

Original Paper

# Euterpe oleracea Mart. (Açaí) Supplementation Attenuates Acute Doxorubicin-Induced Cardiotoxicity in Rats

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## Key Words

Oxidative stress • Apoptosis • Matrix metalloproteinase-2 • Cardiac function

## Abstract

**Background/Aims:** Doxorubicin, a chemotherapy drug used successfully for years, could induce cardiotoxicity. Euterpe oleracea Mart. (açai) is a fruit high in antioxidant properties. The aim of this study was to evaluate doxorubicin-induced cardiotoxicity prevention after açai administration. **Methods:** A total of 64 male Wistar rats were allocated into 4 groups: control (C), açai (A), doxorubicin (D) and açai-doxorubicin (DA). Rats received regular chow (C and D groups) or chow supplemented with açai 5% (A and DA groups) for 4 weeks. Subsequently, rats received doxorubicin 20 mg/kg (D and DA groups) or saline (C and A groups). Euthanasia was performed 48 hours after doxorubicin injection. Left ventricular function was evaluated by echocardiography *in vivo* and by isolated heart study *ex vivo*. Oxidative stress, myocardial metabolism and nitric oxide metabolite were evaluated by spectrophotometry, MMP-2 activity by zymography and caspase-3 and Bcl-2 protein expression by Western blot. **Results:** Doxorubicin induced decreases in body weight, food and water ingestion. We observed decreases in left ventricular fractional shortening in rats treated with doxorubicin. Additionally, the same rats showed lower +dP/dt and -dP/dt during isolated heart study than those who did not receive doxorubicin. Doxorubicin injection increased caspase-3 protein expression, myocardium lipid hydroperoxide concentration, MMP-2 activity, phosphofructokinase and lactate dehydrogenase activity, and decreased  $\beta$ -hydroxyacyl-CoA dehydrogenase, pyruvate dehydrogenase, citrate synthase, complex I, complex II and ATP synthase activity

in myocardium. Açai supplementation improved left ventricular fractional shortening, decreased myocardium lipid hydroperoxide concentration, MMP-2 activity, and improved  $\beta$ -hydroxyacyl-CoA dehydrogenase, phosphofructokinase, citrate synthase, complex II and ATP synthase enzymatic activities. We did not observe differences in nitric oxide metabolite concentrations between groups. **Conclusion:** Doxorubicin induced left ventricular dysfunction, increases in oxidative stress, changes in myocardium metabolism and MMP-2 activation. Açai supplementation was able to prevent these alterations.

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## Introduction

Doxorubicin is a chemotherapy drug extract from *Streptomyces peucetius* fungi and has been used to treat solid tumors and hematologic cancers since the 60s. Nausea, vomiting, alopecia and neutropenia are very common side effects. However, cardiotoxicity is the most serious side effect of doxorubicin treatment and could limit its use in treatment. Cardiotoxicity has several clinical presentations that depend on symptom onset [1].

Chronic cardiotoxicity is the most studied form. The symptoms start weeks or months after treatment and manifest as heart failure and dilated cardiomyopathy. Acute cardiotoxicity is hard to diagnose since it has a less specific clinical presentation. Approximately 11% of patients can develop acute cardiotoxicity hours or days after treatment [2, 3]. Despite these differences in clinical presentation, there is evidence that myocardial damage, including ventricular dysfunction, starts early after doxorubicin administration [4-6].

Many mechanisms are involved in the pathophysiology of doxorubicin-induced cardiotoxicity, and they are not completely understood. Increases in oxidative stress seem to be a cornerstone of myocardial injury [7]. It was recently discovered that increases in reactive oxygen species generation depend on inhibition of topoisomerase 2-beta by anthracyclines [8-10]. An oxidative environment is a potent activator of matrix metalloproteinase (MMP) [11]. Indeed, MMP activation is an early event in doxorubicin-induced cardiotoxicity [5, 6, 12, 13]. In addition, doxorubicin could induce changes in myocardial metabolism and mitochondrial dysfunction which is another important source of reactive species [7, 14]. Doxorubicin administration in rats decreased myocardial lipid metabolism, increased glucose metabolism and decreased enzymatic complex activity of the phosphorylative mitochondrial chain [6], with decreased ATP production [14]. In addition, doxorubicin elevated sympathetic tone, activated the renin-angiotensin-aldosterone system, increased vasopressin and deregulated NO release [7], thereby contributing to vasoconstriction and left ventricular dysfunction.

Considering cardiotoxicity severity can limit the effectiveness of doxorubicin administration and treatment, it is important to discover new strategies or substances that attenuate doxorubicin mediated cardiac injury. Thus, antioxidant rich substances could be of use.

