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Article

Nephroprotective activity of the enriched polyphenol extract of *Euterpe edulis* Martius

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Contrast-induced nephropathy (NIC) is directly related to increased morbidity and mortality, and its treatment and prevention might be achieved by the administration of antioxidant products. The juçara palmetto (*Euterpe edulis* Martius) has fruits rich in phenolic compounds, which are known for their antioxidant activity. This work aimed to evaluate the nephroprotective activity of *E. edulis* pulp in the NIC animal model. The collected fruits were pulped, their contents of polyphenols and anthocyanins were quantified, and their antioxidant activity were evaluated. The nephroprotective effects were determined based on iodine contrast induction and evaluated by biochemical and histological analyses. The results showed that *E. edulis* pulp was rich in polyphenols (811 ± 16.7 mg EAG/g) and anthocyanins (181.25 mg/100 g) and had very strong antioxidant activity, as demonstrated by the DPPH (2,2-diphenyl-1-picryl-hydrazyl) method, which revealed an antioxidant activity index (AAI) of 3.4, and the 2,29-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method, which revealed an IC_{50} of 0.59 ± 0.03 mg/mL. In the in vivo experiments, *E. edulis* pulp tended to provide renal protection and reduce renal dysfunction and tubular morphological lesions in mice after the induction of NIC, and these effects were obtained through the antioxidant activities of the polyphenols in the pulp.

Keywords: Nephroprotection. *Euterpe edulis* Martius. Polyphenols. Anthocyanins. Antioxidant activity.

INTRODUCTION

Acute renal injury, which is also known as contrast nephropathy (NIC), is an iatrogenic condition caused by procedures involving the administration of iodinated contrast agents, which are often used in imaging diagnostics. Moreover, as the third leading cause of renal insufficiency acquired in a hospital environment, NIC is also associated with increased morbidity and mortality (Santos *et al*., 2011). The use of iodinated contrast media during some procedures, including coronary angiography and percutaneous coronary intervention, might cause acute renal injury, the most common cause of which is acute renal failure (Kaye *et al*., 2014). Impaired renal function is often noted due to changes in routine laboratory tests, such as urea and particularly creatinine (Magro, Vattimo, 2007).

The exact pathogenesis of NIC is currently under debate, but it has been confirmed that NIC is caused by a combination of factors (Osthoff, Trendelenburg, 2013; Andreucci *et al*., 2014), such as renal and vascular tubular injury accompanied by direct and indirect influences of reactive oxygen species (EROs) (Heyman *et al.,* 2010). Sufficient histological changes, particularly at proximal tubular lesions, are observed in NIC, and these result in a reduction of the glomerular filtration rate due to the iodinated contrast agent (Billings *et al*., 2008; Andreucci *et al*., 2014; Diogo, Bahlis, Carvalhal, 2014). A factor that has been adopted in the selection of products that can prevent NIC is antioxidant activity (Andreucci *et al*., 2014). As a result, N-acetylcysteine (NAC), ascorbic acid (vitamin C) and α-tocopherol (vitamin E) have been tested as agents for the prevention of NIC (Pattharanitima, Tasanarong, 2014; McCullough, Akrawinthawong, 2013),

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and N-acetylcysteine is the only agent that forms part of the currently used prophylactic regimen.

The fruits of the juçara palmetto (*Euterpe edulis* Martius), which belongs to the Arecaceae family, contain phenolic compounds with antioxidant activity, including anthocyanins (Paredes-López *et al*., 2010; Borges *et al*., 2011a; Inácio *et al*., 2013; Bicudo, Ribani, Beta, 2014). The anthocyanins have been identified in *E. edulis* fruits are cyanidin 3-*O*-glycoside, which is the major anthocyanin in the fruits, and cyanidin 3-*O*-rutinoside, cyanidin-3,5-diglycoside, pelargonidin 3-*O*-rutinoside, peonidin-3-*O*-glycoside, peonidin 3-*O*-rutinoside, cyanidin 3-sambubioside and cyanidin 3-ramnoside, which are found in lower quantities (Bicudo, Ribani, Beta, 2014; Ribeiro *et al*., 2011; Brito *et al*., 2007).

As part of the search for new alternative products for the prevention of NIC that could be used as a functional food, this study aimed to evaluate whether *E. edulis* pulp can be used for the prevention of NIC in an experimental model.

