Free Radicals and Antioxidants 3 (2013) S16–S21

Contents lists available at ScienceDirect

Free Radicals and Antioxidants

journal homepage: www.elsevier.com/locate/fra

Original article

# Effect of the pretreatment with acerola (*Malpighia emarginata* DC.) juice on ethanol-induced oxidative stress in mice – Hepatoprotective potential of acerola juice

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ARTICLE INFO

*Article history:*
Received 25 May 2013
Accepted 11 October 2013
Available online 12 November 2013

*Keywords:*
Antioxidants
Fruits
Free radical scavenging activity
Lipid peroxidation

ABSTRACT

*Background and objectives:* Acerola (*Malpighia emarginata* DC.) is a tropical fruit known for its nutritional and functional properties due to its great contents vitamin C, carotenoids and anthocyanins. The antioxidant potential from plants extracts associated to hepatoprotective activity has been widely studied. However, the effect of antioxidants in natural fruit juice has not been fully searched. The aim of this work was to investigate the antioxidant properties of acerola juice and its hepatoprotective potential against acute ethanol-induced stress.

*Materials and methods:* Ripe acerola were collected, processed into juice and initially analyzed to antioxidant properties *in vitro*. Afterwards, *in vivo* hepatoprotective activity was evaluated in mice. The animals received the juice by gavage as pretreatment for 15 consecutive days and then, were submitted to ethanol-induced stress in single dose (5 g/kg). The activities of serum enzymes as well as lipid peroxidation degree were evaluated. The activities of serum marker enzymes for liver damage as well as lipid peroxidation degree were evaluated.

*Results:* Acerola juice presents great vitamin C (1799.5 mg/100 g FW) and total phenolic (188.4 mg GAE/100 g FW), anthocyanins (9.2 mg/100 g FW), flavonols (7.8 mg/100 g FW) contents and high activity of superoxide dismutase (1053.6 UA/g DM) with a total antioxidant activity of 137.5 μmol Trolox/g FW. The juice treatment inhibited lipid peroxidation and reduced the activities of aminotransferases ($p \leq 0.05$), in mice liver.

*Conclusion:* These results indicate that acerola juice is able to prevent the hepatic damage induced by ethanol, probably as a result of an enhancement of the antioxidant status in the animals.



## 1. Introduction

Acerola (*Malpighia emarginata* DC.), also known as Barbados cherry or West-Indian cherry, is cultivated from Central to Northern South America and well known as a rich source of antioxidants as vitamin C, anthocyanins and carotenoids.[1,2] The antioxidant compounds of acerola were effective scavengers of oxygen ($O_2^-$) and nitrogen (NO) radicals and inhibited NO production.[3]

There is a growing interest in the role of free radical-mediated oxidative stress.[4] Free radicals as reactive oxygen and nitrogen species are capable of attacking many cellular components and causing several human metabolic diseases, including the alcoholic liver disease.[5,6] Alcohol abuse is one of the main causes of liver disease worldwide and has become a social problem. The alcoholic liver injury involves genetic and environmental factors and results of a complex array of patho-physiological events involving various

*Abbreviations:* ABTS, 2.2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt; ALT, alanine aminotransferase; APX, ascorbate peroxidase; AST, aspartate aminotransferase; CAT, catalase; DM, dry mass; GAE, gallic acid equivalent; LDH, lactate dehydrogenase; MDA, malondialdehyde; SOD, superoxide dismutase; TEAC, trolox equivalent antioxidant capacity.

