

Original Paper

Açaí (Euterpe Oleraceae Mart.) Seeds Regulate NF- κ B and Nrf2/ARE Pathways Protecting Lung against Acute and Chronic Inflammation

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Key Words

Acute lung injury • Oxidative stress • Inflammation • Chronic lung injury • Açaí seeds

Abstract

Background/Aims: Respiratory diseases are the world's biggest cause of mortality and disability. Specific nutrients have been proposed to positively affect disease progression as novel therapy alternatives to glucocorticosteroids. There has been a lot of attention in the possible health advantages of dietary assumption of Açaí Seeds, popular native fruit found in the Amazon region which is rich in bioactive compounds. Until today nobody investigated the beneficial property of Açaí Seeds administration in lung disease. **Methods:** In our study we use two model of lung disease: for acute lung disease we use an intrapleural injection of Carrageenan; for chronic disease we used an intratracheal instillation of bleomycin. Açaí Seeds was administered orally dissolved in saline. **Results:** We found that Açaí Seeds was able to reduce histological alteration, cells infiltration, pro inflammatory cytokine release, inflammation, and oxidative stress in both acute and chronic model of lung disease. **Conclusion:** Our data clearly demonstrate for the first time that Açaí Seeds administration was useful against lung disease by the reduction of NF- κ B nuclear translocation and by the stimulation of Nrf2/ARE pathways promoting the physiological antioxidant defense.

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Introduction

Acute and chronic lung diseases are caused by inflammation in the lungs. Acute lung injury (ALI) is defined by a fast inflammatory response in the lungs caused by either communicable (infection) or non-communicable (acid aspiration) disorders [1]. As the lung is exposed to external factors either through the airways such as infectious agents and/or environmental pollutants or through the blood stream like sepsis and/or endotoxin, ALI can result in acute respiratory distress syndrome (ARDS), a spectrum of lung diseases characterized by a severe inflammatory process in the lung parenchyma causing diffuse alveolar damage and respiratory failure [2-4]. When acute inflammation does not resolve completely, chronic inflammation develops. Chronic inflammatory responses clean necrotic debris and apoptotic cells from acute inflammation, guard against and inhibit infection spread, and heal and repair lung tissue damage [5]. Pneumonia and acute respiratory distress syndrome (ARDS) are examples of acute inflammation, whereas asthma and chronic obstructive lung disease (COPD) are examples of chronic inflammation [6].

Most airway pathologies have in common an implementation of inflammatory pathways activation and an increase oxidative stress in various kinds of respiratory cells. Intracellular buildup of reactive oxygen species (ROS) can damage macromolecules such as DNA, proteins, and lipids, resulting in cell abnormalities or death, on the other hands, inflammatory reactions are triggered by unbalanced redox equilibrium and cell damage, which exacerbate oxidative stress [7]. One of the most regulators that protects cells from ROS and xenobiotics is NF-E2-related factor 2 (Nrf2), a cap 'n' collar (CNC)-bZIP family transcription factor. Nrf2 binds to antioxidant response elements (AREs) and activates a battery of antioxidant and detoxifying genes when activated by oxidative and xenobiotic stressors [8]. Nrf2 also activates several developmental genes, allowing it to control cell proliferation, differentiation, cell death, and metabolic programs [9, 10]. Furthermore, Nrf2 interacts with a variety of other proteins and pathways, forming a complex regulatory network for redox homeostasis and other cellular functions. For a variety of human disorders, including respiratory ailments, Nrf2 has been identified as a crucial factor and an important therapeutic target [11]. Additionally, under oxidative stress, complex biological pathways (transcriptional and post-transcriptional) connect Nrf2 and NF- κ B. Nrf2 deficiency increase NF- κ B activity, which promotes cytokine release as well as an increase in molecules adhesion production and more [12].

For these reasons the development of Nrf2-NF- κ B targeting therapeutics will be greatly aided by a thorough knowledge of the molecular partners and downstream activities of Nrf2.

Glucocorticosteroids are often used as an anti-inflammatory therapy for acute and chronic lung inflammation, but specific nutrients have been proposed to positively affect disease progression as novel therapy alternatives for individuals suffering from major side effects or developing resistance [13-15].

In the last years, the foods that has been attracting the attention of researchers in recent years is Acai seeds. Acai Berry is a berry with various nutritional properties and a certain therapeutic potential. This fruit, with a sour and pleasant taste, is produced from a palm (*Euterpe Oleracea*), widespread only in the Amazon. Considered a highly energetic food, the Acai fruit has been consumed by the Indians of the Amazon for centuries, as a food base and natural remedy for various diseases.

Recently, for its antioxidant, energizing and potentially slimming properties, Acai Berry has gained popularity as a functional ingredient, especially following the marketing of its derivatives in the form of juices and food supplements. Currently, the known effects of Acai are mainly determined by the high content of antioxidants, unsaturated fats (omega-3, omega-6 and omega-9), vitamins, minerals such as iron and calcium, that make it a fruit hypocholesterolemic, immunostimulant, anti-inflammatory and antioxidant [16-24].

However, from a scientific point of view, the evidence supporting the positive health effects is still scarce. The research is oriented towards the study of the other possible properties of the Açai Seeds, in particular on its antioxidant activity and on its potential effects in the prevention of diseases caused by oxidative stress. Procyanidins (dimers up to

pentamers), protocatechuic acid, and epicatechin were the most prevalent molecules found in previous investigations and identification of chemicals in Açai Seed extracts. Furthermore, seed extracts had comparable or better scavenging ability against peroxy, peroxy nitrates, and hydroxyl radicals than the pulp, which they linked to the observed procyanidins [19, 25, 26].

With these background in our mind, in this work we tested for the first time the beneficial effects of Açai Seeds assumption in an acute and chronic model of lung inflammation.

Materials and Methods

Animals

CD1 mice (25-30 g, Envigo, Milan, Italy) were employed. The University of Messina Review Board for animal care (OPBA) approved the study (Ethical Code: 89126.24/2021-PR). All animal experiments agree with the new Italian regulations (D.Lgs 2014/26), EU regulations (EU Directive 2010/63) and the ARRIVE guidelines.

Experimental Design and Groups

First experimental design. ALI was made as previously described by Di Paola et al. [27]. Briefly, mice were anaesthetized and saline (0.1 mL) or saline containing 2% λ carrageenan (CAR) (0.1 ml) was injected into the pleural cavity. Açai seeds were washed with water and frozen at -80°C . The frozen seed was lyophilized and stored at -20°C as previously described [28]. Powder obtained were following dissolved in saline.

Mice were casually distributed into the following groups:

- (1) CAR: mice were subjected to the CAR injection described above, and treated with saline solution;
- (2) CAR+Açai Seeds: mice were subjected to the CAR injection described above, and treated orally 1 h after with Açai Seeds dissolved in saline at different doses (100 mg/kg, 300 mg/kg and 500 mg/kg);
- (3) Sham group: animals were subjected to an injection of saline solution;
- (4) Sham groups+Açai Seeds: animals were treated with Açai Seeds dissolved in saline at different doses (100 mg/kg, 300 mg/kg and 500 mg/kg) (data not shown).

In another set of experiments, we compare the highest dose of Açai Seeds with Resveratrol dissolved in saline and administered by oral route at the dose of 50 mg/kg (Supplementary Fig. S1 – for all supplementary material see www.cellphysiolbiochem.com) [29].