MATERIAL AND METHODS

Plant material

Fruits of *E. edulis* were supplied by Incaper and collected in the region of Rio Novo do Sul-ES in August 2013. A voucher specimen was prepared and identified by Prof. M.Sc. Solange Z. Schneider, a botanist at the herbarium at University Vila Velha (UVV), and deposited at this herbarium under the number UVVES2396.

Approximately 1 kg of fruits was pulped according to the pulping technique used by the local population and immersed in water at 40ºC for 60 minutes to release the pulp and seeds. Soon after, the seeds and pulp were separated by sieving. The material was lyophilized (Labconco Freezone® lyophilizer, model 7752020, USA) at a rate of approximately 10 mL/day until the liquid was completely evaporated and a purple powder was obtained. This powder was then stored in a refrigerator at -20°C until use in the experiments.

Evaluation of DPPH antioxidant capacity

The antioxidant activity of *E. edulis* pulp was determined using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical method (Scherer, Godoy, 2009). The minimum inhibitory concentration required to inhibit 50% of the initial free radical concentration (CI_{50}) was calculated using the equation of the straight line obtained from the calibration curve relating the concentration to the potential DPPH radical inhibition. The antioxidant action of the pulp was expressed using the Antioxidant Activity Index (IAA), which is the ratio of the concentration of DPPH (μg/mL) to the CI_{50} (μg/mL), and classified according to the IAA value obtained (weak if IAA <0,5, moderate if the 0.5<AAI<1, strong if 1<AAI<2, and very strong if AAI> 2). Ferulic acid (0.89 to 4.7 μg/mL and R_2 0.99) was employed as a positive control in the antioxidant activity determination.

Evaluation of ABTS antioxidant capacity

The 2,2'-azino-*bis*-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical assay was performed according to the methodology described by Re *et al*. (1999) with some modifications. Specifically, the experiment was performed in 96-well plates, absolute ethanol was used as the blank, and 250 μL of the ABTS radical solution + 20 μL of ethanol was used as the control. The samples consisted of 250 μL of the ABTS radical solution and 20 μL of the *E. edulis* pulp solutions (1.0 to 0.15 mg/mL). The same experiment was performed using BHT antioxidant standards (0.62 to 0.08 mg/mL), TBHQ (0.56 to 0.07 mg/mL) and quercetin (0.3 to 0.006 mg/mL). The percentage of inhibition read at the wavelength of 734 nm was related to the concentration of the pulp or standards, and the CI_{50} was calculated from the equation of the line obtained for each solution. The experiments were performed in triplicate on three different days (Equations of the straight lines of the samples: *E. edulis* pulp, $y = 70.5x + 8.83$, $R^2 = 0.99$; BHT, $y = 95.4x + 7.88$, $R^2 = 0.99$; TBHQ, $y = 179.9x + 3.81$, $R^2 = 0.99$; and quercetin, $y = 299.6x + 1.86$, $R^2 = 0.99$).

Quantification of total polyphenols

The total polyphenols were quantified using the Folin-Ciocalteu reaction, as described by Souza *et al*. (2007) with the modifications described by Mariani *et al.* (2013). A calibration curve with a gallic acid standard was used (10 to 300 μg/mL). The total polyphenols were quantified using the equation of the straight line of the calibration curve, $y = 0.0009x + 0.0021$ and $R^2 = 0.96$. The results are expressed as mg of gallic acid equivalents (EAG) per g of sample. All the analyses were performed in triplicate.

Determination of total anthocyanins

The total monomeric anthocyanin content of *E. edulis* fruit pulp was determined by the differential

pH method described by Giusti and Wrosltad (2001). The final absorbance and the total content of anthocyanins were calculated using the following equations: Final Absorbance = (A_{520} nm - A_{700} nm) at pH 1 - (A_{520} nm - A_{700} nm) at pH 4.5 and Total anthocyanin content = (A x MM x FD x 1000)/(εx1), respectively.

In these equations, A = final absorbance, MM = molecular mass, FD = dilution factor, 1000 = conversion of grams to milligrams, 1 = optical path length in centimeters and ε = 26900 [molar extinction coefficient in L/mol/cm for cyanidin-3-glycoside (C3G)]. The results are expressed as mg of C3G/100 g of dry pulp. The analyses were performed in triplicate.

