ORIGINAL ARTICLE

# Reduced cutaneous inflammation associated with antioxidant action after topical application of the aqueous extract of *Annona muricata* leaves

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## Abstract
*Annona muricata* L. is used in folk medicine for treatment of diseases related to inflammatory and oxidative processes. This study investigated the effect of the aqueous extract of *A. muricata* leaves (AEAM) on TPA-induced ear inflammation and antioxidant capacity, both in vitro and in vivo. The in vitro antioxidant capacity of AEAM was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing/antioxidant power (FRAP) and lipoperoxidation assays. Cytotoxicity and reactive oxygen species (ROS) release were evaluated in the L929 fibroblasts. Swiss mice were submitted to TPA application and were topically treated with AEAM (0.3, 1 or 3 mg/ear). After 6 h, inflammatory and oxidative parameters were evaluated. Quercetin 3-glucoside, rutin, chlorogenic acid, catechin and gallic acid were identified in AEAM. It also presented antioxidant activity in all in vitro assays used. Incubation with AEAM did not cause cell cytotoxicity but reduced ROS release from fibroblasts. Compared with the control group, treatment with AEAM significantly reduced ear oedema and mieloperoxidase activity in inflamed ears, as well as histological parameters of inflammation. These results were associated with the reduction of total hydroperoxides and modulation of catalase, but not superoxide dismutase activity. These findings show the anti-inflammatory effect of AEAM is associated with antioxidant capacity.

## Introducion

*Annona muricata* L. (Annonaceae) is known as soursop or graviola and has been used in folk medicine for the treatment of many pathological processes. This plant species is widely found in tropical and subtropical regions, including Central and South America, Western Africa and Southeast Asia (Wahab et al. 2018). Parts of *A. muricata*, including, fruits, seeds, roots and leaves, are traditionally used as anti-parasite, anti-hypertensive, analgesic, anti-inflammatory, anti-diabetic and anti-cancer agents (Gavamukulya et al. 2017; Wahab et al. 2018).

Some pre-clinical studies have confirmed the anti-inflammatory action of extracts prepared from the leaves of *A. muricata*. The pre-treatment with the ethanol extract of leaves reduced carrageenan-induced paw oedema in rats, which was associated with cyclooxygenase inhibition (Poma et al. 2011). Another study associated the anti-inflammatory effect of the leaf extract with the decrease of TNF-α and IL-1β, besides modulation of NO release (Kim et al. 2016). The aqueous extract of *A. muricata* leaves had anti-inflammatory effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear oedema and carrageenan-induced paw oedema in mice (Quilez et al. 2015).

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Many constituents of *A. muricata* leaves can contribute to its anti-inflammatory effects, since some polyphenols and flavonol triglycosides have been identified in this plant organ (Moghadamtousi 2015). Many acetogenins have also been found in this plant and these substances not only have been associated with toxicity against cancer cell lines and parasites, but also toxic effects on nigral and striatal neurons in rats (Champy et al. 2004; Wahab et al. 2018).

These facts call attention to the possibility of toxicity caused by *A. muricata* preparations, but the study by Quilez et al. (2015) reported no acute toxicity or behavioural changes 48 h after oral administration to mice of the aqueous extract of *A. muricata* leaves in the dosage range of 250–1000 mg/kg. Thus, although controversial, the possibility of toxicity must be considered. However, the anti-inflammatory potential of *A. muricata* can still be useful for topical administration, but this possibility still needs to be better elucidated.

The aim of this study was hence to investigate the effect of topical application of the aqueous extract of *A. muricata* leaves (AEAM) in an acute model of cutaneous inflammation and evaluate the role of its antioxidant activity.

## Materials and methods

### Plant material: collection, processing and extraction

*Annona muricata* L. (Annonaceae) leaves were collected in January 2016 on the campus of Federal University of Sergipe in São Cristóvão, Brazil (11°01′47″ S, 37°20′64″ W). A specimen was deposited under registration number 38149 ASE in the university's herbarium.

The leaves of *A. muricata* were dried in a circulating air oven at an average temperature of 40 °C for 72 h. Then the material was ground in a Wiley knife mill, yielding 300 g of dry powder. This material was submitted to infusion using 100 g of powder/L of distilled water (100 °C for 15 min). After cooling at room temperature (25 °C), the infusion was filtered under reduced pressure, recovering 1.8 L of the solution, which was lyophilised (LS3000, Terroni, São Carlos, Brazil) to obtain 28.2 g of dried AEAM (yield of 9.4% w/w).

### Quantification of total phenolics and flavonoids and characterization of phenolic compounds by HPLC analysis

Total phenolic content and total flavonoids in AEAM were analysed by the methods previously described by Hills and Swain (1959) and Zhishen et al. (1999), with some modifications. The results were plotted based on standard curves of gallic acid (10–200 μg/mL; Sigma Aldrich, USA) or catechin (7.25–145 μg/mL; Sigma Aldrich, USA) respectively,

and were expressed as mg of gallic acid equivalents/g of dried extract or mg of catechin equivalents/g of dried extract.