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2231-2536/$ – see front matter 
http://dx.doi.org/10.1016/j.fra.2013.10.004

types of cells and different factors such as endotoxin, oxidative stress, cytokines, and proteases.[7] Thus, toxic substances generated during alcohol metabolism lead to oxidative stress directly associated with liver pathogenesis.[8]

Antioxidants play a protective role by inhibiting the free radical-induced chain reactions and reduce oxidative damage in the body, preventing the peroxidative deterioration of structural cell membranes lipids and DNA damage.[9] Natural antioxidant products from a wide variety of plant species demonstrate therapeutic effects as anti-inflammatory and hepatoprotective.[10,11] Acerola has not yet been investigated for its anti-inflammatory or hepatoprotective potential, however previous studies have shown this fruit presents anti-hyperglycemic[12] and anti-carcinogenic[13] properties. The crude phenol extract from acerola, when administrated orally was able to reduce UVB-induced hyper-pigmentation.[2] Thus, the aim of this work was to investigate the antioxidant properties of acerola processed into juice and its hepatoprotective potential of against acute ethanol-induced stress.

## 2. Materials and methods

### 2.1. Plant material

Acerola fruits, cultivar *Okinawa* (Fig. 1) provided by a local grower at Paraipaba, Ceará State, Northeast Brazil were harvested ripe and red colored. Fruits were processed immediately with a domestic food processor under low speed and seeds and peel were separated by filtration. The juice was prepared from the processed pulp diluted in distilled water (1:1), taken into consideration when calculating the results, and then, stored at −20 °C until further antioxidant analysis and animal treatments.

### 2.2. Determination of non-enzymatic and enzymatic antioxidants of acerola juice

Total vitamin C was extracted from acerola juice (1:1, g pulp: ml water) and determined by titration with 2, 6-dichloroindophenol according the procedure described by Strohecker and Henning.[14] The results were expressed as mg/100 g FW (fresh weight).

The total phenolic content in acerola juice was measured using a modified Folin–Ciocalteu method.[15] with gallic acid as the standard, and the results were expressed as galic acid equivalents (GAE) mg/100 g FW.

**Fig. 1.** Fruits of acerola (*Malpighia emarginata* DC).

Total anthocyanins and flavonols were extracted from acerola juice (1:1, g pulp: ml water) with 95% ethanol plus 1.5 N HCl (85:15) according to the method of Lees and Francis.[16] The absorbance was measured at 535 nm and 374 nm, respectively. The results were expressed as mg/100 g FW.

The acerola juice was lyophilized and evaluated for activity of antioxidant enzymes. Enzymatic crude fraction was extracted from lyophilized juice (200 mg) homogenized in ice-cold potassium-phosphate buffer 100 mM, pH 7. The homogenate was filtered through a muslin cloth and centrifuged at 12,000 *g* for 15 min. The supernatant fraction was used as a crude extract for enzyme activity assays. All the procedures above were performed in triplicate at 4 °C. Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined spectrophotometrically based on inhibition of the photochemical reduction of nitroblue tetrazolium chloride (NBT).[17] Absorbance of the reaction mixture was measured at 560 nm. One unit of SOD activity (UA) was defined as the amount of enzyme required to cause a 50% reduction in the NBT photoreduction rate. The results were expressed as UA/g of dry mass (DM). Total catalase (CAT, EC 1.11.1.6) activity was measured according to the method of Beers and Sizer.[18] Decrease in $H_2O_2$ was monitored at 240 nm and enzyme activity was quantified by absorptivity coefficient (36 $M^{-1}$ $cm^{-1}$). The results were expressed as μmol $H_2O_2$/min.g of DM. Total ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed according to Nakano and Asada.[19] Reaction was initiated by adding $H_2O_2$ and ascorbate oxidation was measured at 290 nm for 1 min. Enzyme activity was determined using the absorptivity coefficient for ascorbate (2.8 $mM^{-1}$ $cm^{-1}$). The results were expressed in μmol $H_2O_2$/min/g of DM.

### 2.3. Determination of total antioxidant activity of acerola juice

Total antioxidant activity was measured using the 2.2′-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) method as described by Re et al.[20] Once the radical was formed, the reaction was initiated by adding 30 μl of the acerola juice (1:1, g pulp: ml water) extract sample to 3 mL of the $ABTS^+$ radical cation; the absorbance was measured (734 nm) after 6 min. Standard antioxidant Trolox solutions (ranging from 0 to 20 μM) were also evaluated against free radicals. Antioxidant activity was expressed as Trolox equivalent antioxidant capacity (TEAC), μmol Trolox/g FW.