In vivo evaluation of the nephroprotective activity of *E. edulis* pulp

Seventy adult mice (Swiss mice, males, weighing 25-40 g) were used. The animals were obtained from the Laboratory of Experimental Monitoring of the University of Vila Velha (UVV), in Espírito Santo, Brazil. The animals were fed a diet of feed and water and maintained at an average temperature of 22 °C with a 12-h light/12-h dark cycle.

The research was approved by the Committee on Ethics regarding the Use of Animals (CEUA-UVV; protocol # 268/2013).

Prior to the induction of NIC, the animals were subjected to five days of gavage treatment with *E. edulis* pulp at doses of 100 mg/kg, 200 mg/kg and 400 mg/kg. In addition to the synthetic antioxidant *N*-acetylcysteine (NAC) at 100 mg/kg, all the samples were diluted in aqueous solution to a concentration of 50 mg/mL. After 16 hours of water fasting, the synthesis of prostanoids and nitric oxide were inhibited through the administration of indomethacin (10 mg/kg, dissolved in dimethyl sulfoxide, ip) and L-NAME (10 mg/kg, dissolved in 0.9% saline, ip), respectively, and 15 minutes later, NIC was induced through the injection of the second-generation radiological contrast ioversol, which has and low osmolarity (Optiray 320 Mallinckrodt Medical, Inc., St. Louis, MO, USA, 1.5 g iodine/kg). After 24 hours, the animals were euthanized through the administration of thiopental (200 mg/kg, ip). Venous blood was collected from the left ventricle for biochemical measurements of urea and creatinine (Billings *et al*., 2008; Gomes *et al.,* 2014), and the kidneys were removed for histological analysis. These procedures were adapted from protocols previously validated in mice by Lee *et al.* (2006) and Billings *et al*. (2008). The animals used in the evaluation were randomly divided into seven groups (each with n = 7). The control groups were the following: negative control (CN) group, which was administered only water during the treatment; EE400WT group, which was treated with 400 mg/kg *E. edulis* pulp and not administered the agent to induce NIC; NIC group, which was pretreated with water; and the positive control (NAC) group, which was administered 100 mg/kg *N*-acetylcysteine + NIC. The treated groups were the following: EE100 group, which was administered 100 mg/kg *E. edulis* pulp + NIC; EE200 group, which was administered 200 mg/kg *E. edulis* pulp + NIC; and EE400 group, which was administered 400 mg/kg *E. edulis* pulp + NIC.

Cross-linking protein products containing dityrosine (AOPP)

Dityrosine-containing cross-linked protein products, which are referred to as AOPPs, are hypochlorous acid (HClO) products induced by amine chlorination. AOPPs was evaluated as described by Witko-Sarsat *et al.* (1996) with some modifications. Specifically, samples consisting of 200 mg of renal tissue to 1 ml of PBS were prepared. After homogenization, the samples were centrifuged, and the supernatant was separated for use as a protein source. To obtain the standard curve, chloramine T solution (0 to 100 µmol/L), was added to each well of a 96-well microtiter plate (Becton Dickinson Labware, Lincoln Park, NJ, USA), and 10 µL of 1 and 16 mol/L potassium iodide (KI) and 20 µL of glacial acetic acid were added. The absorbance of the reaction mixture was immediately read at a wavelength of 340 nm and compared with that of the blank, which consisted of 200 µL of PBS, 10 µL of KI and 20 µL of acetic acid. The absorbance of chloramine T at a wavelength of 340 nm was linear within the range of 0 to 100 µmol/L (Line equation: $y = 0.1288x + 0.1658$ with $R^2 = 0.98$). The AOPP concentrations were determined when the correlation coefficient was greater than 0.95 and are expressed as µmol/mg total protein, where the total protein was previously quantified using the Bradford method (Bradford, 1976). The equation of the line was $y = 0.6491x + 0.0118$ ($R^2 = 0.98$).

Determination of acute toxicity (LD_{50})

The oral toxicity was assessed using protocol 423 recommended by OECD-423/2001 "Acute Toxicity of Class" (OECD, 2001), which determines the doses to be used in the study. In the present study, the maximum dose of *E. edulis* lyophilized pulp (per gavage in a single dose) used in the protocol, 2000 mg/kg (based on data from previous studies with other species of this plant

(Ribeiro, Seravalli, 2007), was administered directly to the treated group, and purified water was administered to the control group. Three female Swiss mice (nulliparous, nonpregnant, and weighing between 20 and 35 g) were included in each group. The toxicological category was estimated according to the specifications of the protocol, and the experiment was performed in duplicate.