After four hours, animals were killed by cervical dislocation under anesthesia and lungs were conserved for further study. Doses were chosen based on a dose-response study carried out in our lab. Açai Seeds at the doses of 100 mg/kg and 300 mg/kg did not show any advantage in CAR-induced lung inflammation. For this reason, we carried on our experiment only with the dose of 500 mg/kg.

Second experimental design. Bleomycin administration was performed as previously described to reproduce lung fibrosis [30, 31]. In detail, bleomycin sulphate (1mg/kg body weight) was delivered by a single intratracheal administration. A volume of 100 μL was injected at end-expiration to guarantee delivery to the distal airways. This was immediately followed by 300 μL of air [32-34].

Mice were randomly divided into groups:

- (I) Bleomycin: animals that receive one injection of bleomycin.
- (IV) Bleomycin+ Açai Seeds: mice were subjected to the bleomycin injection described above and treated orally with Açai Seeds dissolved in saline at the dose of 500 mg/kg for 14 days.
- (I) Sham: animals that were exposed to the vehicle (saline with 10% of DMSO).

At the end of experiment, mice were sacrificed by cervical dislocation under anesthesia, and lung tissue and bronchoalveolar lavage fluid (BALF) were collected as previously described [35-40].

Exudate and leukocytes count

Measure of exudates and count of leukocytes was made as previously described [41]. Briefly, at the end of the experiment, the chest was opened, and the pleural cavity washed with 1 mL of saline solution containing heparin and indomethacin. The exudate and washing solution were removed by aspiration and the total volume measured. The leukocytes in the exudate were suspended in phosphate-buffer saline (PBS) and counted with after Blue Toluidine staining an optical microscope in a Burker's chamber.

Measurement of Lung Edema

At the end of the experiment, wet lung weights were recorded. The lungs were subsequently dried for 48 h at 180 °C and then weighed again. The water content of the lungs was calculated as the ratio of the wet:dry weight of the tissue [29-31, 42].

Bronchoalveolar Lavage (BAL)

At the end of experiment, mice were euthanized, and the tracheas were cannulated to perform the lavage for cell counting, as previously described [29-31, 42]. Additionally, from BAL, we analyzed the total protein content using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) as previously described [43, 44].

Western Blot Analysis of Cytosolic and Nuclear Extracts

Extracts of the cytosol and nucleus were prepared, as previously mentioned [45-49]. The following primary antibodies were used: anti-iNOS (1:500, Santa Cruz Biotechnology, #sc-7271), anti-NRF-2 (1:500, Santa Cruz Biotechnology, Heidelberg, Germany, #sc-365949), anti-heme oxygenase 1 (HO-1; 1:500, Santa Cruz Biotechnology, Heidelberg, Germany, #sc-136960), anti-(α -sma antibody (1:250, Santa Cruz Biotechnology), anti-I κ b α (1:500, Santa Cruz Biotechnology, #sc-1643), and anti-nf κ b (1:500, Santa Cruz Biotechnology, #sc8414) in 1 \times PBS, 5% w/v non-fat dried milk, and 0.1% Tween 20, at 4 °C overnight [42, 50-52]. For the cytosolic fraction, Western blots were also explored with antibody against β -actin protein (1:500, Santa Cruz Biotechnology, Dallas, TX, USA). The same methods were used for nuclear fraction with lamin A/C (1:500, Sigma-Aldrich Corp., Milan, Italy) [53, 54]. Signals were examined with an enhanced chemiluminescence (ECL) detection system reagent, according to the manufacturer's instructions (Thermo, Monza, Italy). The relative expression of the protein bands was quantified by densitometry with BIORAD ChemiDoc™ XRS+ software [46, 50, 55-57].

Evaluation of Tissue Lipid Peroxidation

Lipid peroxidation were assessed with the consolidated method of malonaldehyde as previously described and expressed as μ M MDA/mg of proteins [58].

Cytokine and Nitrite/Nitrate Measurement

TNF- α or IL-6 or IL-1 β and nitrite/nitrate concentration were measured in the exudates using ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions and expressed as pg/mL or with Griess reaction and express as mmol/mouse [27, 55, 56, 58-64].

Assessment of antioxidant system

The SOD activity assay was measured following the method described by Marklund and Marklund at 420 nm [65]. The results are presented as SOD units/mg protein. Moreover, the activity of CAT was assessed following the method of Aebi in terms of mmoles of hydrogen peroxide (H₂O₂) consumed per min per mg of protein [66]. The results are presented as CAT units/mg protein. Furthermore, the level of the nonenzymatic cellular antioxidant glutathione was measured following the method of Moron et al. [67-69]. The results are presented as GSH units/mg protein.

Measurement of Reactive Oxygen Species (ROS)

2',7'-dichlorofluorescein diacetate (Molecular Probes, Eugene, OR, USA) was used to detect ROS production in lung tissue homogenates as previously described [70-72]. The results were normalized as ROS produced per milligram of protein.

Histopathological Evaluation with Hematoxylin/Eosin and Toluidine Blue and Masson

The lung tissues were stained with hematoxylin and eosin (H&E) for architecture alterations or toluidine blue for mast cells degranulation, or with Masson Thrichrome for collagen deposition and analyzed using a light microscopy (Leica DM6, Milan, Italy) associated with an Imaging system (LasX Navigator, Milan, Italy) [46, 49, 50, 73].

Immunohistochemical localization of Nitrotyrosine, poly(ADP-ribose), ICAM and P-Selectin

At the end of experiments lung tissue were incubated with anti-ICAM-1 murine polyclonal antibody (1/100 in PBS, v/v, Santa Cruz Biotechnology), anti-P-selectin murine polyclonal antibody (1/100 in PBS, v/v, Santa Cruz Biotechnology), anti-PAR murine polyclonal antibody (1/100 in PBS, v/v, Santa Cruz Biotechnology) and anti-nitrotyrosine rabbit polyclonal antibody (1:200 in PBS, v/v, Millipore) as previously described [29-31, 42, 53, 57, 74-77]. Immunohistochemical images were collected using (Leica DM6, Milan, Italy) associated with an Imaging system (LasX Navigator, Milan, Italy). The digital images were opened in ImageJ, followed by deconvolution using the color deconvolution plug-in. When the IHC profiler plug-in is selected, it automatically plots a histogram profile of the deconvoluted DAB image, and a corresponding scoring log is displayed. The histogram profile corresponds to the positive pixel intensity value obtained from the computer program. All immunohistochemical analyses were carried out by two observers blinded to the treatment [29-31, 42, 74-78].

Materials

Unless otherwise stated, all compounds were purchased from Sigma-Aldrich.

Statistical Evaluation

In this study, the data are expressed as the average \pm SEM and represent at least 3 experiments carried out in different days. For *in vivo* studies, N represents the number of animals used. The number of animals used for *in vivo* studies was carried out by G*Power 3.1 software (Die Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). Data were analyzed by an experienced histopathologist, and all the studies were performed without knowledge of the treatments. The results were analyzed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. A *p* value less than 0.05 was considered significant.

Results

Açai Seeds administration reduce histological damage CAR-induced

Architecture alteration, infiltration of neutrophils and oedema are the main characteristic of ALI as showed in Fig. 1 (B-B1 see histological score 1F) compared to sham animals (Fig. 1A-A1 see histological score 1F). Açai Seeds at the doses of 100 mg/Kg (Fig. 1C-C1 see histological score 1F) and 300 mg/Kg (Fig. 1D-D1 see histological score 1F) not showed any significantly decrease in the inflammatory state. On the other hand, the higher dose used, that is 500 mg/kg (Fig. 1E-E1 see histological score 1G), showed a significantly reduction in the degree of injury CAR-induced.