### 2.4. In vivo studies

#### 2.4.1. Animal experiment and sampling

Female Swiss mice, weighing 25–30 g, were housed in standard cages and fed on normal pellet diet and water *ad libitum*. They were maintained under standard experimental conditions of humidity (40–45%), temperature (23–25 °C), 12 h light/dark cycle. The experimental protocol was approved by Ethics Committee of the Federal University of Ceará (protocol number 45/08). Mice were randomly divided into six groups of twenty animals each group. The animals received a pretreatment intra-gastrically once daily for 15 consecutive days: only distilled water (**control**); only acerola juice (**acerola juice**); only ascorbic acid (20 mg/kg), as suggested by the Nutrient Requirements of Laboratory Animals[21] (**ascorbic acid**); distilled water followed to single dose of ethanol (5 g/kg) on the 15th day (**ethanol**); acerola juice followed to single dose of ethanol (5 g/kg) on the 15th day (**acerola juice + ethanol**); ascorbic acid (20 mg/kg) followed to single dose of ethanol (5 g/kg) on the 15th day (**ascorbic acid + ethanol**). This protocol was defined after preliminary kinetic results (data not shown).

#### 2.4.2. Liver lipid peroxidation

The lipid peroxidation of mice liver cells was determined with Thiobarbituric Acid Reactive Species (TBARS) as markers as described by Ohkawa et al.[22] TBARS values were measured 1 h after administration of the acute ethanol dose and expressed as malondialdehyde (MDA) equivalents nmol/g of fresh liver tissue weight.

#### 2.4.3. Biochemistry indicators of liver function

The activity of serum enzymes used as liver damage markers was determined through assay kits (Wiener Laboratories, Rosario, Argentina): lactate dehydrogenase (LDH, EC 1.1.1.27) and aspartate (AST, EC 2.6.1.57) and alanine (ALT, EC 2.7.6.1) aminotransferase. After 6 h from administration of acute ethanol dosage, blood samples were collected from mice retro-orbital plexus and were allowed to clot and centrifuged at 3000 *g* for 10 min to obtain serum. Results were expressed as unit of enzyme activity for litre (U/L). Previously, the kinetic assay was realized to determinate the time used in this protocol (data not shown).

### 2.5. Statistical analysis

Data were initially submitted to Shapiro–Wilk and Bartlett tests to confirm normal distribution and homogeneity of variance, respectively. All data presented both requirements and were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test for comparison of means among groups. Analyses were performed using GraphPad Prism ver. 5.0 (GraphPad Software, La Jolla, CA, USA). Differences were considered statistically significant at $p \leq 0.05$ and results were expressed as the mean ± standard error of means (S.E.M.).

## 3. Results

### 3.1. Non-enzymatic and enzymatic antioxidants of acerola juice

Many of the health-promoting effects of fruits have been attributed to the antioxidant compounds and the antioxidants found in acerola juice are shown in Tables 1 and 2. The total vitamin C content (1799.5 mg/100 g FW) was high indicating that processing did not affect this important nutritional component of acerola. In spite of processing, the total phenolic (188.4 mg GAE/100 g FW), anthocyanin (9.2 mg/100 g FW) and flavonol (7.8 mg/100 g FW) contents of acerola juice was high (Table 1).

The antioxidant status of acerola juice was also investigated by measuring the activities of enzymes involved in free radical neutralization or scavenging (Table 2). The activity of SOD (1053.6 UA/g DM) was high in acerola juice indicating that, in spite of processing there is still a considerable ability to dismutate of superoxide free radicals into less reactive peroxide ($H_2O_2$). The catalasic activity was not detected in acerola juice; however APX activity (3.8 μmol $H_2O_2$/g DM/min) was determined.