Açai (*Euterpe oleracea* Mart.) is a Brazilian fruit from palms of the Arecaceae family. They are rich in fatty acids, including oleic and linoleic acid. In addition, açai has an elevated content of anthocyanins, phenolic compounds with high antioxidant effects [5-17]. Experimental studies have shown that açai has an anti-proliferative effect in neoplastic cells [18, 19], a nitric oxide-mediated vasodilatory effect in mesenteric circulation [20] and was able to reduce genotoxicity induced by doxorubicin [21]. However, the effect of açai in doxorubicin-induced cardiotoxicity has not been studied before.

The aim of study was to evaluate the influence of açai on morphological and functional cardiac alterations, oxidative stress, myocardium metabolism, apoptosis and MMP-2 activity in acute doxorubicin-induced cardiotoxicity in rats.

## Materials and Methods

### *Study design*

This study was previously approved by the local Ethics Committee (protocol number: 1098/2014). Sixty-four male Wistar rats, weighing between 250 and 300 g, were kept in a controlled environment with a 12-hour light-dark cycle at  $23^{\circ} \pm 2^{\circ}\text{C}$ , and free access to food and water. Rats were allocated to 4 groups (16 rats in each group): control (C), doxorubicin (D), açai (A) and doxorubicin/açai (DA). Groups C and D received regular chow and groups A and DA received chow supplemented with açai 5% [19] for 4 weeks. Food and water ingestion were measured during the experiment. Subsequently, rats in D and DA groups received a single intraperitoneal (IP) injection of doxorubicin (20 mg/kg) [5, 6, 22] and rats in C and A groups received an equivalent volume IP injection of saline. Groups D and DA continued to receive açai in the 48 hours between doxorubicin injection and euthanasia. Forty-eight hours after doxorubicin administration, echocardiogram was performed on all animals, followed by euthanasia and isolated heart study. The isolated heart study was performed in 8 rats per group anesthetized with sodium thiopental (80 mg/kg, IP). Rats subjected to isolated heart study were different from rats used to collect hearts for biochemical analyses, as the retrograde perfusion in isolated heart study could interfere with these analyses. Euthanasia was performed by heart excision after thiopental injection (120 mg/kg, IP). Hearts were washed in fresh saline, dissected, weighed and stored at  $-80^{\circ}\text{C}$  until biochemical analysis. Blood was collected, centrifuged and sera stored at  $-80^{\circ}\text{C}$ .

### *Preparation of diet with açai*

All the commercial açai pulp (Icefruit®) used in preparing the diet was homogenized and stored at  $-80^{\circ}\text{C}$ . Pulp was mixed with ground chow Nuvilab (Nuvital®), homogenized, pelleted and stored at  $-20^{\circ}\text{C}$  until administration. The mixture contained 5% açai, as per Fragoso et al. [19].

### *Cardiac function evaluation in vivo: echocardiogram*

Doppler-echocardiograms were performed by one examiner who was blinded to condition. Rats were anesthetized with ketamine (50 mg/kg, IP) and xylazine (1 mg/kg, IP), and exams were performed using a Vivid S6 system (General Electric Medical Systems, Israel) with a 5.0 - 11.5 MHz multifrequency transducer. Measurements were obtained in M-mode. We evaluated the left atrium, left ventricle and aorta diameters, and interventricular septum and left posterior wall thicknesses. Systolic function was analyzed by left ventricular fractional shortening. Diastolic function was analyzed by E and A waves, E/A ratio, E wave deceleration time, and isovolumetric relaxation time (absolute value and corrected by heart rate).

### *Cardiac function evaluation in vitro: isolated heart study*

The isolated heart study was performed as previously described [5]. Briefly, rats were subjected to sternotomy, and the aorta was dissected and cannulated. Subsequently, retrograde perfusion was started with modified Krebs-Henseleit solution (NaCl 118.5 mM/L; KCl 4.69 mM/L;  $\text{CaCl}_2$  2.52 mM/L;  $\text{MgSO}_4$  1.16 mM/L;  $\text{KH}_2\text{PO}_4$  1.18 mM/L; glucose 5.50 mM/L;  $\text{NaHCO}_3$  25.88 mM/L and mannitol 8 mM/L). The solution was kept at  $37^{\circ}\text{C}$ , with a perfusion pressure of 75 mmHg and a controlled  $\text{O}_2$  concentration. Hearts were transferred to the isolated heart study apparatus (Hugo Sachs Elektronik - March-Hugstetten, Germany). A latex balloon connected to a pressure transducer was inserted in the left ventricular cavity. The volume inside the balloon was increased to change diastolic pressure from 0 to 25 mmHg. After each volume variation, we recorded diastolic and systolic left ventricular pressures, maximum left ventricular pressure decrease rate ( $-dP/dt$ ) and maximum left ventricular pressure development rate ( $+dP/dt$ ).

