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Protective effects of *Syzygium cumini* seed extract against methylmercury-induced sistemic toxicity in neonatal rats

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Abstract *Syzygium cumini* (L.) Skeels (Sc) belongs to the medicinal plants with an important source of phenolic compounds. Sc has been shown to possess antioxidant and anti-inflammatory properties. Methylmercury (MeHg), a highly toxic environmental pollutant, induces oxidative stress and dysfunction in many cell types. This study was aimed to evaluate the effect of aqueous seed extract of Sc (ASc) on MeHg-induced toxicity in rats. Two-day-old rats (P2) received a single dose of MeHg (10 mg/kg) and two doses of ASc (0.9 mg/kg) *per os*. After two days, the effects of the treatment were investigated in the cerebral cortex, hippocampus, kidney, liver and urine samples. Our results demonstrated that *N*-acetyl-β-D-glucosaminidase (NAG) activity in the kidney and urine, the lipid peroxidation levels in the liver and kidney samples, as well as the adenosine deaminase (ADA) activity in the hippocampus, kidney and liver were higher in MeHg-group when compared to the control group. The administration of ASc reverted the toxic effects of MeHg. It is noteworthy to observe that the main compounds present in the ASc, as gallic acid (the major component), chlorogenic acid and rutin, might be the responsible for such benefit, since they were found to display antioxidant properties.

Keywords Methylmercury · *Syzygium cumini* · *N*-acetyl-β-D-glucosaminidase · Rat · Adenosine deaminase

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Introduction

Currently, plants and/or their isolated compounds, have received new attention, mainly concerning about the protective effects in relief of diseases (Bhat et al. 2008; Brito et al. 2007). In this context, *Syzygium cumini* (L.) Skeels (Sc) is a well-known plant due to its several pharmacological actions, such as anti-inflammatory, antioxidant, antidiabetic, among others (Prince et al. 2004; Veigas et al. 2008). Another aspect to consider is the fact that Sc protected rats from the carbon tetrachloride-induced hepatotoxicity (Moresco et al. 2007). Sc has shown gastroprotective and anti-ulcerogenic effects after the induction of gastric damage in rats (Ramirez and Roa 2003). Sc also provided protection against the radiation-induced bone marrow death in mice (Jagetia and Baliga

2002). Despite that, the effects of Sc are not completely elucidated.

Methylmercury, a potent hazardous compound, causes acute and chronic damage to multiple organs. The MeHg-exposed individuals exhibit severe forms of neurological disease which include cognitive, sensory and motor disturbances (Clarkson and Strain 2003; Takeuchi et al. 1979). During the early postnatal period the brain is extremely sensitive to external agents, including MeHg (Manfroi et al. 2004; Sakamoto et al. 2004). Furthermore, nephrotoxicity and gastrointestinal effects have been reported. During prolonged exposure, MeHg may accumulate in the kidney and promote renal toxicity (Woods et al. 1991; Zalups and Bridges 2009). MeHg can also affect neuronal and astrocytic functions, cell membrane properties and the cytoskeletal integrity, and can enhance the production of reactive oxygen species (ROS) (Castoldi et al. 2001; Liu et al. 2009; Moretto et al. 2004; Moretto et al. 2005).

Adenosine deaminase (ADA; EC: 3.5.4.4), the enzyme responsible for the degradation of endogenous adenosine to its inactive metabolite inosine, is important in the acute and protracted inflammatory responses (Conlon and Law 2004; Desrosiers et al. 2007). Recent findings of our group demonstrated the intense presence of ADA activity in leukocyte suspension (Abdalla et al. 2010).

An improvement of cell viability in rat hepatocytes was observed with exposure to Sc (Veigas et al. 2008). However, there are few studies in the literature regarding Sc effects on the heavy metal toxicity. Thus, the aim of this study was to investigate whether the treatment with ASc could attenuate MeHg-induced effects on ADA activity, thiobarbituric acid reactive species (TBARS) production in the brain (cerebral cortex and hippocampus), liver and kidney and *N*-acetyl-β-D-glucosaminidase (NAG) activity in the kidney and urine of neonatal rats.