**Table 1**
Non-enzymatic antioxidants in acerola juice.

| Bioactive compounds | mg/100 g FW |
|---|---|
| Total phenolics[a] | 188.4 ± 4.34 |
| Anthocyanins | 9.2 ± 0.48 |
| Flavonols | 7.8 ± 0.16 |
| Vitamin C | 1799.5 ± 52.18 |

Each value is the mean ± standard deviation. GAE = Galic Acid Equivalent, FW = Fresh Weight.
[a] mg GAE/100 g FW.

**Table 2**
Activities of antioxidant enzymes in acerola juice.

| Enzymes | Activity |
|---|---|
| Superoxide dismutase (SOD) | 1053.6 ± 5.21 UA/g DM |
| Ascorbate peroxidase (APX) | 3.8 ± 0.17 μmol $H_2O_2$/g DM/min |
| Catalase (CAT) | Not detected |

DM = Dry Mass, UA = Unit of SOD Activity.

### 3.2. Total antioxidant activity of acerola juice

The total antioxidant activity of acerola juice was determined and compared to that of ascorbic acid, as a reference (Table 3). The results show that total antioxidant activity of acerola juice (137.5 μmol Trolox/g FW) was forty-nine times higher than verified to ascorbic acid (2.8 μmol Trolox/g FW).

### 3.3. Liver lipid peroxidation

The ability of acerola juice to confer hepatoprotection against ethanol-induced stress was evaluated initially through lipid peroxidation degree (Fig. 2). Acute administration of ethanol led to an increase in hepatic MDA levels when compared to control ($p \leq 0.05$), indicating an enhancement in the lipid peroxidation potential of the liver. The previous supplementation with acerola juice to ethanol-treated mice reduced MDA formation in liver ($p \leq 0.05$). Indeed, the animals treated only with acerola juice showed a severe reduction in MDA concentration (approximately 50%) as compared to the control ($p \leq 0.001$). On the other hand, the groups treated with ascorbic acid were similar to respective controls ($p > 0.05$).

### 3.4. Biochemistry indicators of liver function

The activities of serum marker enzymes, such as AST, ALT and LDH increased in ethanol-treated mice compared to the control group ($p \leq 0.05$). Pretreatment with acerola juice diminished AST and ALT levels ($p \leq 0.05$) and did not alter LDH level ($p > 0.05$), although it presented a decrease of 17.0%. Similar results were observed to reference group treated with ascorbic acid (Fig. 3).

## 4. Discussion

Many of the protective effects of fruits have been attributed to the antioxidant properties of compounds, which have ability to scavenge free radicals, preventing DNA damage and cell membrane lipid peroxidation.[23]

Vitamin C, an important water soluble antioxidant, is reported to neutralize ROS and reduce the oxidative stress.[24] Vitamin C content of acerola juice in this study was higher than that of values found for commercial frozen acerola pulp.[25] Acerola is a rich source of vitamin C with values ranging from 500 to 1800 mg/100 g for different clones[26] and higher contents than other traditionally rich

**Table 3**
Total antioxidant activity values of acerola juice and Ascorbic acid.

| Sample | **TEAC**$_{ABTS}$ (μmol Trolox/g FW) |
|---|---|
| Acerola juice | 137.5 ± 28 |
| Ascorbic acid | 2.8 ± 0.12 |

Each value is the mean ± standard deviation. TEAC = Trolox Equivalent Antioxidant Capacity, ABTS = 2.2′-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt, FW = Fresh Weight.