### *Oxidative stress*

Samples of left ventricular tissue (100 mg) were homogenized in sodium phosphate buffer pH=7.4 (0.01 M) and then centrifuged at 12,000 rpm for 30 minutes at  $-4^{\circ}\text{C}$ . The supernatants were used to determine lipid hydroperoxide concentration by spectrophotometry, as a marker of oxidative damage [23]. Antioxidant enzymatic activity was determined by spectrophotometry in the same supernatants. We evaluated glutathione peroxidase (E.C.1.11.1.9.), superoxide dismutase (E.C.1.15.1.1.) and catalase (E.C.1.11.1.6.) activity as per previously described methods [24, 25]. All reagents were obtained from Sigma-Aldrich (Saint Louis, USA).

### *Myocardial metabolism*

All enzymatic activities in myocardium were determined by spectrophotometry. Approximately 100 mg of left ventricular tissue was homogenized in sodium phosphate buffer pH=7.0 (0.1 M) and then centrifuged at 12,000 rpm for 30 minutes at -4°C. Supernatants were used to determine  $\beta$ -hydroxyacyl coenzyme-A dehydrogenase (E.C.1.1.1.35), phosphofructokinase, lactate dehydrogenase (E.C.1.1.1.27), pyruvate dehydrogenase (E.C.1.2.4.1) and citrate synthase (E.C.4.1.3.7) activities [26]. Complex I (E.C.1.6.5.3), complex II (E.C.1.3.5.1) and ATP synthase (E.C.3.6.3.14) activities (enzymes belong to mitochondrial respiratory chain) were measured in the pellet [27]. The pellet was resuspended in sodium phosphate buffer (0.1 M) with sucrose 250 mM and ethylenediamine tetraacetic acid 2 mM. Readings were performed on a  $\mu$ Quant microplate reader (Bio-Tec Instruments, Winooski, VT, USA). All reagents were obtained from Sigma-Aldrich.

### *MMP-2 activity: zymography*

Approximately 30 mg of left ventricular muscle was added to extraction buffer (Tris 50 mM pH 7.4, NaCl 0.2 M, Triton X 0.1% and  $\text{CaCl}_2$  10 mM), crushed and centrifuged. Protein was quantified in supernatants by the Bradford method. Samples (10  $\mu$ g of protein) were diluted in sample buffer (Tris 0.5 M pH 6.8, glycerol 50% and bromophenol blue 0.05%) and electrophoresis was performed on polyacrylamide 8% and gelatin 1% gels. The run was performed at 110 V in a Bio-Rad mini-protean system in the presence of running buffer (Tris-Glycine-SDS pH 8.3). After running, the gels were washed with Triton X-100 2.5% and Tris-HCl 50 mM pH 8.4. The gels were incubated for 17 hours at 37°C with continuous agitation (buffer tris-HCl 50 mM pH 8.4,  $\text{CaCl}_2$  500 mM). The gels were stained with Coomassie brilliant blue 2.5% and discolored by methanol 30% and acetic acid 10%. The gels were photographed by ImageQuant LAS (General Electrics) and analyzed by Gel-Pro 3.2 (Media Cybernetics Rockville, USA). The same control sample was included in each gel to normalize the results. MMP-2 position in the gels was confirmed by recombinant rat/mouse MMP-2 standard (R&D System).

### *Expression of proteins involved in apoptosis: Western blot*

Left ventricular muscle (100 mg) was added to extraction buffer, homogenized and centrifuged. Supernatants were collected and total amount of protein was quantified by the Bradford method. Electrophoresis was performed in acrylamide gels and proteins were transferred to nitrocellulose membranes, which were incubated in 5% skimmed milk. Subsequently, membranes were incubated with primary antibodies for caspase-3 (rabbit monoclonal IgG, Cell Signaling Technology, MA, USA) and Bcl-2 (rabbit monoclonal IgG, Santa Cruz Biotechnology, Europe) for 12 hours, followed by incubation with secondary antibodies. Immunodetection was performed using chemiluminescence in an ImageQuant LAS camera imaging system (General Electrics). Images were analyzed by Gel-Pro 32 (Media Cybernetics Rockville, USA). GAPDH (mouse monoclonal IgG, Santa Cruz Biotechnology, Europe) was used to normalize all proteins.

### *Nitric oxide serum metabolites*

Serum was obtained after blood centrifugation. Dosage was performed as described previously [28].  $\text{NaNO}_2$  dilution curves were made and samples applied in duplicate. Griess reagent (100  $\mu$ l) was added to all wells. Spectrophotometry readings were performed at 540 nm.

### *Statistical analyses*

Variables with normal distribution were shown as means  $\pm$  standard deviation. Variables with non-normal distribution were normalized by mathematical transforming for statistical analysis. Comparisons between groups were by two-way ANOVA. We adopted a statistical significance level of  $p < 0.05$ .

