did not support our hypothesis that the mechanism of AAE-increased osmotic stress resistance requires neither \textit{os-1} downregulation nor glycerol synthesis.

As stress resistance mediated by AAE treatment was dependent on OSR-1, we hypothesized that açaí might act directly by blocking OSR-1 protein activity or may function as a mild stressor (Figure 8). OSR-1 inhibition by AAE in turn activates UNC-43 and SEK-1 and consequently promotes oxidative stress resistance. If AAE treatment generates a hypertonic environment, we could expect increased protein aggregation and proteasomal activity. In agreement with our hypothesis, we observed that AAE treatment increased the number of Q44::YFP protein aggregates. However, proteasomal activity was significantly reduced, which might also contribute to the increased polyglutamine aggregation.

Our study highlights the \textit{in vivo} antioxidant and stress resistance properties of AAE, supporting previous \textit{in vitro} experiments on cultured cells and short-term rodent studies. This work also helps to reveal the underlying molecular mechanisms by which AAE modulates stress responses. We propose that AAE modulates oxidative stress resistance by direct and indirect mechanisms. AAE removes ROS directly and prevents \textit{gs-1} activation and SH level reduction, which in turn protects against oxidative stress. AAE also promotes oxidative stress resistance indirectly through DAF-16 activation and SEK-1 and consequently promotes oxidative stress resistance. AAE also removes ROS directly and prevents \textit{gcs-1} oxidative stress resistance by direct and indirect mechanisms. AAE modulates stress responses. We propose that AAE modulates to reveal the underlying molecular mechanisms by which AAE

\section*{References}