Açai Seeds administration reduce exudates and cytokines release

Injection of CAR stimulated an acute inflammatory response characterized by the accumulation of exudates (Fig. 2A) rich in content of pro-inflammatory cytokines TNF- α (Fig. 2B) as well as IL-1 β (Fig. 2C). Açai Seeds administered orally at the dose of 500 mg/kg was able to significantly reduced exudates formation as well as pro-inflammatory cytokines production.

Fig. 1. Inhibitions of lung injury CAR-induced by Açai Seeds. Sham (Panel A, A1); CAR (Panel B, B1); CAR+ Açai Seeds 100 mg/kg (Panel C, C1); CAR+ Açai Seeds 300 mg/kg (Panel D, D1); CAR+ Açai Seeds 500 mg/kg (Panel E, E1); Histological score (F). As showed in Fig. after carrageenan induction we found architecture alteration, cells infiltration and oedema. Treatment at the lowest doses of Açai Seeds doesn't have any significantly effects. The highest dose showed a significantly protection against acute inflammation. Values are means \pm SEM of 6 mice for all group. Photo shown is representative of the results obtained. See manuscript for further details. *** $p < 0.001$ vs. sham; ### $p < 0.001$ vs. CAR. 20x magnification, scale bar 100 μm ; 40x magnification, 75 μm .

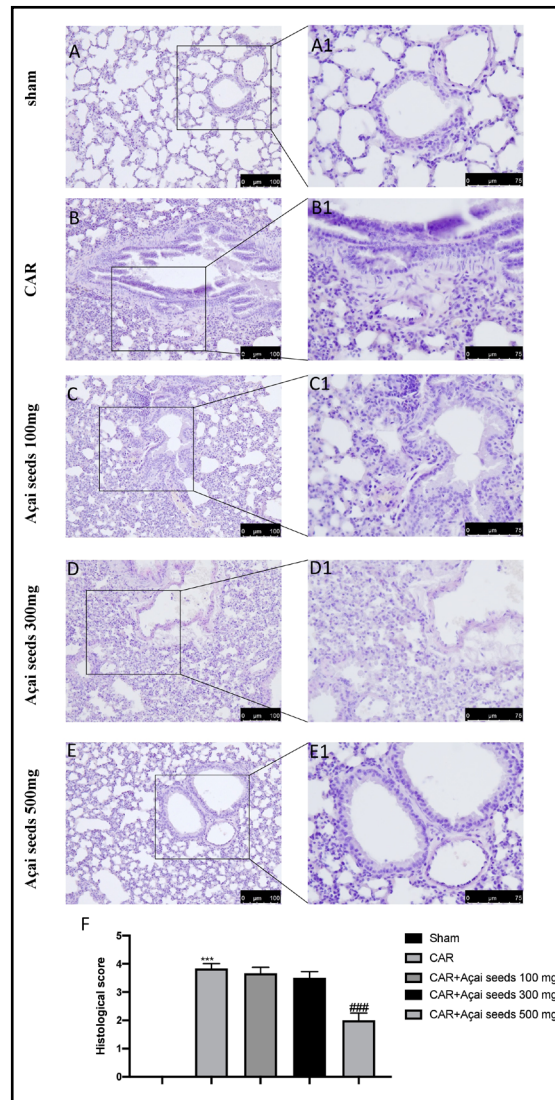
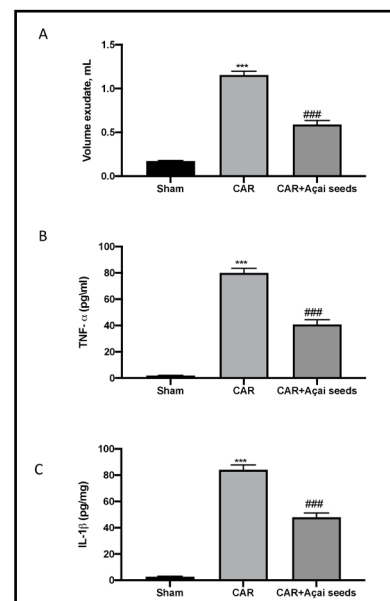


Fig. 2. Açai Seeds administration decreases exudates volumes and pro-inflammatory cytokines. Volume exudates (A); $\text{TNF-}\alpha$ (B) and $\text{IL-1}\beta$ (C). Açai Seeds showed a significantly ability to decrease pro-inflammatory cytokines release and exudates. See manuscript for further details. Values are means \pm SEM of 6 mice for all group. *** $p < 0.001$ vs. sham; ### $p < 0.001$ vs. CAR.



Açai Seeds inhibits mast cells degranulation, PMNs infiltration and MPO production

ALI was also characterized by a significant influx of mast cells, PMNs, and leukocytes into the interstitium and bronchoalveolar space. As believed, after CAR injection we found a significantly increase in mast cell degranulation, PMNs infiltration and MPO levels (Fig. 3B, 3D and 3E) compared to sham animals (Fig. 3A, 3D and 3E) [2]. On the other way the administration of Açai Seeds at the dose of 500 mg/kg significantly decreases all these parameters.

Açai Seeds administration decrease inflammation and improve antioxidant defense

To understand a possible molecular pathway by which Açai Seeds act, we investigated by western blots the activation of NF- κ B pathways. As showed in Fig. 4, we found that following CAR-injection there were a significantly decrease in I κ B- α (Fig. 4A and relative densitometric analysis A1) and therefore a significantly increase in NF- κ B expression compared to sham group (Fig. 4B and relative densitometric analysis B1). Açai Seeds administration at the dose of 500 mg/kg was able to restore in I κ B- α (Fig. 4A and relative densitometric analysis A1) expression as well as to decrease NF- κ B translocation (Fig. 4B and relative densitometric analysis B1). Additionally, considering to anti-oxidative property of Açai Seeds, we investigated Nrf-2 pathways, always by western blot. We found that following CAR-injection into pleural cavity, there were a small but significant increase in Nrf-2 expression (Fig. 4C and relative densitometric analysis C1) as well as in HO-1 expression (Fig. 4 D and relative densitometric analysis D1). After, Açai Seeds administration both expression of Nrf-2 (Fig. 4C and relative densitometric analysis C1) and HO-1 expression (Fig. 4D and relative densitometric analysis D1) were significantly improved.

Fig. 3. Açai Seeds administration decreases cells infiltration. Sham (Panel A); CAR (Panel B); CAR+Açai Seeds 500 mg/kg (Panel C); PMNs infiltration (D); MPO levels (E). Yellow arrow indicates lung mast cells. Values are means \pm SEM of 6 mice for all group. Photo shown is representative of the results obtained. See manuscript for further details. *** $p < 0.001$ vs. sham; ### $p < 0.001$ vs. CAR. 40x magnification, 75 μ m.