Variables that could not be normalized (water and food ingestion, left ventricular diastolic diameter, left ventricular posterior wall thickness, and superoxide dismutase activity) were analyzed by the 't' or Mann-Whitney test. In these cases, p values were adjusted by Bonferroni correction and we considered statistical significance when  $p < 0.008$  (0.05 divided by number of possible comparisons).

## Results

### Morphological characteristics and food and water ingestion

All groups had the same mean body weight at the beginning of the experiment and weight gain was similar between groups. After doxorubicin injection, rats in groups D and DA had decreased body weight with decreased food and water ingestion (Fig. 1).

Left ventricular weight was lower in rats treated with doxorubicin than in rats that did not receive the drug; however, when left ventricular weight was normalized to body weight, rats that received doxorubicin had higher left ventricular weight (Fig. 1). Açai did not interfere with these variables.

### Echocardiography

Group D had greater left ventricular systolic diameter and left atrial diameter than group C. Group DA exhibited lower left ventricular systolic diameter and left atrial diameter than group D. Regarding left ventricular function, group D exhibited less left ventricular fractional shortening than did group C. Group DA showed improvement in left ventricular fractional shortening compared with group D. These results indicate that açai supplementation was effective in improving cardiac structure and function *in vivo* (Table 1).

### Isolated heart study

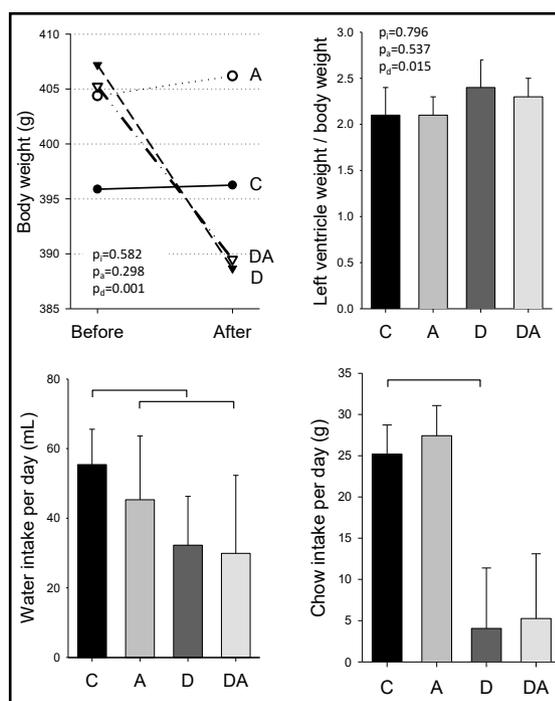
Table 2 displays the isolated heart study results. Doxorubicin decreased maximum +dP/dt, maximum -dP/dt and maximum systolic pressure, suggesting worsening in systolic and diastolic function *in vitro*. We did not observe any influence from açai in these results.

### Apoptosis

Regarding Bcl-2 expression, there were no differences between groups. Doxorubicin administration increased 19KDa caspase-3 expression, and açai supplementation did not change this result (Fig. 2).

### MMP-2 activity

Doxorubicin administration increased both MMP-2 bands in zymography gels. Açai supplementation decreased the intensity of the lower bands both in rats that did and did not receive doxorubicin (Fig. 3).



**Fig. 1.** Body weight and chow and water ingestion. C: control group; A: açai group; D: doxorubicin group; DA: doxorubicin + açai group. Before: body weight immediately before doxorubicin injection; After: body weight 48 hours after doxorubicin injection. Chow and water intake represent daily ingestion after doxorubicin injection. p value: two-way ANOVA, except chow and water intake comparisons, which were analyzed by Mann-Whitney test (in this situation  $p < 0.008$  was considered significant). Brackets link two significantly different groups; pi: p value for doxorubicin vs açai interaction; pa: p value for açai; pd: p value for doxorubicin.

**Table 1.** Morphological variables and cardiac function in vivo evaluated by Echocardiogram. C: control group; A: açai group; D: doxorubicin group; DA: doxorubicin + açai group; HR: heart rate; LVSD: left ventricle (LV) systolic diameter; LVDD: LV diastolic diameter; PW: diastolic posterior wall thickness; IVS: diastolic interventricular septum thickness; LA: left atrium diameter; FS: LV fractional shortening; E: peak velocity of early ventricular filling; A: peak velocity of transmitral flow during atrial contraction; IVRTc: isovolumetric relaxation time corrected by HR. p value: two-way ANOVA, except <sup>®</sup>, which were analyzed by t-test or Mann-Whitney test (in these cases, p values were considered significant when <0.008); pi: p value for doxorubicin vs açai interaction; \*different from C group; <sup>®</sup>different from D group