High-performance liquid chromatography (HPLC) was performed using the conditions described by Oliveira et al. (2017). The AEAM was diluted with methanol (HPLC grade; Merck, Brazil) at 2 mg/mL. This solution was submitted in ultrasound for 15 min and filtered through a 0.45 μm membrane (PTFE; Merck, Brazil) before HPLC injection.

### In vitro antioxidant capacity of AEAM

In vitro experiments were used to determine the antioxidant capacity of AEAM. These experiments were performed twice and in triplicate in microplates. We chose the concentrations of the stock solutions of AEAM (12.5, 25, 50, 100 and 200 μg/mL) and calculated the concentration required for half-maximal inhibition of the parameter evaluated ($IC_{50}$). Trolox (Sigma Aldrich, USA) was used as a positive control.

The antioxidant capacity against 2,2-diphenyl-1-picrylhydrazyl radical (DPPH; Sigma-Aldrich, USA) was measured according to the description of Brand-Williams et al. (1995) with minor modifications (Oliveira et al. 2017). The nitric oxide (NO) scavenging activity was measured using sodium nitroprusside as the NO source (20 nmol/L; Sigma Aldrich, USA), according to Basu and Hazra (2006). Lipid peroxidation was determined according to the method described by Ohkawa et al. (1979), with modifications (Oliveira et al. 2017), in rat brain homogenates in the presence (stimulated) or absence (basal) of a ferrous sulphate solution ($FeSO_4$, 0.145 mmol/L). For these tests, the results were expressed as percentage inhibition [% inhibition = [(control − test)/control] × 100], based on absorbance values, from which the $IC_{50}$ values were calculated.

The reducing potential of AEAM was determined by the ferric reducing/antioxidant power (FRAP) assay, as previously described by Pulido et al. (2000) with modifications (Oliveira et al. 2017).

### Cell viability test and reactive oxygen species (ROS) release test in L929 fibroblasts

Cell viability was assessed using the colorimetric methylthiazolyl-tetrazolium (MTT) assay to measure cell metabolic activity. L929 fibroblasts were cultured in DMEM (Sigma Aldrich, USA) containing 10% fetal bovine serum (Life Technologies, India), 50 UI/mL of penicillin (Life Technologies, India) and 50 μg/mL of streptomycin (Life Technologies, India) in a 5% $CO_2$ atmosphere (37°C). The fibroblasts were seeded in 96-well culture plates ($2 \times 10^4$ cells/well) and then treated with AEAM at the final concentrations of 12.5, 25, 50, 100 and 200 μg/mL for 24 h. Then MTT (0.5 mg/mL in a phosphate-buffered saline (PBS) solution) was placed

in contact with the cells and incubated at 37 °C for 3 h. After removal of MTT, dimethyl sulfoxide (DMSO, 100%; Merck, Brazil) was added for solubilization of the tetrazole salt crystals and the absorbance was measured at 570 nm. The tests were conducted three times and in triplicate and then normalised considering the control absorbance as 100% viability.

The test for ROS release was performed using dichloro-dihydrofluorescein diacetate (H2DCFDA; Sigma Aldrich, USA) reagent according to the manufacturer's instructions. The fibroblasts were cultured and seeded in 96-well plates as described above. After achieving 70% confluence, the cells were pre-treated with the AEAM for 24 h and then exposed to the ROS-inducing stressor ($H_2O_2$, 750 µmol/L; Dinamica, Brazil) for 10 min followed by washing with PBS. The ROS detection reagent, H2DCFDA, was added to each well for 30 min. All experiments included the following groups: control (2% fetal bovine serum); $H_2O_2$ plus vehicle and $H_2O_2$ plus AEAM at the concentrations of 12.5, 25, 50, 100 and 200 µg/mL. Detection for ROS was performed by fluorimetry (Synergy™ H1, BioTek, Winooski, USA) with excitation and emission wavelengths set at 485 and 525 nm, respectively. The experiments were repeated twice with two samples per treatment group.

## Animals

Male Swiss mice (20–30 g) were obtained from the Animal Centre of Federal University of Sergipe. Animals were kept at 21–23 °C with free access to food and water under a 12-h light/dark cycle. All experiments were conducted in agreement with the guidelines of the Brazilian College of Animal Experimentation and the National Institutes of Health and were approved by the Ethics Committee for Animal Use in Research of Federal University of Sergipe (number 9256010719).

## Cutaneous inflammation model

Animals ($n = 6$–8) were anaesthetised with inhalatory isoflurane and 10 µL of TPA (1 µg/ear dissolved in acetone; Sigma Aldrich, USA) was applied to the inner and outer surfaces of the right ear with a polypropylene tip. After 5 min, 10 µL of AEAM (0.3, 1 or 3 mg/ear, dissolved in saline solution) or dexamethasone (0.05 mg/ear; Aché, Brazil) was applied. The left ear received only the vehicles and each animal was used as its own control. After 6 h, the animals were euthanized, and 8-mm diameter ear sites were obtained with a metal punch. The ear weight was measured (in mg) and oedema was calculated by subtracting the mass of the right ear from the left ear (Oliveira et al. 2017).

These tissue samples were submitted to measurement of myeloperoxidase (MPO) activity, histological analysis, quantification of the total hydroperoxides and superoxide dismutase (SOD) and catalase activities.