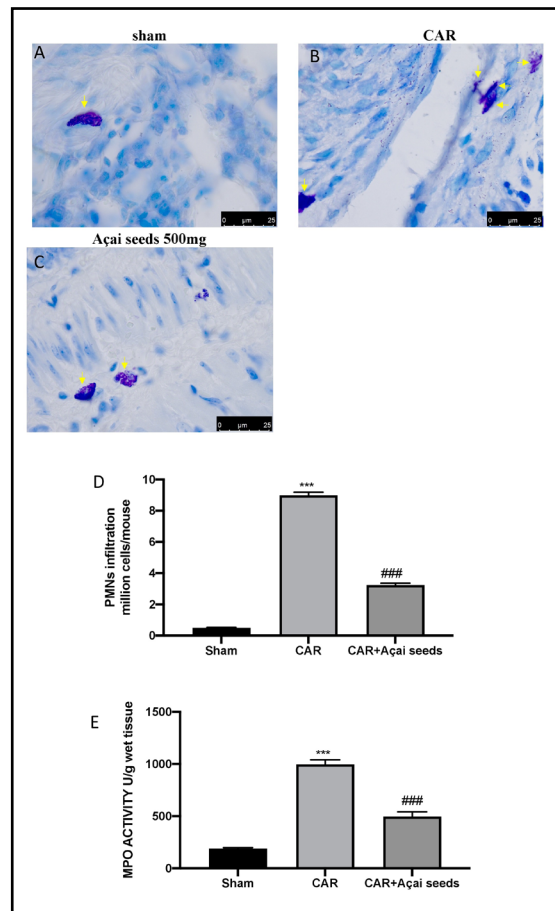
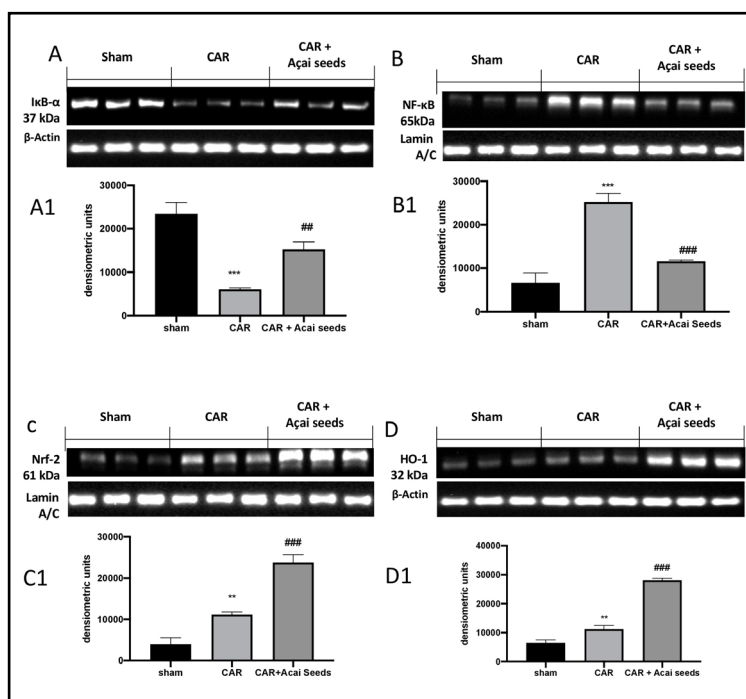


Fig. 4. Açai Seeds decrease NF- κ B traslocation and improve Nrf-2 activation. Western Blots and relative densitometric analysis for I κ B- α (A and A1), NF- κ B (B and B1), Nrf-2 (C and C1) and HO-1 (D and D1). Western Blots of lung samples indicates that after carrageenan injection there was a significantly increase in NF- κ B nuclear translocations as well as a small but significantly increase as a physiological anti-oxidant response in NRF-2 pathways. Açai Seeds at the dose of 500 mg/kg showed anti inflammatory and anti-oxidative properties. Values are means \pm SEM of 6 mice for all group. See manuscript for further details. *** p<0.001 vs. sham; ## p<0.01 vs. CAR; ### p<0.001 vs. CAR.



Açai Seeds reduce nitrosative stress CAR-induced

By western blots, immunohistochemistry and nitrite/nitrate analysis we investigated the effects of Açai Seeds administration on inhibition of nitrosative stress. We found that, after CAR injection there was a significantly increase in nitrotyrosine (Fig. 5B see graph 5D), iNOS (Fig. 5F see densitometric analysis F1) and nitrite/nitrate levels (Fig. 5E) compared to sham group (Fig. 5A see graph 5D for nitrotyrosine expression; Fig. 5F and densitometric analysis F1 for iNOS activity; Fig. 5E for nitrite/nitrate levels). As assumed, Açai Seeds administration protects the lung against nitrosative stress (Fig. 5A see graph 5D for nitrotyrosine expression; Fig. 5F and densitometric analysis F1 for iNOS activity; Fig. 5E for nitrite/nitrate levels).

Açai Seeds reduce adhesion molecules expressions CAR-induced

The intensity of ICAM and P-selectin staining significantly increases in lung tissue slices taken from CAR-treated group (Fig. 6B and 6F, see graph D and H, respectively) compared to sham (Fig. 6A and 6E, see graph D and H, respectively). Açai Seeds at the dose of 500 mg/kg significantly decreases both staining (Fig. 6C and 6H, see graph D and H, respectively).

Açai Seeds administration reduce DNA damage and lipid peroxidation

Immunohistochemical analysis of lung tissue collected from CAR-treated group showed an increased in PARP positive cells (Fig. 7 Panel B, see graph D) compared to sham group (Fig. 7 Panel B, see graph D). Açai Seeds oral administration at the dose of 500 mg/kg was able to significantly decrease PARP expression (Fig. 6 Panel C, see graph D). Additionally, we evaluated lipid peroxidation by MDA activity, and we found that CAR injection in pleural cavity significantly increased lipid peroxidation compared to sham group (Fig. 7E). On the other hand, Açai Seeds oral administration at the dose of 500 mg/kg significantly decrease lipid peroxidation levels.

Fig. 5. Effect of Açai Seeds on nitrosative stress. Sham (Panel A); CAR (Panel B); CAR+Açai Seeds 500 mg/kg (Panel C); graphical analysis (D) for nitrotyrosine. Nitrite/nitrate levels (E); Western Blot and relative densitometric analysis for iNOS (F and F1). Açai Seeds was able to reduce nitrosative stress CAR-induced by the modulation of nitrite/nitrate levels, nitrotyrosine and iNOS expression. Values are means \pm SEM of 6 mice for all group. Photo shown is representative of the results obtained. See manuscript for further details. *** $p < 0.001$ vs. sham; ## $p < 0.01$ vs. CAR; ### $p < 0.001$ vs. CAR. 20x magnification, scale bar 100 μ m.