Variables	C (n=16)	A (n=16)	D (n=15)	DA (n=16)	p <sub>i</sub>	p <sub>açai</sub>	p <sub>doxo</sub>
HR (bpm)	343 ± 51	326 ± 66	286 ± 58	306 ± 60	0.230	0.894	0.013
LVSD (mm)	2.81 ± 0.46	3.04 ± 0.43	3.89 ± 0.88*	3.05 ± 0.71 <sup>®</sup>	0.002	0.110	0.003
LVDD (mm) <sup>®</sup>	6.9 (6.6-7.5)	7.3 (6.9-7.5)	6.9 (6.2-7.4)	6.4 (6.0-7.2)	0.324	0.335	0.324
PW (mm) <sup>®</sup>	1.5 (1.5-1.7)	1.5 (1.5-1.8)	1.5 (1.5-1.5)	1.5 (1.5-1.5)	0.766	0.752	0.502
IVS (mm) <sup>®</sup>	1.5 (1.5-1.8)	1.8 (1.5-1.8)	1.8 (1.5-1.8)	1.8 (1.7-1.8)	0.415	0.138	0.520
LA (mm)	4.9 ± 0.3	5.0 ± 0.4	5.5 ± 0.8*	4.9 ± 0.5 <sup>®</sup>	0.012	0.054	0.037
FS (%)	60 ± 6	58 ± 4	44 ± 9*	54 ± 6 <sup>®</sup>	0.001	0.014	0.001
E/A ratio	1.59 ± 0.26	1.6 ± 0.25	1.67 ± 0.43	1.50 ± 0.22	0.233	0.295	0.905
IVRTc	65 ± 13	66 ± 8.5	70 ± 17	71 ± 8.7	0.959	0.743	0.133

**Table 2.** Left ventricular function in vitro evaluated by isolated heart study. C: control group; A: açai group; D: doxorubicin group; DA: doxorubicin + açai group; V<sub>0</sub>: initial balloon volume; +dP/dt: maximum left ventricular pressure development rate; -dP/dt: maximum left ventricular pressure decrease rate; SP<sub>max</sub>: maximum systolic pressure. Values are expressed as the mean ± standard deviation; p value: two-way ANOVA; pi: p value for doxorubicin vs açai interaction; <sup>#</sup>variable was normalized to statistical analysis

Variables	C (n=8)	A (n=8)	D (n=7)	DA (n=8)	p <sub>i</sub>	p <sub>açai</sub>	p <sub>doxo</sub>
V <sub>0</sub> (μL)	85.6 ± 36.4	90.0 ± 23.0	77.1 ± 32.9	86.9 ± 22.5	0.801	0.508	0.585
+dP/dt <sup>#</sup> (mmHg/s)	2961 ± 1066	3109 ± 978	1768 ± 434	1680 ± 554	0.579	0.912	<0.001
-dP/dt (mmHg/s)	2063 ± 612	2422 ± 619	1275 ± 514	1133 ± 485	0.226	0.596	<0.001
SP <sub>Max</sub> (mmHg)	143 ± 39	137 ± 29	99 ± 21	90 ± 23	0.918	0.499	<0.001

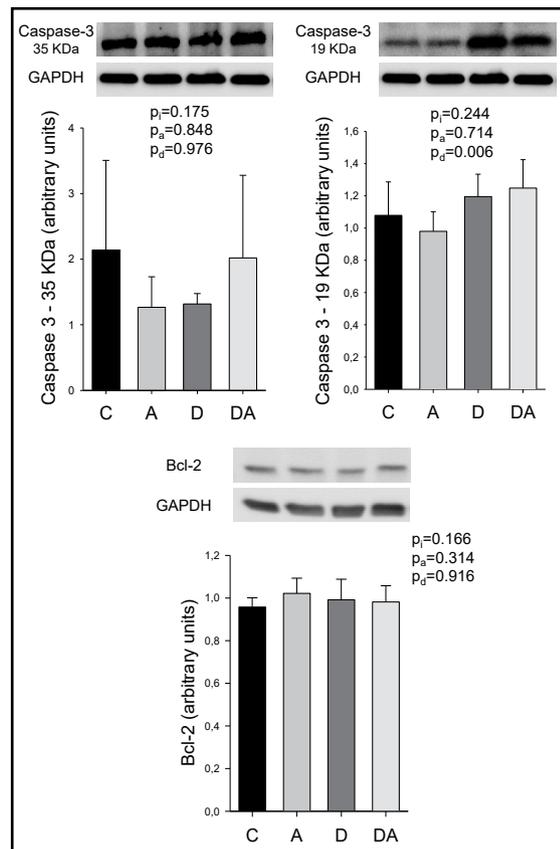
#### Oxidative stress

Rats treated with doxorubicin exhibited higher levels of myocardial lipid hydroperoxide than not treated rats, and açai administration decreased myocardial lipid hydroperoxide levels both in rats that did and did not receive doxorubicin. Additionally, doxorubicin decreased catalase and glutathione peroxidase antioxidant enzyme activity. Açai supplementation increased catalase activity both in rats treated and not treated with doxorubicin. In addition, the DA group exhibited increased glutathione peroxidase activity that achieved values near those of group C (Table 3).