The activity of MPO was determined in ear homogenates as previously described in our laboratory conditions (Oliveira et al. 2017). Results were expressed as units of MPO per site of ear.

The histological analysis was conducted in mouse ear sections of 5 µm stained with hematoxylin and eosin (Laborclin, Brazil). The photographs were evaluated by an expert pathologist who had no information about the group identification, to determine the histopathological parameters. For assessment of the mean number of inflammatory cells, a total of 15 fields per animal were analysed at magnification of 400×. To obtain the thickness of the epidermis, quantitative analysis of randomised images of three histological sections (four measures/section) of each animal was performed using the software UTHSCSA Image Tool14®. The results were expressed as the mean of inflammatory cells or epidermis thickness per histological field.

Quantification of the total hydroperoxides was performed as previously described (Jiang et al. 1992). Ear homogenate was mixed for 30 min with light protected FOX reagent [composed of 0.25 mmol/L of xylenol orange, 0.25 mmol/L of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, and 4.4 mmol/L of butylated hydroxytoluene (BHT), Sigma Aldrich, USA, enriched with methanol and sulfuric acid (97%), Merck, Brazil]. The samples were centrifuged, and the supernatant was measured at 560 nm. Total hydroperoxide was expressed in µmol/L with molar extinction coefficient of $4.3 \times 10^{-4} \, \text{M}^{-1} \, \text{cm}^{-1}$.

The SOD activity was determined as previously described (Madesh and Balasubramanian 1998) and the results were expressed as U/µg of protein. The activity of catalase was determined by measuring the consumption of hydrogen peroxide ($H_2O_2$, 0.3 mol/L) as previously described (Nelson and Kiesow 1972) and was expressed as the extinction of $H_2O_2$ during 1 min at 25 °C ($\Delta E$/min/µg of protein). The protein content of tissues was determined by the Bradford method using the Bio-Rad® protein assay reagent.

## Statistical analysis

The results are expressed as means ± SEM. Statistical evaluation of the data was performed with GraphPad Prism 7.0. Data were tested for normal distribution by the Shapiro–Wilk test. There was no impediment to applying parametric tests, so the results were analysed by one-way analysis of variance (ANOVA) followed by the Tukey test. $p$ values lower than 0.05 were considered significant.

# Results

## Quantification of total phenolics and flavonoids in AEAM and characterization by HPLC

Total phenol content in AEAM was $164.52 \pm 1.13$ mg of gallic acid equivalent/g of extract and total flavonoid content was $77.02 \pm 0.01$ mg of catechin equivalent/g of extract.

The HPLC chromatographic profile of AEAM at 280 nm is shown in Fig. 1. The five major peaks in this chromatogram were identified and quantified as quercetin 3-glucoside ($3.17 \pm 0.01$ µg/mg of extract), rutin ($1.78 \pm 0.04$ µg/mg of extract), chlorogenic acid ($1.40 \pm 0.02$ µg/mg of extract), catechin ($1.11 \pm 0.01$ µg/mg of extract) and gallic acid ($0.27 \pm 0.05$ µg/mg of extract).

## In vitro antioxidant capacity of AEAM

As shown in Table 1, all concentrations of AEAM (12.5, 25, 50, 100 and 200 µg/mL) significantly reduced the DPPH radical formation ($p < 0.001$) and caused significantly higher

NO scavenging activity in comparison with the control group ($p < 0.001$). Trolox (100 µg/mL) also caused these alterations in comparison with the control group ($p < 0.001$).

In addition, significantly higher antioxidant power was detected by FRAP method for the concentrations of 25 ($p < 0.01$), 50, 100 and 200 µg/mL ($p < 0.001$) of AEAM compared to the control group, as was the case of Trolox (100 µg/mL; $p < 0.001$).

The incubation with AEAM significantly reduced the spontaneous ($p < 0.001$ for 50, 100 and 200 µg/mL of AEAM) or $FeSO_4$-induced lipoperoxidation ($p < 0.001$ for 100 and 200 µg/mL) compared to the control group, as did Trolox (100 µg/mL; $p < 0.001$; Table 1).

## Effect of AEAM on cell viability and release of ROS from L929 fibroblasts

We observed no alteration of L929 fibroblasts' viability after incubation with different concentrations of AEAM ($93.3 \pm 7.1$, $95.6 \pm 6.8$, $97.2 \pm 11.9$, $94.0 \pm 4.1$ and $88.8 \pm 4.3\%$, respectively for 12.5, 25, 50, 100 and 200 µg/