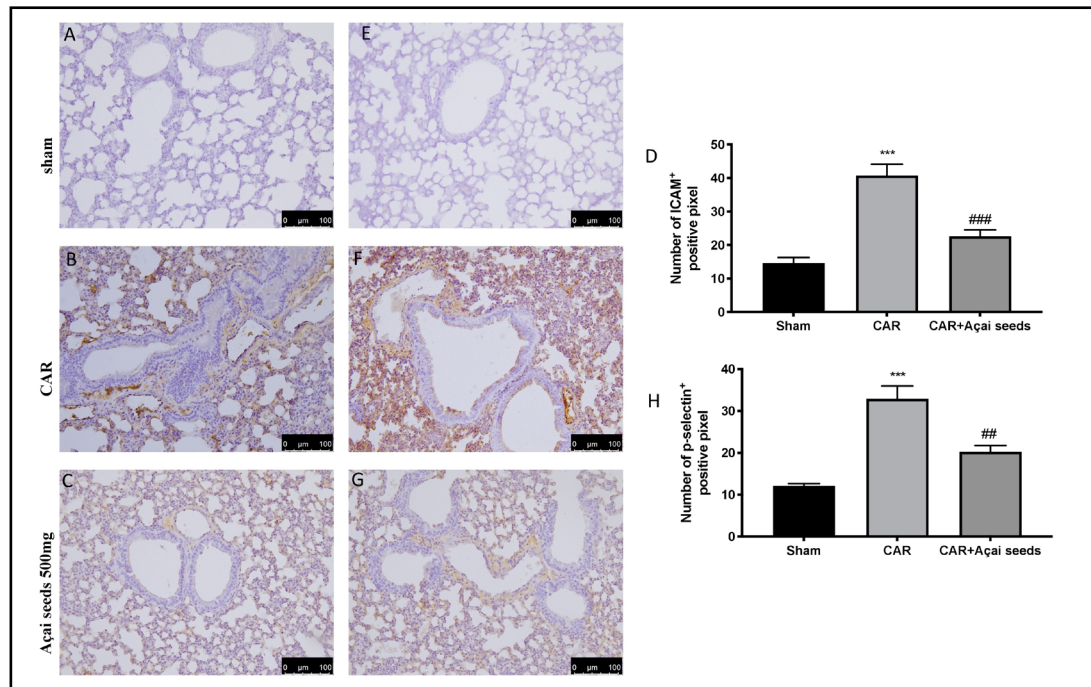
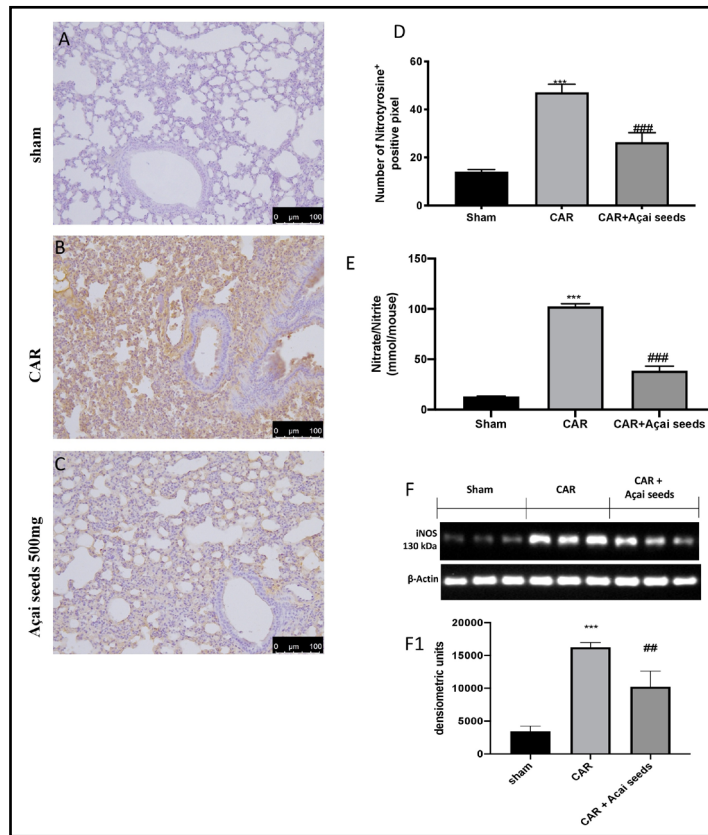
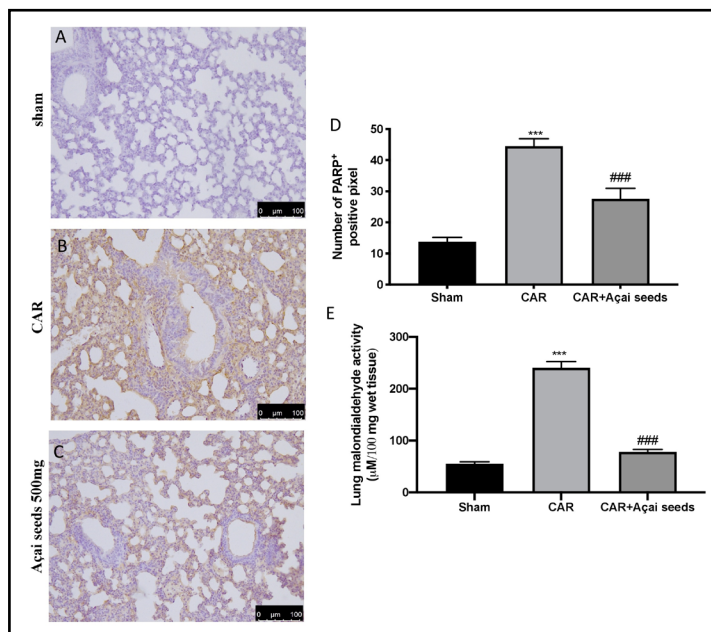


Fig. 6. Açai Seeds administration decreases ICAM and P-Selectin expression. Sham (Panel A); CAR (Panel B); CAR+Açai Seeds 500 mg/kg (Panel C); graphical analysis (D) for ICAM. Sham (Panel E); CAR (Panel F); CAR+ Açai Seeds 500 mg/kg (Panel G); graphical analysis (H) for p-selectin. As showed in Fig. Açai Seeds was able to reduce adhesion molecules expression CAR-induced. Values are means \pm SEM of 6 mice for all group. Photo shown is representative of the results obtained. See manuscript for further details. *** $p < 0.001$ vs. sham; ## $p < 0.01$ vs. CAR; ### $p < 0.001$ vs. CAR. 20x magnification, scale bar 100 μ m.

Fig. 7. Effect of Açai Seeds on PARP expression and MDA levels. Sham (Panel A); CAR (Panel B); CAR+ Açai Seeds 500 mg/kg (Panel C); graphical analysis (D) for PARP. Malondialdehyde activity levels (E). CAR injection induced a strong lipid peroxidation and a significantly expression in PARP. Both inflammatory markers were significantly reduced after Açai seeds administration. Values are means \pm SEM of 6 mice for all group. Photo shown is representative of the results obtained. See manuscript for further details. *** $p < 0.001$ vs. sham; ### $p < 0.001$ vs. CAR. 20x magnification, scale bar 100 μ m.



Açai Seeds reduce bleomycin-induced histological damage

Administration of bleomycin induce a significant alteration in the lung architecture, as highlighted in inflamed areas with notable presence of neutrophils and thickening of the lung tissue (Fig. 8B-B1 see Ashcroft score 8D) compared to the sham group (Fig. 8A-A1 see Ashcroft score 8D). Daily administration of Açai Seeds at the dose of 500 mg/kg for 14 days significantly decreases histological damage bleomycin-induced (Fig. 8C-C1 see Ashcroft score 8D). Additionally, the increase in the wet:dry lung weight ratio due to infiltration of inflammatory cells and edema founded in bleomycin group where significantly reduced after Açai Seeds administration (Fig. 1F).

Açai Seeds administration reduced bleomycin-induced inflammatory cell migration

Bleomycin damage is characterized by damage to the endothelial lining of small vessels and capillaries, accompanied by vascular congestion and increased microvascular permeability, which leads to an inflammatory response [79]. After bleomycin injection, we found an increase in cellular density in BAL (Fig. 9 from A to E for total cells, neutrophils, lymphocytes, and macrophages, respectively) as well as an increase in protein content (Fig. 9E) compared to the sham group. After 14 days of oral administration of Açai Seeds at the dose of 500 mg/kg we found a significantly decrease in all parameters evaluated.

Açai Seeds decrease bleomycin-induced fibrosis

Masson's trichrome staining and western blot analysis of α -SMA were used to evaluate the pulmonary fibrosis in lung tissue. After bleomycin injection, we found a significantly increase in fibrotic lesions and collagen accumulation in the lungs of mice (Fig. 10B and graph 10D) compared to the sham group (Fig. 10A and graph 10D). On the other hand, daily administration of Açai Seeds at the dose of 500 mg/kg significantly decreases collagen deposition and fibrotic scar. Another consolidated marker of fibrosis in the literature is represented by the expression of the α -SMA [30, 80]. As presumed, we found a significant increase in the group subjected to bleomycin when compared to the sham group (Fig. 10E and densitometric analysis 10-E1). A significantly decrease in α -SMA expression were found after Açai Seeds administration.