Histological analysis

For histopathological examination, the kidneys were preserved in 10% formaldehyde, and the liver was preserved in Bouin's fixative solution. The samples were maintained in 70% alcohol until preparation of the histological sections and then contrasted with hematoxylin and eosin (HE). The tissues were embedded in paraffin and sectioned at 4 mm (Mattos *et al*., 2009).

The slides were qualitatively analyzed through optical microscopy by an experienced pathologist. All the cuts were blindly examined (Billings *et al.,* 2008).

Statistical analyses

The values obtained from the biological evaluations are expressed as the means ± standard deviations of the mean (EPMs), and the values obtained from the chemical evaluations are expressed as the means ± standard deviations (DPs). These values were then analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc text, with $p < 0.05$. The correlation coefficient of the standard curves was obtained through linear regression using the least squares method, and the data were also analyzed by ANOVA with a 95% LC and n-1 degrees of freedom. The limits of detection (LD) and quantification (LQ) were calculated by dividing the standard deviation of the linear coefficients of the calibration curves generated in the assays. Excel (Microsoft Office 2010) and Prism software (Prism 6, GraphPad Software, Inc., San Diego, CA, USA) were used.

RESULTS

Evaluation of the antioxidant capacity of *E. edulis* pulp

According to DPPH method, which was performed as described by Scherer and Godoy (2009), the antioxidant activity index (AAI) of *E. edulis* pulp was 3.4, and the CI_{50} was 11.4 µg/mL. Ferulic acid had an AAI of 5.49 and a CI_{50} of 7.12 µg/mL, and the R^2 of the assay was 0.994.

The antioxidant capacity of the tested compounds, as determined based on the inhibition of the ABTS cation radical, could be ranked in the following order: quercetin (0.16 ± 0.005 mg/mL) > TBHQ (0.25 ± 0.005 mg/mL) > BHT (0.44 ± 0.1 mg/mL) > *E. edulis* pulp (0.59 ± 0.03 mg/mL).

Quantification of total polyphenols and anthocyanins

The total polyphenol content of lyophilized *E. edulis* was 811 ± 16.7 mg EAG/g), and in the present study, the monomeric anthocyanins were quantified using the differential pH method, which revealed a content of 181.25 ± 5.36 C3G mg/100 g.

In vivo evaluation of the nephroprotective activity of *E. edulis* pulp based on the creatinine and urea levels

The present study constitutes the first investigation of the effects of *E. edulis* pulp on both renal function and structure in response to oxidative stress in mice with radiological contrast-induced nephrotoxicity.

In the present study, the serum creatinine levels of the NIC group (0.60 ± 0.14 mg/dl) were increased compared with those of the control group (0.14 ± 0.01 mg/dL), and this increase in creatinine content was greater than 25%. No difference was found between the control group (0.14 ± 0.01 mg/dL) and the 400 mg/kg *E. edulis* group (0.17 ± 0.0 mg/dL). The animals treated with *E. edulis* pulp at concentrations of 100 mg/kg (0.27 ± 0.02 mg/dL), 200 mg/dL (0.34 ± 0,10 mg/dl) and 400 mg/dL (0.38 ± 0.09 mg/dL) presented creatinine levels close to those of the control animals (0.14 ± 0.01 mg/dL) and the animals in the N-acetylcysteine-treated group (0..1 ± 0.00 mg/dL), but their levels were not different from those of the animals in the NIC group (0.60 ± 0.14 mg/dL) (Figure 1).

The serum values of urea in the animals that received 100 mg/kg *E. edulis* (93 ± 0.6 mg/dL) pulp prior to the induction of contrast nephropathy were similar to those of the control group (71 ± 2.8 mg/dL). *E. edulis* pulp at a dose of 100 mg/kg yielded superior renal protection to treatment with N-acetylcysteine (226 ± 9.3 mg/dL), as indicated by urea values after the induction nephropathy similar to those found for the NIC group (256 ± 21 mg/dL). No difference was found between the groups administered the highest dosages of *E. edulis* pulp, 200 mg/dl (237 ± 48 mg/dL) and 400 mg/dl (323 ± 40 mg/dL), and the NIC group (Figure 2).