**Fig. 1** Analysis of the aqueous extract of *Annona muricata* (AEAM) leaves by high-performance liquid chromatography ($y = 280$ nm)
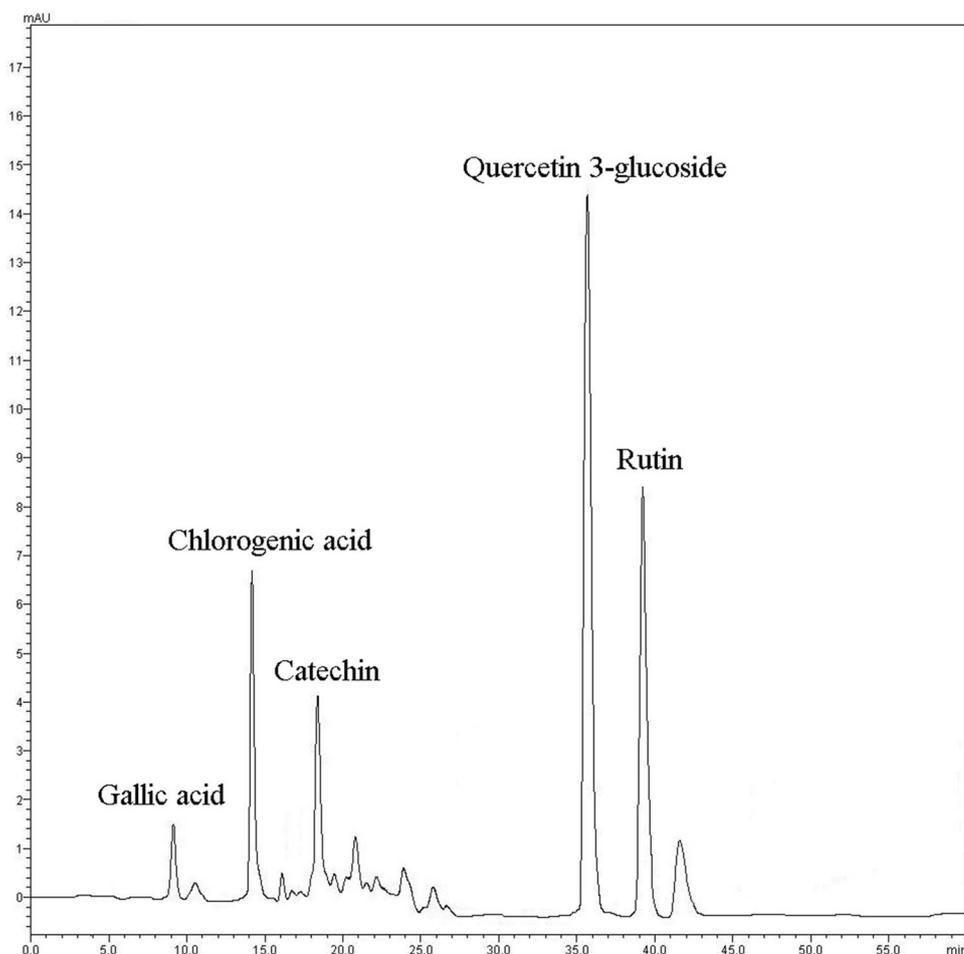
**Table 1** In vitro antioxidant activity of aqueous extract of *Annona muricata* leaves (AEAM)

| Group | DPPH (%) | NO (%) | FRAP ($Fe^{3+}/Fe^{2+}$) | TBARS (%) | |
|---|---|---|---|---|---|
| | | | | Basal | $FeSO_4$ |
| Control | $100.0 \pm 1.9$ | $100.0 \pm 3.9$ | $4.6 \pm 0.8$ | $100.0 \pm 2.8$ | $100.0 \pm 1.2$ |
| AEAM (12.5 µg/mL) | $78.9 \pm 0.9$*** | $74.8 \pm 1.4$*** | $30.8 \pm 6.0$ | $94.6 \pm 0.6$ | $98.8 \pm 0.6$ |
| AEAM (25 µg/mL) | $70.4 \pm 1.3$*** | $57.2 \pm 0.9$*** | $53.8 \pm 5.0$** | $92.3 \pm 1.9$ | $96.4 \pm 0.7$ |
| AEAM (50 µg/mL) | $51.3 \pm 0.2$*** | $35.8 \pm 0.6$*** | $99.5 \pm 4.4$*** | $80.3 \pm 1.1$*** | $94.5 \pm 0.7$ |
| AEAM (100 µg/mL) | $45.5 \pm 2.0$*** | $29.8 \pm 0.1$*** | $224.1 \pm 5.2$*** | $51.1 \pm 1.6$*** | $88.1 \pm 2.1$*** |
| AEAM (200 µg/mL) | $28.2 \pm 0.5$*** | $24.5 \pm 0.2$*** | $472.8 \pm 2.3$*** | $22.4 \pm 1.1$*** | $75.0 \pm 1.9$*** |
| Trolox (100 µg/mL) | $22.5 \pm 2.0$*** | $28.4 \pm 0.1$*** | $402.8 \pm 10.0$*** | $44.1 \pm 1.0$*** | $57.3 \pm 0.3$*** |
| $IC_{50}$ (µg/mL) | 97.1 | 44.1 | – | 122.8 | 401.5 |

AEAM was tested in the α, α-diphenyl-β-picrylhydrazyl (DPPH) radical assay, ferric reducing/antioxidant power (FRAP), nitric oxide (NO) scavenging activity and thiobarbituric acid reactive substances (TBARS) tests with spontaneous or $FeSO_4$-induced lipid peroxidation. The concentrations are shown of the stock solutions. The results are the mean $\pm$ SEM of the percent inhibition; $n = 3$ experiments performed in duplicate. $IC_{50}$ represents the concentration required for 50% maximum inhibition