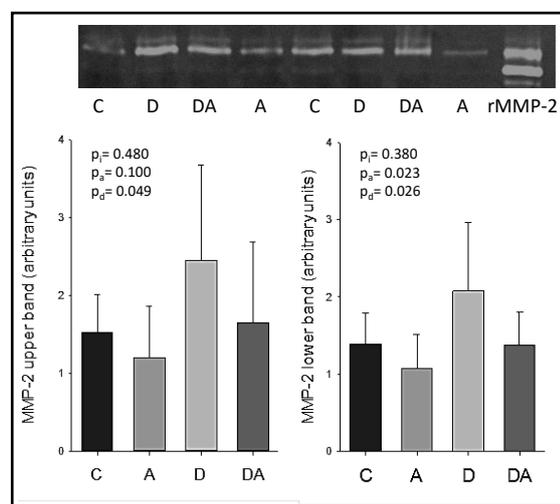
#### Myocardial metabolism

Doxorubicin increased phosphofructokinase and lactate dehydrogenase activity, along with decreased β-hydroxyacyl CoA dehydrogenase, pyruvate dehydrogenase and citrate synthase activity. The DA group showed greater activity of phosphofructokinase, β-hydroxyacyl CoA dehydrogenase and citrate synthase than group D. Açai administration decreased lactate dehydrogenase and increased pyruvate dehydrogenase activity both in rats that did and did not receive doxorubicin. Regarding the respiratory mitochondrial chain, doxorubicin decreased complex I, II and ATP synthase activities. Complex II activity was better in DA than D, and the DA group exhibited similar ATP synthase activity to group C (Table 4).

**Fig. 2.** Apoptosis-related protein expression. Figure shows Bcl-2 and caspase 3 protein expression evaluated by Western blot. All values of protein expression were normalized by GAPDH. C: control group; A: açai group; D: doxorubicin group; DA: doxorubicin + açai group. p value: two-way ANOVA; pi: p value for doxorubicin vs açai interaction; pa: p value for açai; pd: p value for doxorubicin.



**Fig. 3.** Matrix metalloproteinase-2 activity. Figure shows zymography gel image. White bands represent gelatin degradation by matrix metalloproteinase (MMP)-2. C: control group; A: açai group; D: doxorubicin group; DA: doxorubicin + açai group. rMMP-2: recombinant mouse/rat MMP-2 used as positive control. p value: two-way ANOVA; pi: p value for doxorubicin vs açai interaction; pa: p value for açai; pd: p value for doxorubicin.



### Nitric oxide metabolites

We did not observe differences in nitric oxide metabolite serum concentrations between groups (C:  $10.8 \pm 6.0$ , A:  $9.62 \pm 4.7$ , D:  $9.74 \pm 4.6$ , DA:  $10.9 \pm 6.7$  nM/ $\mu$ L;  $p_{\text{doxo}}=0.863$ ,  $p_{\text{açai}}=0.771$ ,  $p_{\text{interaction}}=0.536$ ).

**Table 3.** Oxidative stress marker and activity of antioxidant enzymes. C: control group; A: açai group; D: doxorubicin group; DA: doxorubicin + açai group; LH: lipid hydroperoxide; CAT: catalase; SOD superoxide dismutase; GSH-Px: glutathione peroxidase. Values are expressed as the mean  $\pm$  standard deviation or median and quartile interval (SOD). p value: two-way ANOVA, except <sup>®</sup>, which was analyzed by Mann-Whitney test (in this situation, p value was considered significant when  $<0.008$ ); pi: p value for doxorubicin vs açai interaction; \*different from C group; &different from D group

Variables	C (n=8)	A (n=8)	D (n=8)	DA (n=8)	p <sub>i</sub>	p <sub>açai</sub>	p <sub>doxo</sub>
LH (nmol/g tissue)	213 $\pm$ 40	180 $\pm$ 31	321 $\pm$ 38	240 $\pm$ 32	0.068	<0.001	<0.001
CAT ( $\mu$ mol/g tissue)	78.4 $\pm$ 19.2	104.4 $\pm$ 25.0	42.9 $\pm$ 10.5	62.2 $\pm$ 10.3	0.596	0.001	<0.001
SOD <sup>®</sup> (nmol/mg protein)	11.4 (9.9-13.1)	9.5 (9.0-11.6)	10.5 (9.6-12.9)	10.0 (9.4-11.2)	0.195	0.815	0.102
GSH-Px (nmol/mg tissue)	75.0 $\pm$ 8.2	69.9 $\pm$ 6.7	55.2 $\pm$ 8.1*	77.1 $\pm$ 5.1&	<0.001	0.002	0.018