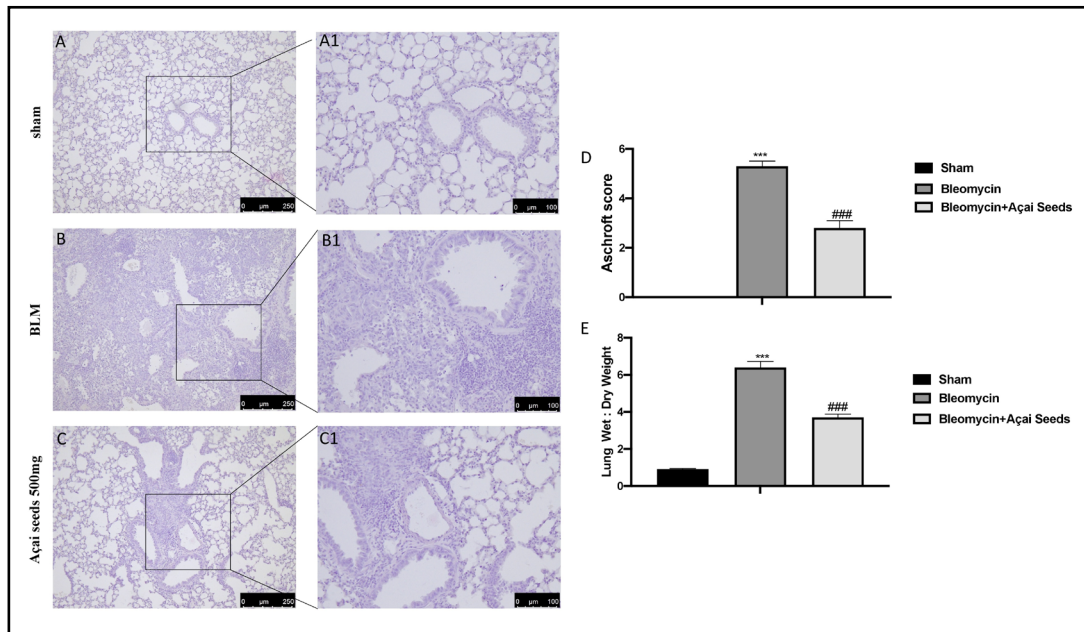


Fig. 8. Açai Seeds reduce bleomycin-induced histological damage. (A) Sham, (B) Bleomycin, (C) Bleomycin+Açai Seeds 500 mg/kg, and (D) Ashcroft score; (E) wet:dry lung weight ratio. 14 days of BLM injection we found a markedly increased lung inflammatory cells infiltration, edema, thickening of the alveolar wall and fibrosis of alveolar septa as well as an increase in the wet:dry lung weight ratio. Daily administration of Açai Seeds was able to reduce alterations BLM-induced as well as wet:dry lung weight ratio. Data are expressed as the mean \pm SEM of $n = 6$ animals for each group. *** $p < 0.001$ vs. sham; ### $p < 0.001$ vs. Bleomycin. 10x magnification, scale bar 250 μm . 20x magnification, scale bar 100 μm .

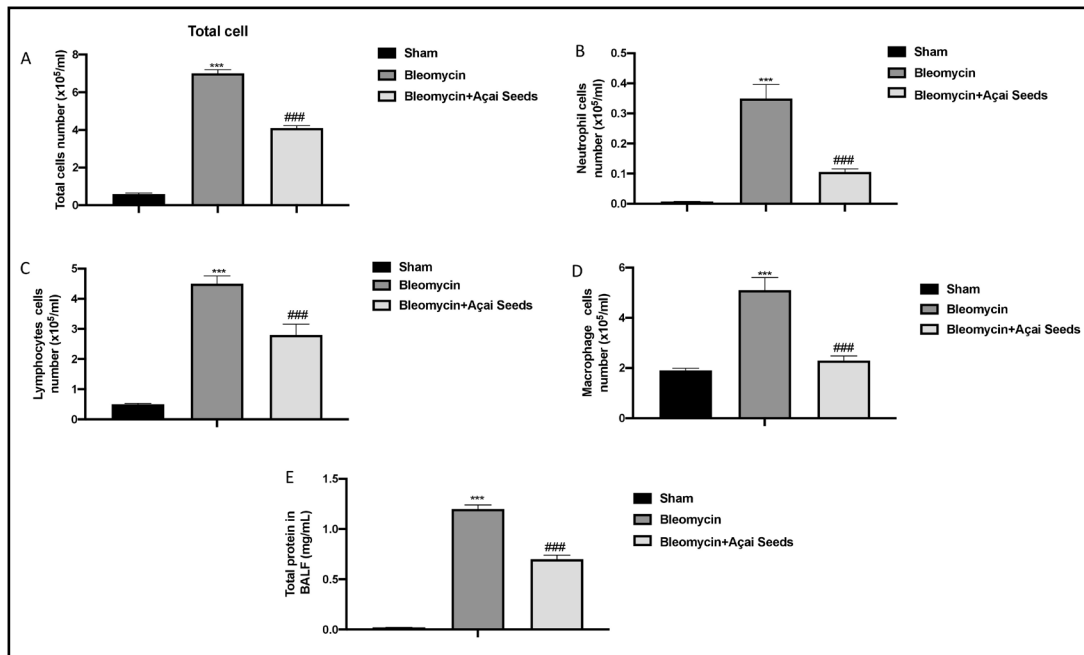
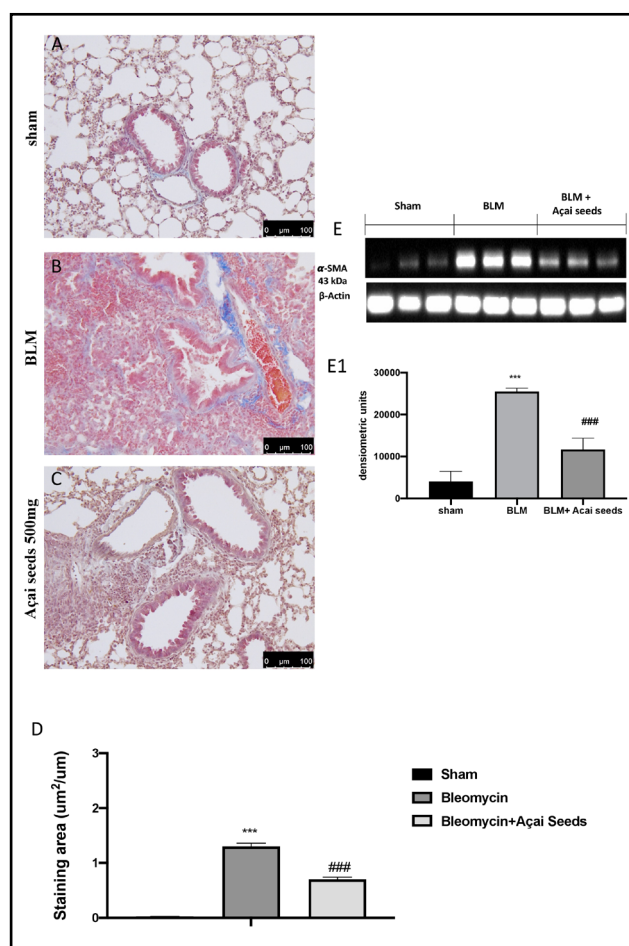


Fig. 9. Açai Seeds decrease bleomycin-induced inflammatory cell migration. Total (A) and differential cell counts (B for neutrophils; C for lymphocytes; D for macrophages) in bronchoalveolar lavage (BAL) fluid; total protein concentration in BAL (E). Daily administration of Açai Seeds at the dose of 500 mg/kg was able to reduce cellular migration in BAL as well as the increase in protein content BLM-induced. Data are expressed as the mean \pm SEM of $n = 6$ animals for each group. *** $p < 0.001$ vs. sham; ### $p < 0.001$ vs. Bleomycin.