** $p < 0.01$ or *** $p < 0.001$ vs. respective control

**Table 2** Inhibition of reactive oxygen species (ROS) release in L929 fibroblasts treated with aqueous extract of *Annona muricata* (AEAM)

| Group | ROS (fluorescence) |
|---|---|
| Control (vehicle) | $1467 \pm 138$ |
| $H_2O_2$ (750 µmol/L) $\pm$ vehicle | $34{,}033 \pm 1392$### |
| $H_2O_2$ (750 µmol/L) $\pm$ AEAM (12.5 µg/mL) | $28{,}941 \pm 1038$** |
| $H_2O_2$ (750 µmol/L) $\pm$ AEAM (25 µg/mL) | $7365 \pm 608$*** |
| $H_2O_2$ (750 µmol/L) $\pm$ AEAM (50 µg/mL) | $3619 \pm 594$*** |
| $H_2O_2$ (750 µmol/L) $\pm$ AEAM (100 µg/mL) | $3103 \pm 331$*** |
| $H_2O_2$ (750 µmol/L) $\pm$ AEAM (200 µg/mL) | $1784 \pm 203$*** |

ROS release in the absence or presence of AEAM leaves. The mean $\pm$ SEM of the fluorescence values are shown for $n = 2$ experiments performed in duplicate. ### $p < 0.001$ vs. control and ** $p < 0.01$ or *** $p < 0.001$ vs. ROS-inducing stressor ($H_2O_2$)

mL of AEAM) for 24 h in comparison with the control group ($100.0 \pm 0.1\%$).

Fibroblasts treated with a stressor agent ($H_2O_2$, 750 µmol/L) released higher amounts of ROS than the control cells ($p < 0.001$). Table 2 shows that the treatment with

AEAM at 12.5, 25, 50, 100 and 200 µg/mL impaired this release ($p < 0.01$ for 12.5 µg/mL or $p < 0.001$ for other concentrations compared to vehicle-treated group).

## Effect of AEAM on TPA-induced ear oedema

The topical administration of TPA increased ear weight by $22.1 \pm 1.1$ mg/site (Fig. 2a). In the ears treated with AEAM (1 and 3 mg/ear), we observed lower oedema ($p < 0.001$) in comparison with the vehicle group. The MPO activity was significantly higher in ears that received TPA (Fig. 2b, $p < 0.001$) than acetone (control). This activity was decreased by AEAM at 1 and 3 mg ($p < 0.001$). Dexamethasone (0.05 mg/site) administration also reduced oedema ($p < 0.001$) and MPO activity ($p < 0.001$) compared to the vehicle group.

The histological sections of ears that received topical administration of TPA showed massive presence of leukocyte infiltrate, oedema and hyperaemia (Fig. 3a, e), but the ears that received acetone as vehicle presented normal tissue architecture (Fig. 3b, f). The representative

**Fig. 2** The aqueous extract of *Annona muricata* (AEAM) leaves reduces oedema (**a**) and myeloperoxidase (MPO) activity (**b**) induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mice. Dexa (dexamethasone). Data are expressed as mean $\pm$ SEM ($n = 8$). ### $p < 0.001$ vs. Ctr group and *** $p < 0.001$ vs. vehicle group
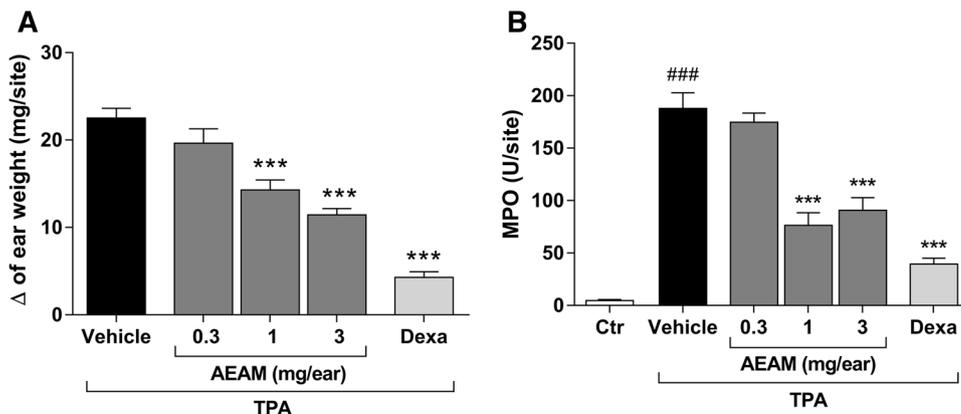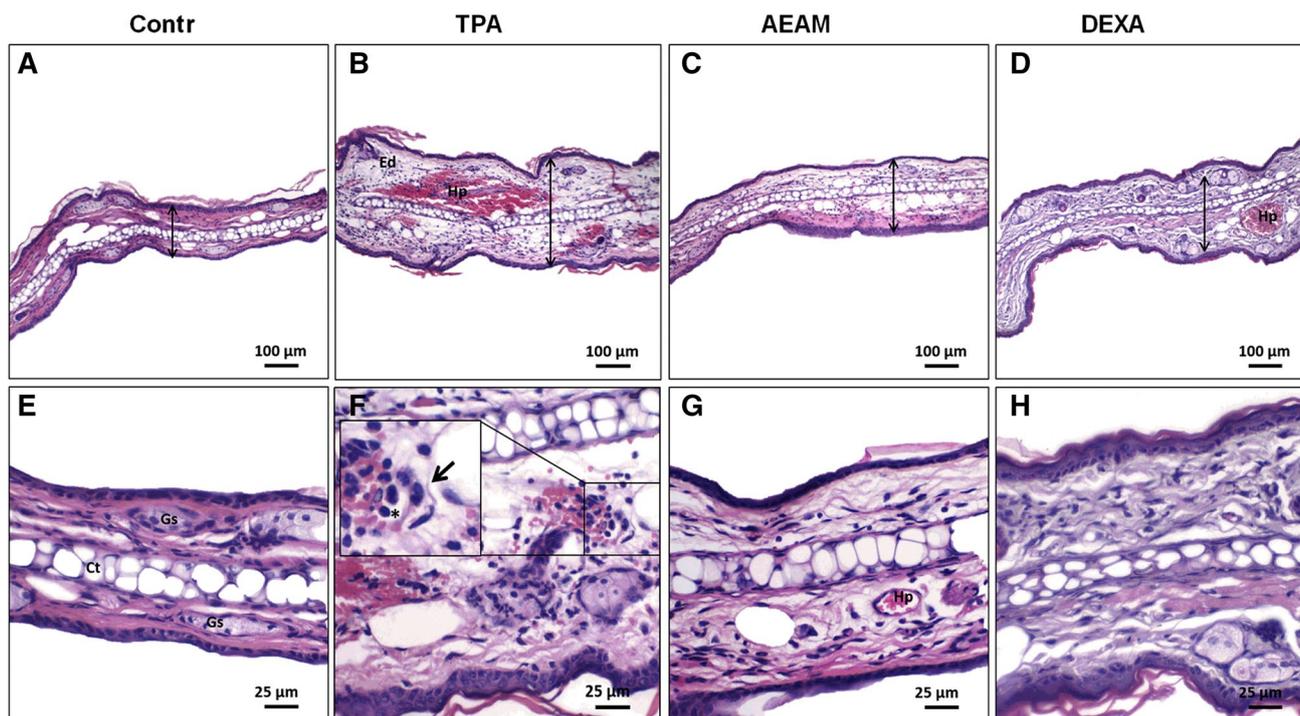
**Fig. 3** Representative images of the histopathological alterations in mice ears inflamed by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and treated with the aqueous extract of *Annona muricata* L (AEAM) leaves. Representative histological images of the ear at ×100 (**a–d**)