**Table 4.** Activity of enzymes related to myocardium energy metabolism. C: control group; A: açai group; D: doxorubicin group; DA: doxorubicin + açai group. Values are expressed as the mean  $\pm$  standard deviation; p value: two-way ANOVA; pi: p value for doxorubicin vs açai interaction; \*different from C group; &different from D group; %different from A group

Variables	C (n=8)	A (n=8)	D (n=8)	DA (n=8)	p <sub>i</sub>	p <sub>açai</sub>	p <sub>doxo</sub>
$\beta$ -hydroxyacyl-CoA- dehydrogenase (nmol/mg protein)	31.5 $\pm$ 5.0	26.2 $\pm$ 3.9*	13.0 $\pm$ 2.8*	21.4 $\pm$ 2.4&%	<0.001	0.251	<0.001
Phosphofructokinase (nmol/g tissue)	145 $\pm$ 28.8	138 $\pm$ 19.8	176 $\pm$ 25.6*	133 $\pm$ 22.7&	0.047	0.008	0.156
Lactate dehydrogenase (nmol/mg protein)	58.3 $\pm$ 8.5	48.5 $\pm$ 6.7	111 $\pm$ 30.3	77.1 $\pm$ 25.6	0.263	0.002	0.001
Pyruvate dehydrogenase (nmol/g tissue)	267 $\pm$ 41.4	348 $\pm$ 33.2	152 $\pm$ 34.2	268 $\pm$ 37.2	0.206	<0.001	<0.001
Citrate synthase (nmol/mg protein)	49.0 $\pm$ 7.2	42.6 $\pm$ 5.7	26.6 $\pm$ 6.5*	36.3 $\pm$ 6.5&	<0.001	0.150	<0.001
Complex I (nmol/mg protein)	13.4 $\pm$ 4.3	13.9 $\pm$ 3.4	9.1 $\pm$ 2.5	10.9 $\pm$ 2.1	0.560	0.308	0.003
Complex II (nmol/mg protein)	4.8 $\pm$ 1.1	4.1 $\pm$ 0.9	1.6 $\pm$ 0.4*	2.5 $\pm$ 0.6&%	0.009	0.786	<0.001
ATP synthase (nmol/mg protein)	22.7 $\pm$ 3.2	22.4 $\pm$ 2.9	13.0 $\pm$ 2.7*	23.8 $\pm$ 3.0&	<0.001	<0.001	<0.001

## Discussion

The aim of this study was to investigate the role of açai supplementation in prevention of cardiac dysfunction and biochemical changes in acute doxorubicin-induced cardiotoxicity. Our results showed that doxorubicin administration was able to induce cardiotoxicity, with morphological changes, impairment of left ventricular function, increases in MMP-2, oxidative stress and changes in cardiac metabolism compatible with pathological conditions. When açai was administered in tandem, we observed improvements in left ventricular function and cardiac metabolism and decreases in oxidative stress and MMP-2.

Açai is composed of proteins, fibers, minerals, unsaturated fatty acids and high levels of high antioxidant phenolic compounds, such as anthocyanin [15]. Anthocyanin may be absorbed as an intact molecule in the stomach or small bowel, as these intact molecules have been found in plasma and urine [29]. Rats ate 25 g of the chow açai mix per day, which is equivalent to 1.25 g of açai pulp per day. This amount is equivalent to 39 g of açai for humans (2.5 tablespoons per day) [30].

Doxorubicin can induce generalized side effects, including alopecia, neutropenia, nausea, vomiting and decreased appetite [3]. Indeed, we observed decreased food and water ingestion, followed by decreases in body weight in rats that received doxorubicin. Reduced

body weight was probably due to decreases in intravascular volume as the period was very short (48 hours) for decreases in body mass. As a result of intravascular volume depletion, rats could have tissue hypoperfusion. To maintain organic perfusion, the rats may have had peripheral vasoconstriction and increased afterload. In hearts with dysfunction, such as hearts with acute cardiotoxicity, increased afterload could contribute to cardiac function impairment.

Rats that received doxorubicin showed worse systolic and diastolic left ventricular function on echocardiography than rats that did not receive doxorubicin. The same results were observed in terms of cardiac function evaluation *in vitro* (isolated heart study), where rats that received doxorubicin exhibited lower  $+dP/dt$  (that represents systolic function) and lower  $-dP/dt$  (that represents diastolic function). When açai was also administered, we observed improved left ventricular function *in vivo*, but not *in vitro*. We believe that these differences could result from vasodilatation induced by açai administration [31], as the afterload component was absent in the isolated heart study.

Doxorubicin could induce vasoconstriction mediated by several mechanisms, including the renin-angiotensin-aldosterone system and adrenergic stimuli [7]. Another mechanism involved in doxorubicin-induced vasoconstriction is nitric oxide mediation [32] and açai could affect the nitric oxide system [33-35]. To investigate the vasodilator effect of açai, we evaluated nitric oxide metabolite serum levels. However, results were similar between groups.