Fig. 10. Açai Seeds decrease bleomycin-induced fibrosis. (A) Sham, (B) Bleomycin and (C) Bleomycin+ Açai Seeds 500 mg/kg; (D) staining area; Western Blot and relative densitometric analysis of α -SMA (E and E1). By Masson Trichrome and western blot of α -SMA we found that Açai Seeds administration was able to reduce fibrotic lesion BLM-induced. The photos are demonstrative of at least three experiments carried out on different experimental days. Data are expressed as the mean \pm SEM of $n = 6$ animals for each group. *** $p < 0.001$ vs. sham; ### $p < 0.001$ vs. Bleomycin. 20x magnification, scale bar 100 μ m.



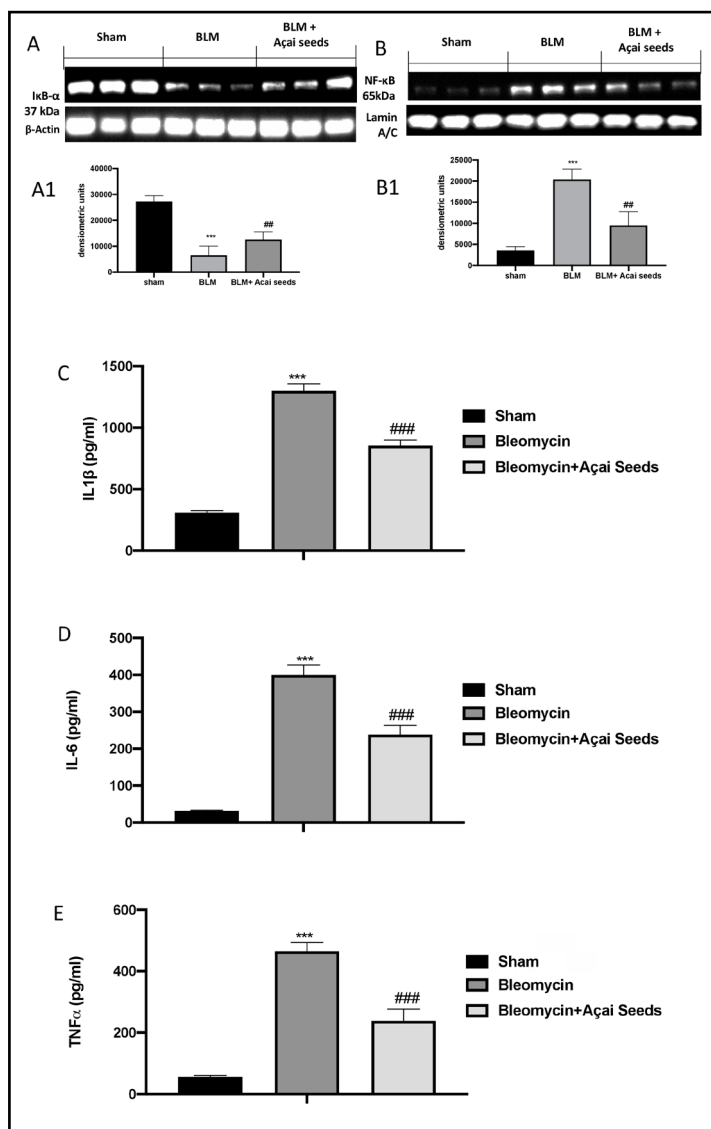
Açai Seeds administration decrease bleomycin-induced inflammation

To confirm our previous data on ALI, we investigated again by western blot the activation of NF- κ B pathway. As assumed, we found a significantly decrease in I κ B- α expression (Fig. 11A and A1), and consequently a significantly increase in NF- κ B translocation (Fig. 11B and B1) in bleomycin group compared to sham group. On the other hand, we found a significantly restored in I κ B- α expression (Fig. 11A and A1), and a significantly decrease in NF- κ B translocation (Fig. 11B and B1). The same trend was observed in the analysis of IL-1 β (Fig. 10C), IL-6 (Fig. 10D) and TNF- α (Fig. 10E).

Açai Seeds administration increase physiological antioxidant defense and decrease lipid peroxidation in lung

As is well known, administration of bleomycin causes cellular toxicity via production of ROS, which leads to serious damage to the lungs (Fig.12A). By western blot we investigated Nrf-2 (Fig. 12B and B1) and HO-1 (Fig. 12C and C1) expressions, and we found that they were significantly increased after bleomycin injection compared to sham. After daily Açai Seeds administration at the dose of 500 mg/kg we found a significantly increase in Nrf-2 (Fig. 12B and B1) and HO-1 (Fig. 12C and C1) expressions. Considering that Nrf-2 is involved in the physiological regulation of the oxidative defense of the cells, we also investigated the activity of SOD (Fig. 11D) and CAT (Fig 11E). As assumed, we found that Açai Seeds administration was able to increase the physiological antioxidant response compared to bleomycin group. Therefore, in a similar way, the overproduction of free radicals resulted in an increase in lipid peroxidation, as reflected by the MDA levels induced by bleomycin administration, were significantly reduced after Açai Seeds administration (Fig. 11F).

Fig. 11. Açai Seeds administration decrease inflammatory pathway and cytokines release. Western Blots and relative densitometric analysis for I κ B- α (A and A1), NF- κ B (B and B1), ELISA quantification for IL-1 β (C), IL-6 (D) and TNF- α (E). By western blots and ELISA kit we found that the activation of NF- κ B as well as the production of pro inflammatory cytokines were significantly reduced after Açai Seeds administration. Data are expressed as the mean \pm SEM of n = 6 mice/group. *** p<0.001 vs. sham; ## p<0.01 vs. Bleomycin; ### p<0.001 vs. Bleomycin.