and ×400 (**e–h**) magnifications. *Ct* hyaline cartilage, *Gs* sebaceous glands, *Ed* interstitial oedema, *Hp* hyperemia, *Arrow* leukocyte infiltrate, *Double arrow* dermoepidermal tissue thickness, *Dexa* dexamethasone. Control (Ctr) ears received only vehicle treatment

images of ears treated with AEAM (3 mg/ear; Fig. 3c, g) or dexamethasone (Fig. 3d, h) showed that leukocyte infiltrate and oedema were less frequently observed. The quantification of these parameters in both ears treated with AEAM or dexamethasone indicated a lower number of leukocytes ($p < 0.001$ Fig. 4a) and decreased dermal thickness ($p < 0.001$ Fig. 4b) compared to the vehicle group.

## Antioxidant effect of AEAM in TPA-induced ear oedema

Ears administered with TPA presented higher total hydroperoxide content in comparison to the ears that received only acetone. In contrast, there were significantly lower total hydroperoxide contents in the ears treated with AEAM at 0.3 ($p < 0.05$), 1 ($p < 0.001$) and 3 mg/ear ($p < 0.001$; Fig. 5a).

The activities of SOD and CAT were significantly reduced by TPA administration in comparison with the control ears ($p < 0.05$ and $p < 0.001$). The treatment with AEAM