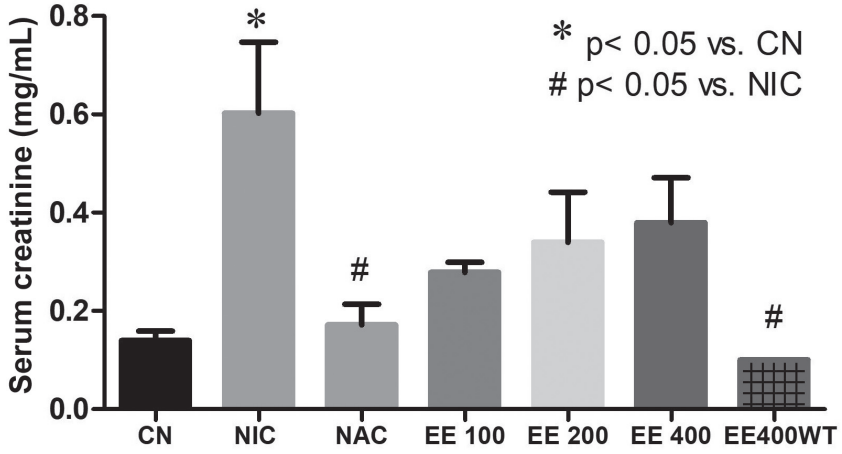

FIGURE 1 - Serum creatinine level in mice treated for 5 days before the induction of NIC. Groups: CN, negative control; NIC, contrast nephropathy; NAC, 100 mg/kg *N*-acetylcysteine; EE100, 100 mg/kg *E. edulis* + NIC; EE200, 200 mg/kg *E. edulis* + NIC; EE400, 400 mg/kg *E. edulis* + NIC; and EE400WT, control 400 mg/kg *E. edulis*.

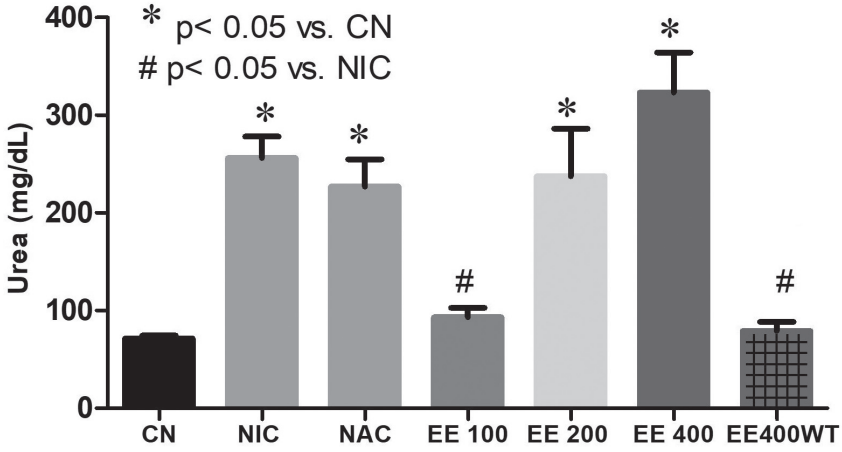

FIGURE 2 - Serum urea level in mice treated for 5 days before the induction of NIC. Groups: CN, negative control; NIC, contrast nephropathy; NAC, 100 mg/kg *N*-acetylcysteine; EE100, 100 mg/kg *E. edulis* + NIC; EE200, 200 mg/kg *E. edulis* + NIC; EE400, 400 mg/kg *E. edulis* + NIC; and EE400WT, control 400 mg/kg *E. edulis*.

Cross-linking protein products containing dityrosine (AOPPs)

In the present study, the degree of protein oxidation of the animals belonging to the NIC group (0.27 ± 0.01 µmol/mg) was increased compared with the control group (0.16 ± 0.0 µmol/mg). The animals treated with *E. edulis* pulp at concentrations of 100 mg/kg (0.21 ± 0.02 µmol/mg) and 200 mg/dL (0.27 ± 0.09 µmol/mg) showed a similar AOPP level to the control animals and to the group treated with NAC, which represents the conventional treatment (0.25 ± 0.01 µmol/mg). Only the group treated with 400 mg *E. edulis* (0.35 ± 0.07 µmol/mg) presented elevated protein oxidation compared with the control animals. The group that received only *E. edulis* pulp presented AOPP values equal to those of the control group (Figure 3).

