Syzygium cumini Leaf Extract Protects Against Ethanol-Induced Acute Injury in Rats by Inhibiting Adenosine Deaminase Activity and Proinflammatory Cytokine Production

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ABSTRACT

The consumption of a large quantity of alcohol over a relatively short time is an increasingly important public health issue. Syzygium cumini has anti-inflammatory and antioxidant properties. Hence, this study aimed to investigate the activity of the enzyme adenosine deaminase and the release of inflammatory mediators in an animal model of acute ethanol intoxication. We also investigated the possible beneficial effects of an aqueous leaf extract of S. cumini (ASc). To address this issue, two groups of rats received a single oral dose of ethanol (5 g kg\(^{-1}\)). After 30 min, a group was treated with 0.4 g kg\(^{-1}\) of ASc. A control group and a group treated only with ASc were also included. After 6 h, we found that the acute administration of ethanol increased ADA activity in serum and in spleen lymphocytes and the IL-1, IL-6, TNF-\(\alpha\), INF-\(\gamma\) and NO\(_x\) serum levels. Moreover, ethanol decreased the levels of IL-10 and ADA activity in the cerebral cortex. The ASc was effective in reversing the changes in ADA activity and in the levels of NO\(_x\) and IL-10. In conclusion, our results suggest that the effect on the purinergic system is one of the mechanisms by which ethanol exerts its effects on the inflammatory mediators and on the central nervous system. The results also highlight the potential effect of S. cumini treatment in ameliorating changes caused by acute ethanol intoxication.

Key words: Adenosine deaminase activity, acute ethanol intoxication, inflammation, Syzygium cumini

INTRODUCTION

Ethanol is a substance that is widely used. When consumed in excess, ethanol causes harmful health effects; therefore, policies to reduce and prevent its use are considered high-priority public health issues (WHO., 2014). In particular, the acute ethanol intoxication is a clinically harmful condition that results from the ingestion of a large amount of alcohol. This intoxication can promote heterogeneous clinical manifestations and involve different organs and apparatuses, with behavioral, cardiac, gastrointestinal, neuronal, pulmonary and metabolic effects (Vonghia et al., 2008). Ethanol consumption also exerts influence on the inflammatory response and dependent mechanisms of this response (Molina, 2014; Goodman et al., 2013; Li et al., 2011). Among the changes produced by high doses of alcohol are those involving the adenosinergic system. In fact,
adenosine signaling has been implicated in the pathophysiology of many Central Nervous System (CNS) disorders, including, alcoholism (Burnstock, 2008; Dunwiddie and Masino, 2001; Fredholm et al., 2005a).

The adenosine system is considered a powerful mechanism for regulation of various processes related to inflammatory response and protection of tissues from injury (Sitkovsky and Ohta, 2005; Antonioli et al., 2008). Furthermore, adenosine contributes to the resolution of inflammation by interacting with adenosine receptors at later stages of immune or inflammatory processes (Lawrence et al., 2002). When combined with other pathological mechanisms, the inflammation is suggested to contribute to the development of hepatic disease associated with excessive ethanol consumption (Beier and McClain, 2010).

The inflammatory changes caused by ethanol differ according to its consumption. Acute or moderate ethanol use causes an attenuated inflammatory response, whereas the heavy use is related to increased inflammation (Goral et al., 2008). In spite of the benefits of the development of immune and inflammatory responses of the body to an external injury, when in excess, they can cause significant tissue damage (Rodriguez-Molinero et al., 2007). In this sense, the demand for mechanisms of regulation of these processes is highlighted. By interacting with immune cell receptors, adenosine plays a powerful role in immunosuppression, regulating immune responses and limiting inflammatory responses to harmful external insults (Sitkovsky and Ohta, 2005; Gessi et al., 2008). Whereas, the biological functions regulated by adenosine depend on its concentration in the biophase receptors, the catabolic enzyme adenosine deaminase (ADA), thus, plays a pivotal role in the modulation of purinergic responses (Antonioli et al., 2012). Several lines of evidence support a complex regulatory role played by ADA in different immune-cell functions, as well as, a significant involvement of this catabolic enzyme in the pathophysiology of several inflammatory diseases. Metabolically, ADA is an enzyme that catalyzes the conversion of adenosine to inosine, thus the increase in ADA activity may affect adenosine levels available for stimulation of adenosine receptors expressed on T-cell surface, contributing to impaired immune regulation (Hershfield, 2005; Mandapathil et al., 2010; Gessi et al., 2007) and protracted inflammatory responses (Conlon and Law, 2004).