**Fig. 4** The aqueous extract of *Annona muricata* L (AEAM) leaves reduces inflammatory cell number per field (**a**) and thickness of dermis (**b**) in mice ears inflamed by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) Dexa (dexamethasone). Data are expressed as mean ± SEM ($n = 6–7$). [###]$p < 0.001$ vs. Ctr group and [***] $p < 0.001$ vs. vehicle group
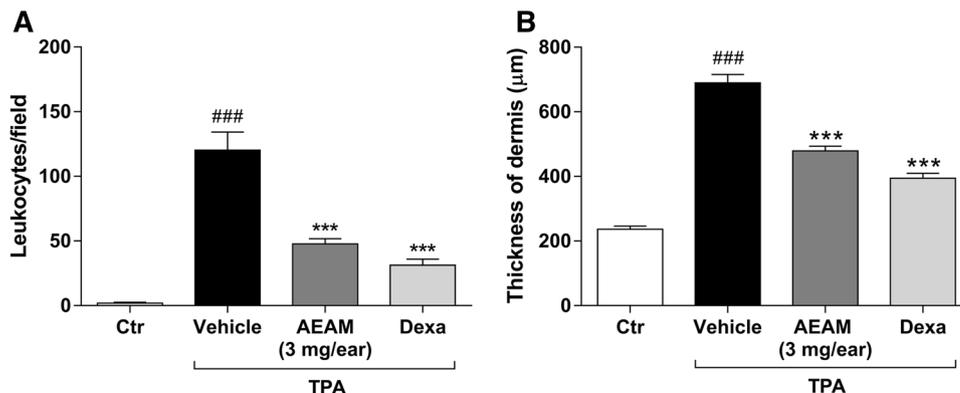
**Fig. 5** In vivo antioxidant activity of the aqueous extract of *Annona muricata* (AEAM) leaves in ears inflamed with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Total hydroperoxide concentration (**a**) and catalase (CAT; **b**) and superoxide dismutase (SOD; **c**) enzyme activities were measured. Data are expressed as mean $\pm$ SEM ($n = 8$). $^{\#}p < 0.05$ or $^{\#\#\#}p < 0.001$ vs. Ctr group and $^{*}p < 0.05$ or $^{***}p < 0.001$ vs. vehicle group
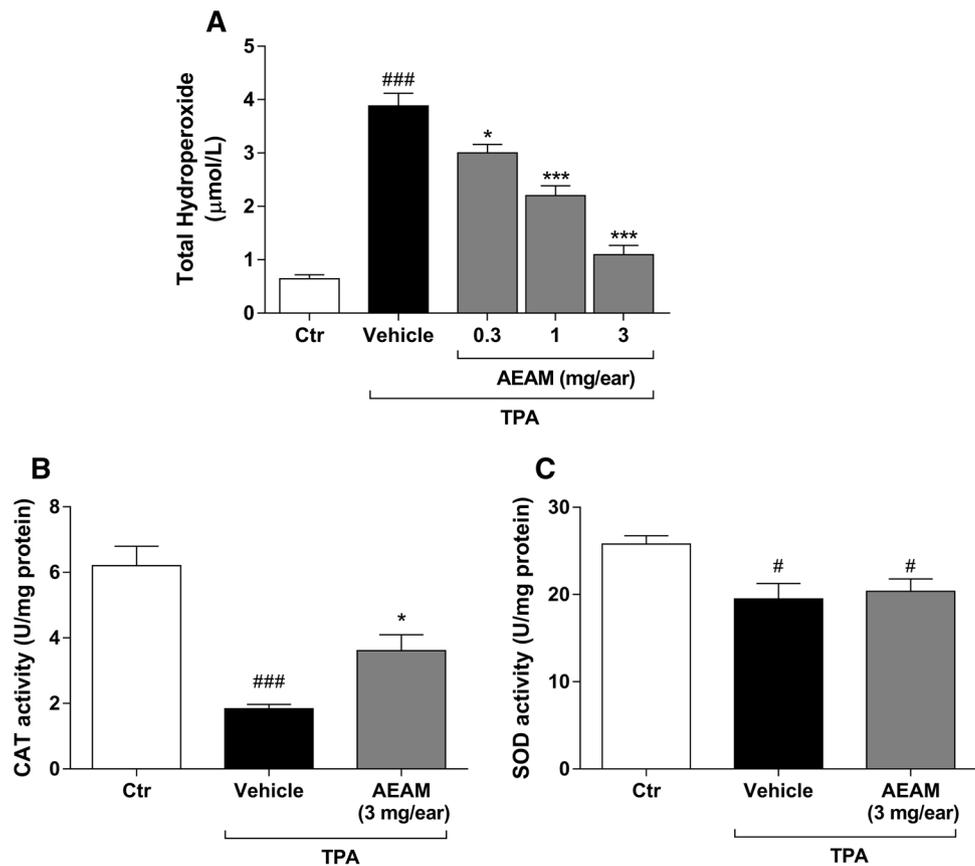


at 3 mg/ear significantly ($p < 0.001$) attenuated the alteration caused by TPA in CAT ($p < 0.05$), but not SOD activity compared to the vehicle group (Fig. 5b, c).

## Discussion

In the current study, we prepared the AEAM and described its phytochemical profile and in vitro antioxidant effect against radicals, lipoperoxidation or cells submitted to an oxidant condition, as well as evaluated the topical anti-inflammatory and antioxidative effect of AEAM in a murine model of cutaneous inflammation.

The total phenol and flavonoid contents found in the AEAM through colorimetric assays might have been caused by the compounds such as the five substances identified through HPLC analysis (quercetin-3-glucoside, rutin, chlorogenic acid, catechin and gallic acid). These compounds were previously described in preparations from *A. muricata* (Wahab et al. 2018). They are commonly found in foods, such as fruits and vegetables, and the dietary intake of polyphenols is usually associated with benefits such as reduction of inflammation (Tresserra-Rimbau et al. 2018). Furthermore, polyphenols are capable of reducing oxidative stress and maintaining the redox-system homeostasis (Hussain

et al. 2016). Hence, the presence of phenolic compounds in AEAM might be responsible for the antioxidant activity of this extract.