Materials and methods

Chemicals

Methylmercury (II) chloride was obtained from Aldrich Chemical Co (98% of purity; Milwaukee, WI). *N*-acetyl-β-D-glucosaminidase, kaempferol, rutin, gallic and chlorogenic acids reference standards were acquired from Sigma Chemical Co. (St. Louis, MO, USA). Methanol and acetonitrile were of HPLC grade, and they were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Plant material and aqueous seed extract of *Syzygium cumini* (L.) Skeels preparation

The seeds of Sc were collected fresh locally and they were cleaned, dried and powdered. Eighty (80 g) grams of the seed powder were extracted with 400 ml of distilled water for 1 h under reflux as described by Prince et al. (1998). The extract was lyophilized and stored at −20°C until further use. The yield of the extract was 6.2% w/w.

HPLC–DAD characterization of the extract

HPLC was carried out in a Shimadzu Prominence Auto-Sampler (SIL-20A), equipped with UV–Vis detector DAD/SPD-M20A. The mobile phase was methanol:acetonitrile:water (40:15:45) containing 1.0% acetic acid in isocratic condition at a flow rate of 0.8 ml/min in a RP-C18 column. The mobile phase and the samples were filtered through a 0.45 μm membrane filter (Millipore) and degassed by an ultrasonic bath prior to use. Chromatographic peaks were confirmed by comparing their retention time and DAD–UV spectra with those of the reference standards at 257 nm. Rutin, kaempferol, chlorogenic and gallic acids were diluted in the same mobile phase to obtain the standard curves in the concentration range of 0.0125 to 0.200 mg/ml. Calibration curves for rutin, chlorogenic and gallic acids were, respectively: $Y = 19217x–16949$ ($r = 1.0000$), $Y = 30153x–214576$ ($r = 0.9998$) and $Y = 16324x–661582$ ($r = 0.9967$). All chromatographic operations were performed in triplicate at room temperature.

Experimental design

The animals were used according to the guidelines of the Ethics Committee for Animal Research of the Federal University of Santa Maria approved the experimental protocol (23081.007418/2007-75). Two-day-old Wistar male rats, weighing 6–9 g were obtained from our own breeding colony. They were divided into four homogeneous groups and kept in separate

animal rooms, on a 12-h light/dark cycle, at room temperature and with free access to food and water. The sequence of treatment was:

Group 1(*G*1) Control animals was given, orally, 0.9% NaCl.
Group 2(*G*2) Two-day-old pups were treated with one dose of MeHg (10 mg/kg) according with Sakamoto et al. (2004).
Group 3 (*G*3) Two-day-old rats received only ASc as follow: in the begin of the experiment, oral of Asc (0, 9 mg/Kg) was administrated. The other dose of Asc (0, 9 mg/Kg) was given twelve hours after. Therefore, two oral doses of ASc according Moresco et al. (2007).
Group 4 (*G*4) Received a single oral dose of MeHg (10 mg/kg). Immediately after, oral administration of Asc was given (0, 9 mg/Kg).Twelve hours after the pups received other dose of ASc (0.9 mg/kg). All the procedures were made with the aid of a Microman pipette (Gilson Medical Electronics). Wistar rats were euthanized two days after the final administration, when were with four-day-old (P4).

Sample preparation

Wistar rats were euthanized and their brains were promptly removed. Hippocampus, cerebral cortex, liver and kidney of neonatal rats (P4) were carefully separated and processed as described previously by Bellé et al. (2009) for ADA assay and by Robic et al. (1995) for NAG assay, with some modifications. All the procedures described above were performed at 0–4°C. A urinary sample was withdrawn by direct puncture of the bladder during euthanasia, with the aid of a syringe with modifications according to Kavlock et al. (1981).