The use of plants for medicinal purposes is widespread throughout the world. Because of this widespread use, traditional medicinal plants have been studied in relation to its various pharmacological properties (Bhuyan et al., 2010). In this context, Syzygium cumini (L.) skeels (Myrtaceae) has been shown to possess bioactive compounds such as flavonoids, glycosides, tannins, anthocyanins and ascorbic acid to which are assigned the pharmacological activities of this plant. In particular, the leaves have antibacterial and anti-inflammatory properties and have been extensively used for the treatment of diabetes (Pepato et al., 2001; Jagetia and Baliga, 2002; Pari and Saravanan, 2002; Sharma et al., 2006). In addition to these pharmacological actions, the leaves already showed effect against liver damage produced by carbon tetrachloride (Moresco et al., 2007). Indeed, Hossain et al. (2011) were the first to identify the hepatoprotective effect of the extract of S. cumini in an animal model of chronic ethanol exposure. Furthermore, previous studies have demonstrated that the aqueous leaf extract of S. cumini (ASc) exhibits protective properties to the increase in ADA activity observed in serum and cells of diabetic and hyperglycemic patients in vitro (Bopp et al., 2009; De Bona et al., 2010).

Thus, considering the numerous pathophysiological changes caused by excessive ethanol consumption, among which are inflammatory alterations and also the wide use of S. cumini in folk medicine against inflammation and its known effects on the adenosine system, the present study
was aimed at investigating the activity of ADA and the release of inflammatory mediators in acute ethanol intoxication using rats as an experimental model. The possible beneficial effect of an ASc in this model was also evaluated.

MATERIALS AND METHODS

Chemicals: Adenosine was obtained from Sigma Chemical Co (St. Louis, MO, USA). Ethanol was obtained from Vetec (Rio de Janeiro, Brazil). All other chemicals were of analytical grade and were obtained from standard commercial suppliers.

Plant material and ASc preparation: Leaves of *S. cumini* were freshly locally collected, cleaned, dried and powdered. They were identified by the Laboratory of Botanic and Pharmacognosy of the Franciscan University Center, Santa Maria, Brazil. The leaves were dried in a greenhouse with air circulation at 40°C for approximately 48 h. Then, they were ground in a knife mill. The products were submitted to extraction in a Soxhlet apparatus until exhaustion. After extraction, the solvent was evaporated in a rotavapor to give the crude extract. The stock solution was made by dissolving 1 g of the crude extract in 100 mL NaCl 0.9%. A voucher specimen (SMDB 14.001) was identified and deposited at the Herbarium of the Federal University of Santa Maria.

High-performance liquid chromatography characterization of the ASc: Reverse phase chromatographic analyses were carried out under gradient conditions using C18 columns (4.6×150 mm) packed with 5 μm diameter particles. The mobile phase was: (A) Acetonitrile: Water (95:5, v/v) and (B) Water: Phosphoric acid (98:2, v/v) and the composition gradient was: 5% of A until 10 min and changed to obtain 20, 40, 50, 60, 70 and 100% A at 20, 30, 40, 50, 60 and 80 min, respectively, following the method described by Kamdem et al. (2013), with slight modifications. The ASc was analyzed at a concentration of 1 mg mL⁻¹. The presence of eleven antioxidant compounds was investigated, namely gallic, chlorogenic, caffeic and ellagic acid, catechin, epicatechin, quercetin, quercitrin, isoquercitrin, kaempferol and rutin. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.6 mL min⁻¹, injection volume was 40 μL and wavelength was 254 nm for gallic acid, 280 nm for catechin and epicatechin, 325 nm for chlorogenic, ellagic and caffeic acids and 366 nm for rutin, isoquercitrin, quercitrin, kaempferol and quercetin. The samples and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standard references were prepared in the HPLC mobile phase at a concentration range of 0.025-0.300 mg mL⁻¹ for quercetin, quercitrin, isoquercitrin, kaempferol and rutin; 0.040-0.250 mg mL⁻¹ for gallic, chlorogenic, caffeic and ellagic acids and 0.03-350 mg mL⁻¹ for catechin and epicatechin. The chromatography peaks were confirmed by comparing their retention time with those of reference standards and by DAD spectra (200-600 nm). All chromatographic operations were carried out at ambient temperature and in triplicate. The Limit Of Detection (LOD) and Limit Of Quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as defined by Boligon et al. (2012). LOD and LOQ were calculated as, 3.3 and 10 c/S, respectively, where o is the standard deviation of the response and S is the slope of the calibration curve.