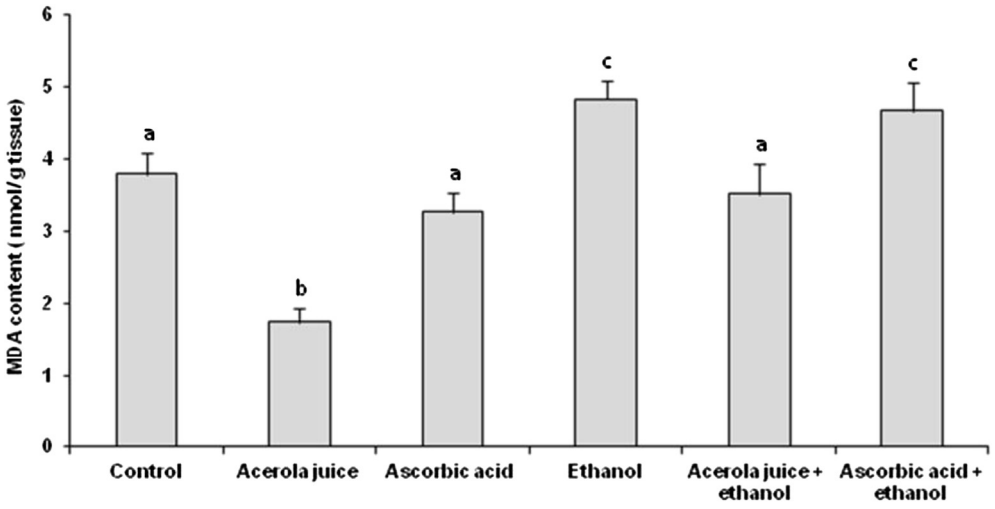


**Fig. 2.** Lipid peroxidation of hepatic cells from mice supplemented with acerola juice and submitted ethanol stress. Results are expressed as means ± S.E.M. ($n = 10$). Different small letters indicate significant difference among groups ($p < 0.05$).

fruit as orange and guava.[27] However, a tropical fruit from the Amazon region, camu–camu has even higher vitamin C contents than acerola.[28]

The total phenolic content of acerola juice was high, when compared to others fruits as papaya[27] and passion fruit.[29] However, it was lower than that found others acerola cultivars.[30] Polyphenols have been associated to prevention of degenerative diseases in human and this was mainly attributed to their antioxidant properties.[31] Among the phenolics, anthocyanin and flavonol contents were further determined in acerola juice. The major anthocyanins identified in acerola varieties were cyanidin-3-rhamnoside and pelargonidin-3-rhamnoside.[32–34] Regarding the flavonol, the acerola juice presented lower levels than other acerola varieties,[35] ranging from 9.31 to 20.22 mg/100 g and others fruits as cashew apple and açai.[28] However, acerola juice still has more flavonol than kiwi and avocado.[36]

In this study, SOD activity was high in acerola juice, thus SOD might play an important role in dismutation of superoxide radicals. In spite of this, CAT activity was not detectable. Probably, the high contents of non-enzymatic antioxidants contribute to maintain low levels of ROS, such as peroxide ($H_2O_2$), and as consequence to inhibit CAT activity. It was also related that under low concentrations, the peroxidases play a important role in $H_2O_2$ detoxification.[37] Thus, APX activity can be main responsible for $H_2O_2$ detoxification in acerola.

A complex antioxidant system, including enzymes and non-enzymatic antioxidants is activated to protect cellular membranes and organelles against the damaging effects of reactive species.[38] Thus, the total antioxidant activity corresponds to a synergistic action of different classes of antioxidant[39] as verified in acerola juice. The antioxidant activity of acerola juice was higher than found for guava and strawberry[29] and other tropical fruits.[40] These results indicate that among the non-enzymatic antioxidants, vitamin C is the major contributor to the total antioxidant activity in acerola juice.

Taking into account all the presented data concerning all of the non-enzymatic and enzymatic antioxidant properties in acerola juice, it was selected for the great potential as functional food. The effect of acerola juice ingested by Swiss mice challenged with a single dose of ethanol (5 g/kg) was evaluated for its protection against hepatotoxicity. It has been shown that ethanol administration induces an oxidative stress that could be prevented by antioxidant compounds.[41] This beneficial effect is due to their action as free radical-neutralizing agents and reduction of oxidative damage in the body.[6]