Measurement of NAG activity

The samples were added to an enzyme reaction mixture that consisted of a substrate (p-nitrophenyl-*N*-acetyl-β-D-glucosaminide) dissolved in sodium citrate buffer (pH 4.4). Following incubation at 37°C for 15 min, the reaction was stopped by adding 2-amino-2-methyl-1-propanol (AMP) buffer (pH 10.25), and the reaction product was measured spectrophotometrically at 405 nm. NAG activity in the kidney homogenate and urine was proportional to the absorbance of the p-nitrophenylate ion liberated during enzymatic hydrolysis of the substrate, after correction of absorbance of the blank sample. Results were expressed as units per liter per gram of tissue and units per liter, respectively (Horak et al. 1981).

Determination of TBARS levels

Tissues were homogenized in tenfold volume of 10 mM Tris–HCl buffer solution, pH 7.4. The homogenates were centrifuged at $1000\times g$ for about 10 min. Lipid peroxidation levels were measured as thiobarbituric acid reactive species (TBARS) by a modification of the method of Buege and Aust (1978).

Measurement of ADA activity

Tissue ADA activities were estimated spectrophotometrically by the method of Giusti (1974), which is based on the direct measurement of the ammonia produced when ADA acts in excess of adenosine. Results were expressed as units per gram protein in the tissue, and expressed as means and standard error. All samples were assayed in duplicate.

In vitro assay

Animals were euthanized and their brains were promptly removed. The cerebral cortex of young rats (P4) were carefully separated and processed as described above. The homogenates of cerebral cortex were treated with hydrogen peroxide (H_2O_2) in two different concentrations (0.5 and 1 mM), and or not 200 μg/ml of ASc for 60 min at 30°C in a water bath; thereafter, TBARS levels were determined spectrophotometrically.

Statistical analysis

Data were analyzed using two-way ANOVA for multiple group comparison. Post hoc analysis was carried out using the Duncan multiple range test. Values of $P < 0.05$ were considered statistically significant.

Results

Chemical characterization of the extract

HPLC chromatogram of ASc showed the presence of gallic acid, kaempherol, chlorogenic acid, rutin and

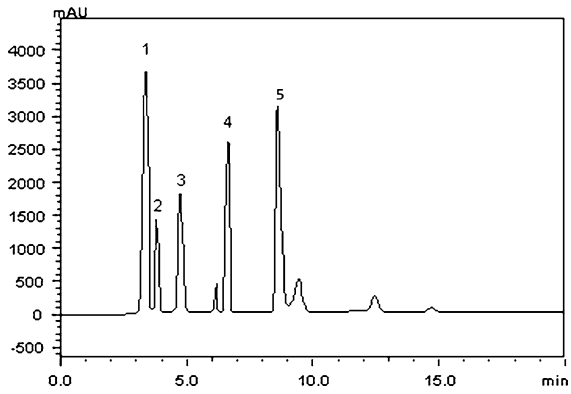

Fig. 1 Chemical characterization of the aqueous seed extract of *Syzygium cumini* (ASc). HPLC fingerprint of the ASc showing typical patterns of gallic acid (*1*) kaempherol (*2*), chlorogenic acid (*3*), rutin (*4*), and an unknown pick (*5*) at 257 nm

an unknown peak (Fig. 1). Gallic acid, an hydrolysable tannin, was the major component (2.63%), followed by rutin (0.084%) and chlorogenic acid (0.057%). Kaempherol was not quantified. The results were similar to other studies (Damasceno et al. 2002; Mahmoud et al. 2001; Sharma et al. 2003; Timbola et al. 2002).

NAG activity in the kidney and urine

We observed that NAG activity in the kidney and urine was higher in MeHg-group when compared to the control group. NAG activity was decreased in the urine of neonates exposed to MeHg and treated with ASc (Fig. 2a). However, the administration of ASc did not alter the effects caused by MeHg in the kidney of neonatal rats (Fig. 2b).

TBARS levels in the hippocampus, cerebral cortex, liver and kidney

Our experiments showed that TBARS levels did not change in the hippocampus and cerebral cortex of MeHg-treated neonatal rats (Table 1). In contrast, TBARS levels were higher in the liver and in the kidney when compared to the control group. However, the administration of ASc completely reverted the MeHg-induced increase of TBARS levels in the hepatic and renal tissue (Fig. 3a, b).