Histological analysis

The histological analysis of the kidneys collected from the animals belonging to the NIC group showed vacuolar degeneration and cortical vacuolization (Figure 4B, I, P). These lesions were not observed in any of the CN (Figure 4A, H, O) or EE400WT (Figure 4D, K, R) slides, which showed renal cells with a preserved renal architecture.

The animals that received 100 mg/kg *E. edulis* pulp (Figure 4E, L, S) and *N*-acetylcysteine (Figure 4C, J, Q) presented better preserved renal cell structures with a normal tubular and glomerular appearance without pathological findings. However, the toxicity caused by the iodate contrast agent was increased in the animals administered 200 mg/kg (Figure 4F, M, T) and 400 mg/kg *E. edulis* (Figure 4G, N, U). In these laminae, discrete to

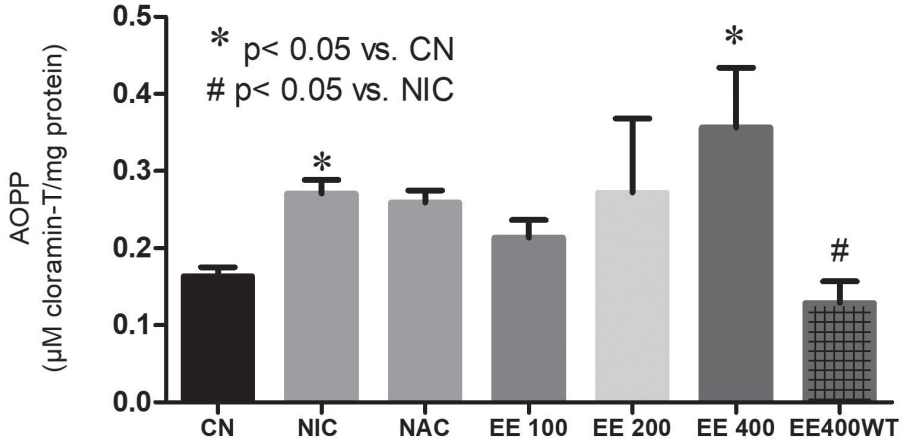

FIGURE 3 - AOPP level of mice treated for 5 days prior to the induction of NIC. Groups: CN, negative control; NIC, contrast nephropathy, NAC, 100 mg/kg *N*-acetylcysteine; EE100, 100 mg/kg *E. edulis* + NIC; EE200, 200 mg/kg *E. edulis* + NIC; EE400, 400 mg/kg *E. edulis* + NIC; and EE400WT, control 400 mg/kg *E. edulis*.

intense vacuolar degeneration, the presence of perivascular and inflammatory infiltrates, and dilation of the proximal tubule were observed (Figure 4).

Determination of acute toxicity (LD_{50})

Considering the results of the biochemical analyses performed in the present study, which demonstrated that *E. edulis* pulp does not exert toxic effects at a dose lower than 400 mg/kg in the absence of radiocontrast, we started the LD_{50} protocol with a dose of 2000 mg/kg. No morbidity or mortality was recorded in the evaluated groups, which confirms that *E. edulis* pulp exhibits acute oral toxicity at a dose higher than 2000 mg/kg and classifies this fruit as category 5 according to the criteria of the experimental protocol adopted (OECD, 2001). The histological analysis of the renal and hepatic tissues revealed no pathological findings in either organ.

The biochemical results from the determination of renal and hepatic function markers showed no difference between the groups treated with *E. edulis* pulp and the control group, with the exception of the creatinine level (Table I).