This possibility was confirmed in vitro for AEAM, since it was able to reduce radicals, such as DPPH and NO, indicating that AEAM compounds can either act as donors of $H^+$ ions in reaction with DPPH or present NO scavenging capacity. Furthermore, AEAM also showed radical reducing potential in the FRAP assay, a method in which the antioxidant substance is capable of reducing $Fe^{3+}$-ferricyanide complexes into the ferrous ($Fe^{2+}$) form (Pulido et al. 2000). This finding is relevant because the oxidant reactions catalysed by $Fe^{2+}$ can generate hydroxyl radicals (e.g., by Fenton's reaction), which are highly damaging to cells (Valko et al. 2016). Finally, the antioxidant effect of AEAM was also demonstrated by the reduction of lipid peroxidation, which is formed from ROS and other secondary oxidation products (Moon and Shibamoto 2009). Altogether, our findings indicate the antioxidant action of AEAM, which corroborates previous observations for *A. muricata* preparations by other authors (Gavamukulya et al. 2017).

In agreement with these findings, our data show that AEAM reduced the release of ROS from L929 fibroblasts, which reinforces the possibility that AEAM can have protective effects in cells by preventing ROS release. Both

excessive ROS and reactive nitrogen species (RNS) production can contribute to the inflammatory process, with the activation of intracellular mechanisms that lead to the release of pro-inflammatory cytokines and other mediators, e.g., from the nuclear factor-kappa B (NF-κB) signalling pathway (Becker et al. 2014).

In addition, AEAM did not affect the viability of L929 fibroblasts in culture. This is important because some researchers have suggested a toxic effect caused by the acetogenins presented in *A. muricata* preparations (Wahab et al. 2018). However, in agreement with our findings, Quilez and co-workers (2015) showed that decoction extracts of *A. muricata* leaves at concentrations of 500, 250 and 100 μg/mL did not induce cytotoxicity in isolated murine macrophages.

These results led us to propose that AEAM may produce anti-inflammatory effects that are related with antioxidant action in vivo. Thus, we used the TPA-induced ear inflammation model to evaluate this possibility. We observed that treatment with AEAM reduced ear oedema and MPO activity. These findings were corroborated by the histological analysis, which showed reduced thickness of dermis and leukocyte counts in ears treated with AEAM. These results are in line with a previous study that showed anti-oedematogenic effect of the decoction extract of *A. muricata* at doses of 2.5 and 5 mg/ear, accompanied of reduction of MPO activity in the same model used in our study (Quilez et al. 2015). However, these authors did not discuss any mechanism for the effects described. Other studies have also reported anti-inflammatory activity of *A. muricata*. De Sousa and co-workers (2010) showed that treatment with ethanol extract of *A. muricata* leaves reduced carrageenan-induced paw oedema and reduced pleural exudate and leukocyte infiltration in carrageenan-induced pleurisy in rats. Topical treatment with ethyl-acetate extract of *A. muricata* improved the wound healing induced by uninfected excision, by reducing neutrophil and macrophage infiltration and increasing collagen deposition (Moghadamtousi 2015).

Interestingly, previous studies have shown that compounds identified in AEAM caused anti-oedematogenic effect on mice ears. Topical administration of quercetin-3-glucoside (isoquercitrin) reduced ear oedema induced by croton oil (at approximately 0.14 and 0.46 mg/ear; Sosa et al. 2007) or xylene (at 0.02 mg/ear; Fu et al. 2020); as did rutin (at 0.01 and 0.03 mg/ear; Camponogara et al. 2020) or catechin (at 0.6 mg/ear; Pietrovski et al. 2008) in the ear oedema induced by croton oil. On the other hand, in the model of xylene-induced ear oedema, the anti-oedematogenic effect of chlorogenic acid and rutin was demonstrated after oral administration of doses of 10–15 mg/kg and 2.5–10 mg/kg, respectively (Torres-Rêgo et al. 2016). These findings make it reasonable to attribute the anti-inflammatory action of AEAM to the compounds present in this extract.

The reduction of MPO activity by the treatment with AEAM in the present study suggests there was reduction of neutrophils at inflamed ear sites. It is widely known that neutrophils participate in the first defence line against pathological agents and release granules containing peroxidases, such as MPO. These enzymes can produce ROS using $H_2O_2$ produced by NADPH oxidase, which leads to lipid peroxidation (Osawa 2017). Thus, the MPO activity reduction by AEAM may be directly linked to oxidative stress inhibition and it might be that AEAM compounds directly reduce ROS/RNS formation. Treatment with AEAM decreased total hydroperoxide content in inflamed ears, which can be considered a marker of oxidative alteration in membranes (Osawa 2017). Interestingly, we observed that treatment with AEAM also modulated CAT, but not SOD, activity, which might have contributed to the reduction of oxidative stress, since CAT catalyses the conversion of hydrogen peroxide into water and $O_2$ (Bresciani et al. 2015). Thus, the increase of CAT activity may be other protection mechanism against oxidative damage. Our findings partially agree with those of Moghadamtousi (2015), who reported the wound healing activity of an *A. muricata* preparation due to the modulation of CAT, SOD and glutathione peroxidase activity enzymes.

## Conclusions

The AEAM prepared led to radical scavenging, reduced lipoperoxidation and protected fibroblasts from oxidant alterations and also had an anti-inflammatory effect after topical application on inflamed ears, associated with the inhibition of oxidative stress and augmentation of CAT activity. Thus, we suggest AEAM is a potential preparation for treating cutaneous inflammatory diseases, by maintaining redox homeostasis.

## Compliance with ethical standards

**Conflict of interest** The authors report no conflicts of interest.

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