Considering the in vitro assay, both H_2O_2 concentrations (0.5 and 1 mM) caused an increase in TBARS levels ($P < 0.01$). Notwithstanding, ASc was able to inhibit the H_2O_2 effects in the tissue homogenates ($P < 0.01$) (Fig. 4).

ADA activity in the hippocampus, cerebral cortex, liver and kidney

We observed that ADA activity was increased in hippocampus, kidney and liver, but no alterations were found in cerebral cortex (Table 1). The administration of ASc inhibited ADA activity in the kidney of G4, although no effect was observed in the liver (Fig. 5a, b).

The body weight gain of the animals

Table 2 shows that MeHg caused a significant decrease in the body weight gain and in the weight of the hippocampus and cerebral cortex at the end of the treatment, when compared to the control group ($P < 0.05$). The administration of ASc did not affect the body weight and the weights of hippocampus and cerebral cortex. ASc was able to prevent the weight

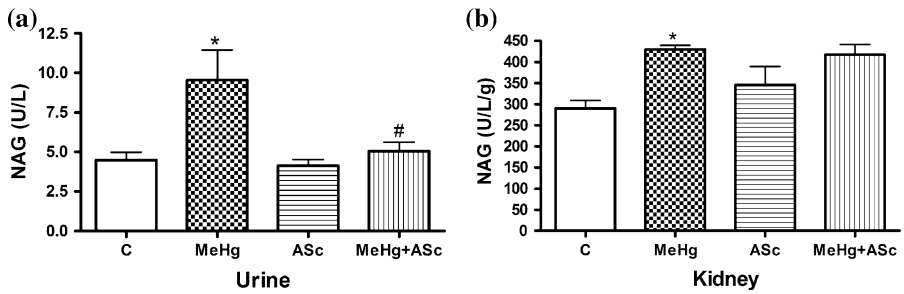

Fig. 2 NAG activity in the urine (**a**) and kidney samples (**b**) of neonatal rats treated with 10 mg/kg of MeHg and/or 0.9 mg/Kg of ASc. Results are expressed as means ± SEM of ten animals and represented as units per l for urine and units per l per g of tissue for kidney. *Statistically significant differences from control group ($P < 0.05$); #Statistically significant differences from MeHg ($P < 0.05$), as determined by two-way ANOVA followed by the Duncan post hoc test

Table 1 Effects of MeHg and ASc administration on ADA activity and TBARS levels in the hippocampus and the cerebral cortex of neonatal rats

Groups	ADA (U/mg protein)		TBARS (nmol/g tissue)	
	Hippocampus	Cerebral cortex	Hippocampus	Cerebral cortex
Control	26.73 ± 2.17	8.23 ± 0.35	57.76 ± 1.82	10.85 ± 1.30
MeHg	40.55 ± 1.92*	7.81 ± 0.48	64.41 ± 9.88	11.52 ± 3.55
ASc	40.85 ± 4.70*	6.07 ± 0.37	72.10 ± 9.76	8.59 ± 2.48
MeHg + ASc	47.75 ± 6.91*	6.57 ± 0.48	63.13 ± 7.24	8.10 ± 2.11

Values are expressed as means ± SEM for ADA activity and TBARS levels after the MeHg treatment ($n = 10$ rats/group). * Values statistically different compared to control group ($P < 0.05$), by two-way ANOVA, followed by the Duncan post hoc test