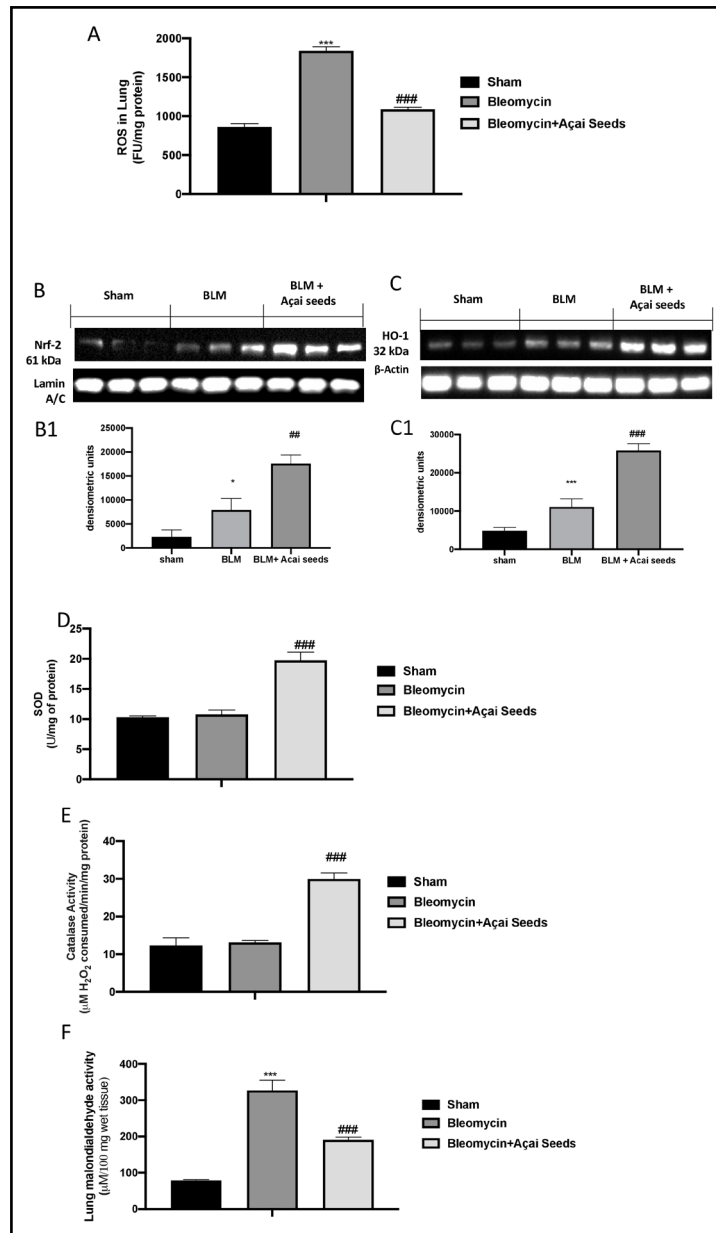
Açai Seeds showed protection like Resveratrol

To compare the effects of Açai Seeds administration with the other well know antioxidants, we choose to make a new experiment with Resveratrol at the dose of 50 mg/kg. We found that the administration of Açai Seeds showed a significantly protection like resveratrol, reducing inflammation, architecture alteration, edema, and cells infiltration (Supplementary Fig. S1).

Discussion

In recent years, the use of natural compounds used to counter the onset of inflammatory events or even as a supplement to accompany drug treatments is enjoying great success [55, 56, 74, 81, 82]. Dietary supplements are in fact extensively utilized and have the potential to enhance health when given to people who need them. Although better diet quality is necessary to address these difficulties, dietary supplements and/or food fortification may be useful for those who are at risk of deficiency or have other medical disorders [83]. The *Euterpe oleracea* Mart. palm (*Aceraceae*), often known as "Açai" is a promising new source

Fig. 12. Açai Seeds administration increase physiological anti-oxidant defense and decrease lipid peroxidation in lung. ROS measurement in lung homogenate (A) Western Blot and relative densitometric analysis of Nrf-2 (B and B1) and HO-1 (C and C1), SOD (D), CAT (E) and Malondialdehyde activity levels (F). Oxidative stress after BLM induction stimulated the physiological antioxidant response put in place by the activation of Nrf-2. We found that Açai Seeds administration stimulated the activity of anti-oxidant enzymes and reduce lipid peroxidation. Data are expressed as the mean \pm SEM of n = 6 mice/group. *p<0.05 vs. Sham; *** p<0.001 vs. sham; ## p<0.01 vs. Bleomycin; ### p<0.001 vs. Bleomycin.



of natural antioxidants. This plant may be found across northern South America, having the highest concentration in Brazil's Amazonian flood basins [19]. Despite the recent advances, Açai seeds warrant supplementary exploration as to their potential application for health benefits. Açai seeds extracts have previously demonstrated substantial antioxidant activity against the oxidation of linoleic acid as well as a potent scavenging ability against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and superoxide anion in a study of antioxidant activities of extracts from tropical and oriental medicinal plants [84]. Açai seeds extracts could thus provide benefits like grape seed or pine bark extracts, which are particularly high in oligomeric procyanidins. They have exhibited not only equivalent to or higher *in vitro* radical scavenging capability than butylated hydroxytoluene (BHT), but also cataract-prevention and antibacterial capabilities [19].

Lung damage, both acute and chronic, is a serious and rising worldwide health problem. The damage to many of these lung illnesses is irreversible, depleting the host's ability to grow new lung, and existing medicines are just supportive rather than rehabilitative [85].

Both acute and chronic lung disease have in common several kinds of inflammatory cells that are activated during inflammation. Each one secretes cytokines and mediators that influence the behavior of other inflammatory cells. Inflammation progresses due to the orchestration of these cells and chemicals. Excessive inflammation in the lungs, which is a key organ for gas exchange, can be fatal. Because hazardous microorganisms are continually present in the lungs, a prompt and powerful defensive response is essential to eradicate the inflammation as quickly as possible [4].

In our experimental model of acute lung injury, we found a significantly alteration of lung architecture accompanied with an increase in cytokines release, mast cells degranulation, PMNs infiltration and MPO production after the injection in the pleural cavity of CAR. After the oral treatment with Açai Seeds we detected a considerably improvement in all these parameters.

Another key pathway involved during ALI development is oxidative and nitrosative stress. Phagocytic cells are the most prominent source of harmful oxidants. Although endothelial and alveolar epithelial cells may both produce oxidants, the predominance of oxidants in the lungs come from activated phagocytic cells [86, 87]. Nrf2, a critical nuclear transcriptional factor with significant antioxidative properties, has been frequently employed as a promoter to reduce oxidative stress and inflammation [88-91]. In our study, after CAR injection into the pleural cavity, we found a physiological increase in Nrf-2 expression as well as in HO-1, that were increased after Açai Seeds administration. As a consequence of oxidative stress, we found also a significantly increase in DNA damage and lipid peroxidation. Açai Seeds administration significantly reduce lipid peroxidation as well as DNA damage. Oxidative stress and the impairment in the physiological antioxidant response are a key pathway involved in bleomycin-induced lung injury. In our experiment after the induction of lung injury with an instillation of bleomycin we found a significantly increase in lung alteration due to an increase in fibrosis, as demonstrated with the increase in α -SMA expression. After 14 days of oral daily treatment with Açai Seeds, we notice a significantly decrease in histological damage as well as in fibrosis. As demonstrated in acute model, we also found that Açai Seeds administration was able to reduce inflammatory pathways and increase physiological endogenous response.

Conclusion

Our study demonstrated not only that Açai Seeds nutritional supplementation have a beneficial effect on acute and chronic lung disease but also that it's probably act by the modulation of NF- κ B and Nrf-2 pathways.

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Author Contributions

Conceptualization, Marika Cordaro; Data curation, Alessio Peritore; Investigation, Livia Interdonato and Tiziana Genovese; Methodology, Ramona D'Amico, Rosalia Crupi and Enrico Gugliandolo; Project administration, Salvatore Cuzzocrea and Rosanna Di Paola; Supervision, Tiziana Genovese; Validation, Roberta Fusco and Daniela Impellizzeri; Writing – original draft, Marika Cordaro; Writing – review & editing, Rosalba Siracusa. All authors have read and agreed to the published version of the manuscript.

Statement of Ethics

The University of Messina Review Board for animal care (OPBA) approved the study (Ethical Code: 89126.24/2021-PR). All animal experiments agree with the new Italian regulations (D.Lgs 2014/26), EU regulations (EU Directive 2010/63) and the ARRIVE guidelines.

Disclosure Statement

The authors declare that no conflicts of interest exist.

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