Free radicals are byproducts of normal respiratory process, however under stressful conditions they are over-produced leading to an imbalance between pro-oxidant and antioxidant systems. In excess, these chemical species will react indiscriminately with membrane lipids, proteins and nucleic acids resulting, at last, in cell death. Free radical-induced lipid peroxidation causes a decrease in membrane fluidity and usually culminates in rupture and leakage of intracellular electrolyte material, as the tissue disintegrates. The effect of acerola juice on ethanol-induced membrane lipid peroxidation measured in the liver of challenged mice is shown in Fig. 2. The acerola juice treatment reduced significantly lipid peroxidation, over 50%, as determined by lower MDA levels in mice liver when compared to control treatment with distilled water ($p \leq 0.001$). However, mice treated with ascorbic acid, showed no statistical difference in lipid peroxidation degree when compared to control ($p > 0.05$). These results suggest that, although acerola juice presents high contents of vitamin C associated to a high total antioxidant activity, this compound is not the main responsible for the inhibition of lipid peroxidation, here reported.

When mice were subjected to an acute administration of ethanol, there was a significant increase in hepatic lipid peroxidation degree when compared to control ($p \leq 0.05$), indicative of an undergoing oxidative stress which enhanced lipid peroxidation of liver cells membranes. Liver of animals fed with acerola juice previously to acute ethanol administration presented a significant reduction in lipid peroxidation, similar to control levels, when compared to the only ethanol-treated group. Moreover, the pretreatment with ascorbic acid previously to ethanol administration did not prevent lipid peroxidation and did not differ statistically from the ethanol-treated group.

Thus, there is evidence that acerola juice promotes beneficial effects to human health under normal physiological and ethanol-induced oxidative stress conditions. The main nutraceutical characteristic of acerola is the high antioxidant activity usually attributed to its high vitamin C content, however results show that vitamin C is not the main responsible for liver cells membrane protection against free radicals, but it will probably depend on other components as phenolic compounds to play such role. Righetto et al[39] also reported that vitamin C will only induce oxidative protection only when associated synergistically with others antioxidant components. These results also agree with