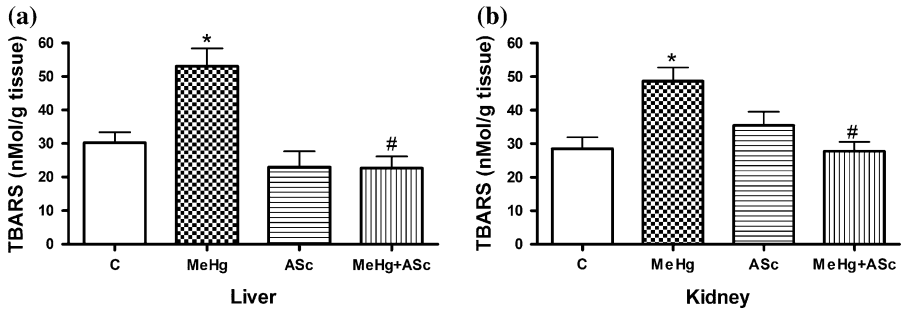

Fig. 3 TBARS levels in the liver (**a**) and kidney (**b**) of neonatal rats. Results are expressed as means ± SEM of ten animals and represented as nmol per g of tissue. *Statistically significant differences from control group ($P < 0.05$); #statistically significant differences from MeHg ($P < 0.05$), as determined by two-way ANOVA followed by the Duncan post hoc test

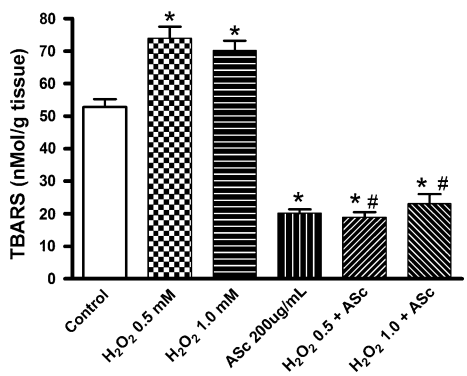

Fig. 4 TBARS levels in the cerebral cortex of young rats in vitro. Results are expressed as means ± SEM of ten animals expressed as nmol per g of tissue. *Statistically significant differences from control group ($P < 0.01$); #statistically significant differences from H_2O_2 0.5 mM or H_2O_2 1.0 mM ($P < 0.01$), as determined by two-way ANOVA followed by the Duncan post hoc test

loss in the liver and in the kidney of G4, and no deaths were recorded; whereas, a death rate of 30% was observed in G3 rats.

Discussion

The results of this study showed that the acute treatment with MeHg affected all tissues analyzed. *N*-acetyl-β-D-glucosaminidase (NAG; EC: 3.2.1.30) is a lysosomal enzyme present in high concentrations in renal proximal tubular cells. Increase of urinary NAG excretion is one of the most sensitive markers of renal disease (Bosomworth et al. 1999; Nordberg et al. 2009). In fact, the significant increase observed in urinary excretion of NAG activity, indicates the occurrence of tubular cell injury in MeHg-treated rat kidneys. Also, these high enzyme activities may indicate possible cell and nucleic acid damage destruction due to MeHg toxicity in the renal tissue.

It is well established that adenosine modulates the proliferation, survival and apoptosis of many different cell types (Jacobson et al. 1999; Wardas 2002). Changes in ADA activity may modify the local concentration of adenosine, and as a consequence, influence adenosine actions (Abbracchio and Ceruti 2007; Fredholm 2007). ASc administration

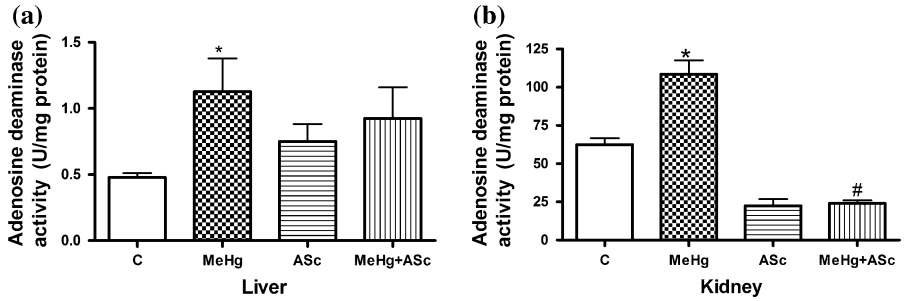

Fig. 5 Effects of acute exposure to MeHg and/or ASc on ADA activity in the liver (**a**) and in the kidney (**b**) of neonatal rats. Results are expressed as means ± SEM of ten animals expressed as U per mg of protein. *Statistically significant differences from control group ($P < 0.01$); #statistically significant differences from MeHg ($P < 0.01$), as determined by two-way ANOVA followed by the Duncan post hoc test