Increases in oxidative stress are frequently implicated in the pathophysiology of doxorubicin-induced cardiotoxicity. The myocardium is more susceptible to oxidative damage than other organs for several reasons. First, the heart has lower levels of catalase and superoxide dismutase [36]; second, glutathione peroxidase is quickly inactivated by doxorubicin in the myocardium [36]; third, the heart is rich in mitochondria, an important source of reactive oxygen species production and a target for doxorubicin action [32]. Indeed, in animals that received doxorubicin we observed decreased glutathione peroxidase and catalase activities and increased myocardial lipid hydroperoxide, a cellular lipid damage marker. Lipid peroxidation disrupts the cell barrier, cellular membrane channels and membrane pumps, which contributes to worsening cardiac function. Açai administration reduced lipid hydroperoxide in both the A and DA groups, thus reinforcing its antioxidant properties.

A pro-oxidative environment is one of the most potent stimuli for MMP-2 activation, including intracellular activation, where MMP-2 has a high affinity for contractile proteins and could contribute to myocardium dysfunction [11]. MMP activation is essential for the remodeling process. Increased MMP-2 gene expression and activation are early events in cardiotoxicity and follow heart dilatation [5, 6, 12, 13]. Indeed, our results showed higher MMP-2 activity in doxorubicin-treated animals combined with increases in oxidative stress and increases in cardiac left chambers. It could be a potential target for preventing dilated cardiomyopathy induced by doxorubicin. In our study, açai supplementation reduced MMP-2 activation and it was accompanied by decreased left ventricle systolic and left atrium diameters.

There are several mechanisms that can generate reactive species, one of which is the mitochondrial phosphorylative respiratory chain. A previous study showed that doxorubicin interfered with all mitochondrial enzymatic complex activities [37]. However, another study showed that the effect of doxorubicin on mitochondrial complex activity was not homogenous, with more intense effects in complex III and IV, lower intensity in complex I and no effect in complex II [38]. We observed that doxorubicin decreased complex I, II and ATP synthase activity in rats. Açai attenuated mitochondrial dysfunction because of increased ATP synthase activity near group C levels, and considerably improved complex II activity. Additionally, açai supplementation increased citrate synthase activity, which could have improved the Krebs cycle function. By increasing ATP synthase activity, açai may have collaborated in increasing ATP generation.

In physiological situations, fatty acids are the main substrate for energy generation in the myocardium. However, in pathologic situations, the myocardium starts to use mainly glucose, as fatty acids require more energy to be metabolized than glucose [39]. Acutely, it is a protective strategy for the myocardium, but chronically, the accumulation of fatty acids inside myocytes could be damaging [40]. In our study, doxorubicin induced changes compatible with pathological situations. We observed increased activity in enzymes related to the glucose metabolism and decreased activity in  $\beta$ -hydroxyacyl CoA dehydrogenase enzyme related to fatty acid metabolism. When açai was also administered, we observed that enzyme activity related to the energetic metabolism was maintained close to normal.

Despite the importance of apoptosis in the doxorubicin antitumor effect, apoptosis can also contribute to cardiotoxicity. Doxorubicin could interfere with p53 and p38 regulation, PI3K/Akt pathway inhibition, activated caspases and deregulated Bcl-2 and Bax protein expression [32, 41-44]. In contrast to previous studies showing that doxorubicin decreased Bcl-2 expression [43, 44], we did not observe differences in Bcl-2 expression between groups. Perhaps the Bcl-2/Bax ratio is more important than Bcl-2 expression alone in this experimental model [45]. As expected, our study showed doxorubicin-induced increases in caspase-3 protein expression, suggesting that apoptosis is an important mechanism in doxorubicin-induced cardiotoxicity, but açai had no effect on this injury mechanism.

It is important to emphasize that our experimental model utilized a high single dose of doxorubicin and did not exactly reproducing doxorubicin administration in humans. However, this model is fast, less expensive and reduces animal suffering. In addition, chronic cardiotoxicity shares pathophysiological mechanisms with acute cardiotoxicity and is a good model for studying potential substances and strategies that can prevent cardiotoxicity. Despite our encouraging results, clinical studies are necessary to determine the role of açai in this severe complication.

## Conclusion

Acute doxorubicin administration induced *in vivo* and *in vitro* left ventricular dysfunction, increased oxidative stress, caspase-3 protein expression, MMP-2 activation, and induced changes in myocardium energy metabolism compatible with pathological conditions. Açai supplementation attenuated cardiotoxicity with improved cardiac function *in vivo*, decreased oxidative damage and MMP-2 activation, and modulated myocardial metabolism changes including improvement in the mitochondrial phosphorylative respiratory chain. Açai can therefore potentially prevent or attenuate doxorubicin-induced cardiotoxicity.

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## Disclosure Statement

The authors declare that they have no competing interests.

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