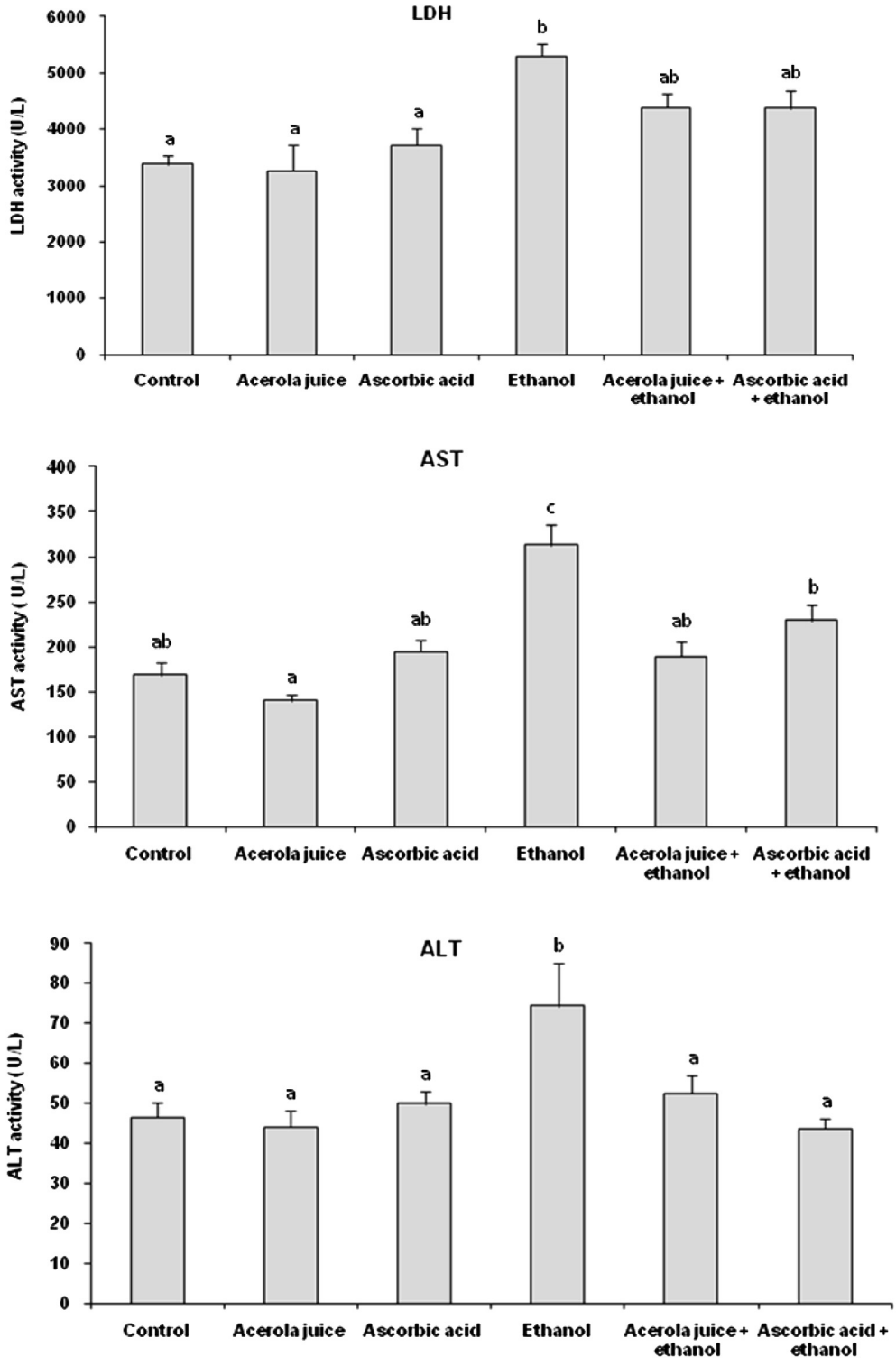


**Fig. 3.** Biochemical indicators of liver function in the serum from mice supplemented with acerola juice and submitted ethanol stress. LDH: Lactate Dehydrogenase; AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase. Results are expressed as means ± S.E.M. ($n = 10$). Different small letters indicate significant difference among groups ($p < 0.05$).

others studies using different experimental models of ethanol exposure, confirming the pathogenic role of oxidative stress in mice liver and indicating that antioxidants found in diet may provide protective effects against ethanol-induced liver damage.[7,8,42]

The enzymes AST, ALT and LDH are well known biomarkers of liver toxicity as their leakage into the blood stream is mainly attributed to hepatic damage.[43,44] Thus, the levels of these serum marker enzymes for hepatic damage were evaluated (Fig. 3) and under normal physiological condition, mice fed with acerola juice and ascorbic acid did not differ statistically from control regarding their activities ($p \leq 0.05$). However, mice subjected to an acute administration of ethanol presented a significant increase in activity for all evaluated liver enzymes, when compared to control ($p \leq 0.05$) corroborating with the idea that ethanol stress induces a

patho-physiological disorder in the liver. Both pretreatments with acerola juice and ascorbic acid previously to ethanol administration reduced significantly AST and ALT levels ($p \leq 0.05$) when compared to the ethanol-treated group. In spite of the acute ethanol dosage, AST and ALT activity levels were similar to control group, thus acerola juice acted as a liver-protecting agent against ethanol-induced damage.

The hepatoprotective effect of fruit or plant extracts has been strongly associated with their antioxidant potential.[6,9,11,41] The antioxidants present in acerola juice play an important role in the observed hepatoprotective effect as vitamin C seems involved in the stability of normal marker enzymes levels; meanwhile phenolics seem involved in liver cell lipid peroxidation inhibition. Globally, the acerola juice antioxidants of non-enzymatic or enzymatic nature are capable of scavenging or neutralizing reactive species excessively produced under alcohol-induced stress and as a result, enhancing the total antioxidant status of the liver.

## 5. Conclusion

In conclusion, our results reveal that antioxidants from acerola juice prevent the hepatic damages induced by ethanol. Thereby, these results provide a strong scientific basis for the use of acerola juice as functional food.

## Conflicts of interest

All authors have none to declare.

## Acknowledgments

We thank the Brazilian agencies CAPES, CNPq and FUNCAP for the financial support.

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