Animals: Twenty four male Wistar rats weighing 190-210 g were used for the experiment. The procedures followed the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria (protocol number 072/2013). All efforts were made to minimize the number of animals used and their suffering.
Experimental protocol: After the acclimation period, the animals were randomly separated into four groups of six animals each: Control, ethanol (EtOH), ASc and ethanol plus ASc (EtOH+ASc). Initially, the control and ASc groups received water by gavage. The remaining groups received an acute (5 g kg\(^{-1}\) of body weight) dose of 40% (w/v) ethanol in aqueous solution (Portari et al., 2008). After thirty minutes, animals from control and EtOH groups received water by gavage, whereas animals from ASc and EtOH+ASc groups received 400 mg kg\(^{-1}\) of ASc by gavage (Hossain et al., 2011). The animals were left to recover with free access to water and 6 h after ethanol administration were anesthetized using isoflurane and euthanized to collect serum and tissues. The cerebral cortex tissue was homogenized, centrifuged and the supernatant was stored at -80°C. The spleen was used for isolation of lymphocytes.

Isolation of rat spleen lymphocytes: Lymphocytes were isolated from the rat spleens under aseptic conditions as described by SaiRam et al. (1997). Briefly, spleens were taken out, washed with cold PBS, cut into several pieces and gently crushed in PBS. Subsequently, cells were collected by centrifugation. Erythrocytes were lysed with lysis buffer (0.15 M NH\(_4\)Cl, 1 mM NaHCO\(_3\), 0.1 mM EDTA, pH 7.4) and washed and resuspended in PBS. Cell number and viability were determined by trypan blue exclusion. More than 95% of the cells were found to be viable. Final cell suspension was performed in PBS (pH 7.4) and 3×10\(^6\) cells mL\(^{-1}\) were used for each analysis.

Measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and Advanced Oxidation Protein Products (AOPP) serum levels: The levels of ALT and AST in the serum were detected using enzymatic methods with commercial kits from Labtest Diagnóstica S.A. (Brazil), using a semi-automatic biochemical analyzer (LABQUEST). The results were expressed as U L\(^{-1}\). AOPP was measured by the semiautomated method as described by Witko-Sarsat et al. (1998), with results expressed as, µM L\(^{-1}\).

ADA activity assay in serum, lymphocytes and cerebral cortex: ADA activity was estimated spectrophotometrically using the method of Giusti and Gakis (1971), which is based on the direct measurement of the formation of ammonia produced when ADA acts in excess of adenosine. The results were expressed in U L\(^{-1}\) for serum and lymphocytes and in U g\(^{-1}\) protein for the tissues.

Measurement of IFN-δ, TNF-α, IL-1, IL-6, NOx and IL-10 serum levels: Inflammatory cytokine quantification was assessed by ELISA using commercial kits for human IFN-δ, TNF-α, IL-1, IL-6 and IL-10 (R and D Systems, Minnesota, USA), according to manufacturer’s instructions. Briefly, 96-well microplates were sensitized with the primary antibody at room temperature for 30 min. Then, the samples were added and incubated at 37°C for 30 min. After washing, the secondary antibody conjugated with peroxidase was added and incubated. The presence and concentration of the cytokines were determined by the intensity of the color measured by spectrometry in a micro-ELISA reader. Nitric Oxide (NOx) was determined indirectly by quantifying serum NOx (Tatsch et al., 2011), measured by the modified Griess method, using the Cobas Mira® automated analyzer. The results were expressed as, µM L\(^{-1}\).