Table 2 Effects of MeHg exposure and ASc administration on body weight gain of neonatal rats

Groups	Control	MeHg	ASc	MeHg + ASc
Initial body weight (g)	6.85 ± 0.23	6.57 ± 0.29	6.85 ± 0.23	7.35 ± 0.19
Hipoccampus weight (g)	0.021 ± 0.0009	0.018 ± 0.0008*	0.017 ± 0.0009*	0.018 ± 0.0008*
Cortex weight (g)	0.200 ± 0.004	0.170 ± 0.003*	0.194 ± 0.007	0.189 ± 0.006
Kidney weight (g)	0.100 ± 0.002	0.059 ± 0.0032*	0.093 ± 0.004	0.096 ± 0.004#
Liver weight (g)	0.30 ± 0.018	0.20 ± 0.000*	0.30 ± 0.011	0.30 ± 0.014#
Body weight gain (g)	10.54 ± 0.29	6.53 ± 0.31*	8.06 ± 0.33	8.64 ± 0.38#

Values are expressed as means ± SEM for body weight gain between P2 and P4 days of treatment ($n = 10$ rats/group). * Values statistically different compared to Control group, # is different compared to MeHg group ($P < 0.01$), by two-way ANOVA, followed by the Duncan post hoc test

significantly attenuated the increase of renal ADA activity, urine NAG activity and the lipid peroxidation caused by MeHg exposure. The main compounds present in the ASc (gallic acid, chlorogenic acid and rutin) may be responsible for these effects, since their antioxidant capacity is reported elsewhere (Damasceno et al. 2002; Mahmoud et al. 2001; Timbola et al. 2002). As being evidenced by chemical characterization of the extract, rutin, which is one of the major representatives of flavonoids present in the extract, has an antioxidant capacity (Gong et al. 2010; Korkmaz and Kolankaya 2009). The protective effect of ASc observed in the present study is consistent with the beneficial results found by Moresco et al. (2007) and Stanley et al. (2003).

Furthermore, we did not observe the peroxidation of lipid membranes in the cerebral cortex and in the hippocampus in the MeHg-group. It is possible that the acute treatment (two days) was not long enough to cause the loss of plasma membrane integrity. Nevertheless, the hippocampus was more sensible to MeHg and ASc treatment because ADA activity increased after both administrations. It is possible to consider that alterations of ADA activity in different brain regions are related with the maintenance of adenosine levels. Also, the role of adenosine in the regulation of synaptic transmission can be responsible for this response of the enzyme activity to injury caused by MeHg exposure. Also, preliminary assays have been performed to check the antioxidant property of ASc. There are evidences indicating antioxidant activity in vitro and in vivo for ASc. In fact, ASc has been identified as a potent inhibitor of ROS formation and lipid peroxidation (Prince et al. 1998). Corroborating with the studies, we observed that the administration of ASc completely reverted the MeHg-induced increase of TBARS levels in the hepatic and renal tissue (Fig. 3).

It is worth of interest to note that the ASc avoided the loss of weight observed in the MeHg group. Moreover, we did not observe mortality in the MeHg-ASc group, which may be related to the systemic protective effect presented by the ASc administration.

In conclusion, the results of this study revealed that lipid peroxidation, NAG and ADA activity could be early markers of renal toxicity. It is also important to point out that the protective effect of ASc against the MeHg-induced effects could be related to its ability to detoxify H_2O_2. Our findings, together with those from previous research (Moresco et al. (2007) and Stanley et al. (2003) suggest the potential value of ASc effects. However, further studies should be conducted to investigate the perspective of the use of ASc as a potent antioxidant against MeHg-induced toxicity.

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