Statistical analysis: The analyses were performed using STATISTICA for Windows, version 6.0 (StatSoft. Inc., Tulsa, OK, USA). All data were analyzed, using two way ANOVA, followed by
the Duncan multiple range test and presented as Mean±Standard Deviation (SD). A value of p<0.05 was considered statistically significant for all analyses.

RESULTS

HPLC analysis: HPLC analysis HPLC fingerprinting of ASc revealed the presence of gallic acid ($\lambda = 245$ nm, $t_R = 11.56$ min, peak 1), catechin ($\lambda = 280$ nm, $t_R = 17.09$ min, peak 2), chlorogenic acid ($\lambda = 325$ nm, $t_R = 23.64$ min, peak 3), caffeic acid ($\lambda = 325$ nm, $t_R = 25.13$ min, peak 4), ellagic acid ($\lambda = 325$ nm, $t_R = 30.15$ min, peak 5), epicatechin ($\lambda = 280$ nm $t_R = 35.84$ min, peak 6), rutin ($\lambda = 366$ nm, $t_R = 39.96$ min, peak 7), quercetin ($\lambda = 366$ nm, $t_R = 44.83$ min, peak 8), isoquercitrin ($\lambda = 366$ nm, $t_R = 46.57$ min, peak 9), quercetin ($\lambda = 366$ nm, $t_R = 51.23$ min, peak 10) and kaempferol ($\lambda = 366$ nm, $t_R = 60.34$ min, peak 11) (Table 1).

Effect on ADA activity: Initially, we observed that a high dose of ethanol caused a significant increase in the (a) ADA activity in serum and (b) Lymphocytes, when compared to the control (p 0.001) (Fig. 1). However, ADA activity in the serum and lymphocytes of rats treated with ASc

### Table 1: Composition of the aqueous leaf extract of Syzygium cumini

| Compounds         | Concentration (mg g$^{-1}$) | Percentage | LOD (µg mL$^{-1}$) | LOQ (µg mL$^{-1}$) |
|-------------------|----------------------------|------------|-------------------|--------------------|
| Gallic acid       | 29.82±0.01$^a$             | 2.98       | 0.027             | 0.089              |
| Quercetin         | 19.07±0.02$^b$             | 1.90       | 0.010             | 0.034              |
| Caffeic acid      | 17.50±0.02$^b$             | 1.75       | 0.016             | 0.054              |
| Isoquercitrin     | 15.46±0.05$^b$             | 1.54       | 0.025             | 0.082              |
| Rutin             | 11.31±0.01$^b$             | 1.13       | 0.009             | 0.030              |
| Ellagic acid      | 10.62±0.01$^b$             | 1.06       | 0.022             | 0.074              |
| Quercitrin        | 9.84±0.04$^b$              | 0.982      | 0.031             | 0.102              |
| Chlorogenic       | 6.15±0.03$^b$              | 0.61       | 0.018             | 0.059              |
| Epicatechin       | 4.98±0.03$^b$              | 0.49       | 0.007             | 0.023              |
| Kaempferol        | 4.58±0.01$^b$              | 0.45       | 0.043             | 0.141              |
| Catechin          | 4.37±0.01$^b$              | 0.45       | 0.036             | 0.119              |

Results are expressed as Mean±SD of three determinations, Means followed by different letters differ (p<0.01) by the Tukey test, LOD: Limit of detection, LOQ: Limit of quantification.

Fig. 1(a-b): Acute ethanol intoxication and/or ASc treatment on, (a) ADA activity in serum and (b) Lymphocytes. Data are reported as, Mean±SD and expressed as, units per liter. Statistically significant differences were determined by two-way ANOVA followed by the duncan multiple range test, *p<0.05 in comparison with the control group (n = 6), C: Control, EtOH: Ethanol, ASc: Aqueous leaf extract of Syzygium cumini
Fig. 2: Acute ethanol intoxication and or ASc treatment on ADA activity in the cerebral cortex. Data are reported as, Mean±SD and expressed as units per liter. Statistically significant differences were determined by the Duncan test, *p<0.01 in comparison with the control group, #p<0.01 in comparison with the EtOH group (n = 6), C: Control, EtOH: Ethanol, ASc: Aqueous leaf extract of *Syzygium cumini*

### Table 2: Effect of the acute ethanol intoxication on AST, ALT and AOPP levels

| Parameters | Control | EtOH | ASc | EtOH+Asc |
|------------|---------|------|-----|----------|
| AST (U L⁻¹) | 120.4±16.8 | 131.5±23.4 | 123.00±13.3 | 120.90±19.3 |
| ALT (U L⁻¹) | 48.6±9.6 | 44.4±4.9 | 0.52±7.1 | 47.50±9 |
| AOPP (µM L⁻¹) | 12.0±2.2 | 10.6±1.8 | 9.67±2.2 | 9.28±1.31 |

Data are reported as Mean±SD (n = 6). ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, AOPP: Advanced oxidation protein products, EtOH: Ethanol, ASc: Aqueous leaf extract of *Syzygium cumini*

30 min after ethanol administration returned to values comparable to those of control animals. In contrast to the changes caused by ethanol in serum and lymphocytes, ADA activity was reduced in the cerebral cortex after acute ethanol administration, when compared to the control group (p<0.01). Such effect was also reversed by ASc treatment (Fig. 2).

**Effect on inflammatory parameters:** As shown in Fig. 3, acute ethanol intoxication has the potential to change inflammatory cytokines and mediator levels. The administration of ethanol was able to cause a significant increase in the serum levels of the proinflammatory mediators IL-1, IL-6, TNF-α, INF-δ and NOx and a decrease in the anti-inflammatory cytokine IL-10 (p<0.001). Although, ASc treatment did not prevent the increase in the levels of the proinflammatory cytokines IL-1, IL-6, TNF-α and INF-δ, the beneficial effect of ASc was observed in preventing the ethanol-induced NOx increase. Moreover, ASc partially prevented the reduction in IL-10 levels caused by ethanol intoxication (p<0.01).

**Effect of the acute ethanol intoxication on the levels of AST, ALT and AOPP:** After six hours of ethanol administration, no significant alterations were observed in the activity of the liver enzymes AST and ALT and in the AOPP level (Table 2). The results section should provide details of all of the experiments that are required to support the conclusions of the paper. There is no specific word limit for this section. The section may be divided into subsections, each with a concise subheading. Large datasets including; raw data, should be submitted as supporting information files; these are published online alongside the accepted article. We advise that the results section be written in past tense.
DISCUSSION

The present study demonstrated that after six hours of acute intoxication, although ethanol did not change serum AST, ALT and AOPP levels, we observed significant immunological and inflammatory changes. Ethanol promoted an increase in the serum levels of proinflammatory mediators (IL-1, IL-6, TNF-α, IFN-δ and NOx) and a reduction in the anti-inflammatory cytokine IL-10. A significant finding in this study was the fact that ethanol caused an increase in ADA activity in serum and splenic lymphocytes. Despite increasing amounts of knowledge regarding the
physiological role of ADA activity, there is currently no conclusive information on this enzymatic function under acute ethanol intoxication conditions, particularly under inflammatory conditions. It has been shown that augmented ADA activity reflects accelerated purine turnover and high salvage pathway activity which may lead to reduce adenosine levels (Gulec et al., 2003; Kather, 1990) and to further tissue damage by interfering with the role of this nucleoside in the regulation of inflammation (Ohta and Sitkovsky, 2001). Moreover, this increased ADA activity may be related to changes in the levels of IL-1, IL-6, IL-10, TNF-α, IFN-δ and NOx, observed after acute exposure to high doses of ethanol. Acute inflammation is a critical homeostatic mechanism for protecting a host against noxious conditions, including tissue injury (Wang et al., 2012). In fact, many of the activities of effector cells of innate and adaptive immunity are mediated by cytokines. In this regard, several studies have demonstrated the pivotal involvement of adenosine in lymphocyte proliferation as well as in cytokine production and differentiation (Csoka et al., 2008; Nemeth et al., 2006), thus confirming a critical role played by ADA activity in lymphocyte action. Alcohol consumption, whether acute or chronic, has a significant impact on phenotypic and functional aspects of immune cells. Hence, the ethanol-induced increase in the ADA activity in spleen lymphocytes can also harm this control exercised by ADA in the cells of the immune system.

Alcoholism has been associated with a disruption in the balance and function of cytokines (Crews et al., 2006; Gonzalez-Quintela et al., 2000). Changes, such as; increased levels of IL-4 and IFN-α in the stomach, systemic changes, such as; increased synthesis of IL-4 and decreased production of IL-10 by splenocytes are observed (Andrade et al., 2006). Patients with alcoholic cirrhosis present increased concentrations of TNF-α and IL-6 as well as decreased production of IL-10 and IL-2 (Romeo et al., 2007). Interestingly, we observed these changes with only one exposure to a high ethanol dose.

Considering the exacerbation of proinflammatory mediators and the reduction in the levels of anti-inflammatory mediators, it is necessary to find alternatives to prevent these harmful effects triggered by the use of ethanol. In this sense, numerous studies are dedicated to evaluate the protective effects of drugs or nature compounds on binge drinking (Zhang et al., 2012; Lv et al., 2010; Song et al., 2006). In this context, ASc was able to prevent the increase in ADA activity in serum and lymphocytes. It also reduced the serum levels of NOx and partially re-established levels of the anti-inflammatory cytokine IL-10. It is assumed that the mechanisms described in this study are advantageous in situations of acute high-dose ethanol exposure, since the reduction of ADA activity can result in increased levels of adenosine, which affect various physiological and pathological processes in cells, such as the release of inflammatory cytokines (Hasko and Cronstein, 2004).

This promising therapeutic effect may be related to the various phytochemicals present in the ASc. Furthermore, polyphenolic compounds including flavonoids and phenolic acids are known to have various physiological activities, such as; antioxidant, anti-inflammatory, antimicrobial and anticancer properties (Ushida et al., 2008). Many neuronal responses to ethanol are suggested to be mediated by adenosine. Our results suggest that the reduction in ADA activity observed after acute exposure to ethanol can lead to accumulation of adenosine in the cerebral cortex of rats. In fact, the nucleoside adenosine is an important neuromodulator in the CNS having a key role in the control of excitatory and inhibitory functions (Fredholm et al., 2005b). Indeed, ataxia is one of the most significant depressant effects observed after excessive intake of ethanol (Choi et al., 2004; Clark and Dar, 1989). Therefore, these results may corroborate other study showing that ethanol acts directly on the brain to increase extracellular levels of adenosine (Sharma et al., 2010). It is
consolidated that these metabolites play a key role in the brain mediating some actions of ethanol (Deitrich, 2004; Israel et al., 1994). Thus, the reduction of ADA activity in the cerebral cortex could be mediated by these metabolites. ASc was able to restore ADA activity to levels observed in the control animals. This action caused by ASc treatment can establish the homeostasis of adenosine in the CNS, preventing its accumulation and hence, avoiding the effect of excess adenosine in the cerebral cortex. In fact, previous studies have also shown that, in addition to their potent antioxidant/anti-inflammatory effects, the beneficial properties of polyphenols might occur through alterations in stress signaling and improve cognition reducing oxidative stress by modulating protein and lipid oxidation and by improving total antioxidant status (Joseph et al., 2006, 2007; Gutierrez et al., 2012).

The acute ethanol intoxication in rats caused changes in the release of inflammatory mediators and in ADA activity. The ASc was effective in protecting changes in ADA activity and partially prevented the changes in the levels of the inflammatory mediators. These activities might be attributed, at least in part, to the high phenolic and flavonoid contents present in the ASc. Our results also suggest that the effect on the purinergic system is one of the mechanisms by which ethanol exerts its effects on the inflammatory mediators and on the CNS.

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