DISCUSSION

Evaluation of the antioxidant capacity of *E. edulis* pulp

The results of the present study provide the first demonstration of the renal protective effect of *E. edulis* pulp, and the mechanism of action likely involves the antioxidant effect of polyphenolic substances and resulting in decreased renal dysfunction and

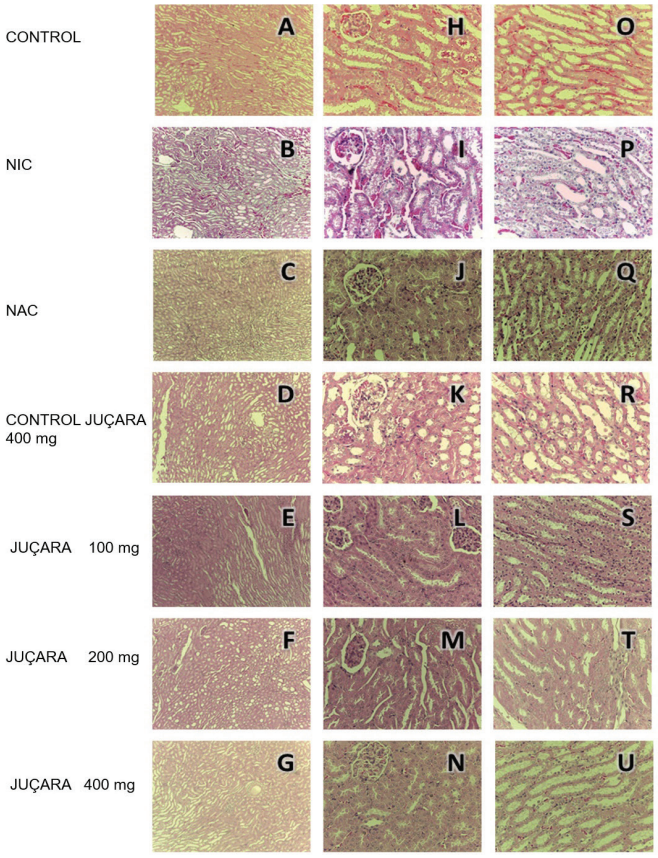

FIGURE 4 - Representative micrographs showing the renal histology of the mice after the induction of NIC. Groups: CN, negative control; NIC, contrast nephropathy; NAC, 100 mg/kg N-acetylcysteine; EE100, 100 mg/kg *E. edulis* + NIC; EE200, 200 mg/kg *E. edulis* + NIC; EE400, 400 mg/kg *E. edulis* + NIC; and EE400WT, control 400 mg/kg *E. edulis*. Images A to G: cortex-medulla section (100x), demonstrating changes in the typical renal architecture; Images H to N: cortical region (400x); and images O to U: medulla region (400x) showing tubulointerstitial lesions.

TABLE I - Biochemical results of renal and hepatic acute toxicity of animals treated with 2,000 mg/kg of *E. edulis* (T) and control (C) pulps

Biochemical target	Groups	Average (mg/dL) ± standard error
AST	C	239 ± 67.5
	T	204 ± 34.5
FA	C	176 ± 44.7
	T	89.2 ± 10.3
GGT	C	3.83 ± 0.87
	T	2.6 ± 0.67
Urea	C	41.1 ± 1.93
	T	49.6 ± 5.75
Creatinine	C	0.23 ± 0.03
	T	0.36 ± 0.03 *
Total protein	C	5.51 ± 0.44
	T	5.16 ± 0.36
Albumine	C	1.93 ± 0.11
	T	3.12 ± 1.2

morphological tubular lesions in mice after the induction of NIC.

The *E. edulis* fruit is considered a source of polyphenols and anthocyanins, and the antioxidant activity of an extract or fruit pulp, as previously discussed by several authors, is directly associated with the amount of these compounds present in the chemical composition (Vedana *et al.,*2008; Silva *et al.*, 2010; Koolen *et al.*, 2013; Silva *et al.,* 2013).

The results obtained with the DPPH method indicated a very strong AAI of 3.4 and a CI_{50} of 11.4 µg/mL, which suggests that *E. edulis* pulp exhibits antioxidant activity under the conditions used in this experiment. In addition, these values are close to those obtained in this study for ferulic acid (AAI = 5.49 and CI_{50} = 7.12 µg/mL), demonstrating the antioxidant potential of *E. edulis.*

Using the ABTS method, the CI_{50} of *E. edulis* pulp was compared with the concentrations obtained for the standard solutions (BHT, TBHQ and quercetin), and significant ($p < 0.05$) differences were found in all pairwise comparisons. In addition, if a product has a higher antioxidant potential, a lower concentration is required for the inhibition of free radicals (Stajčić *et al.*, 2012). It should be noted that the observed CI_{50} of *E. edulis* pulp, which is a nonconcentrated product from the edible part of the fruit, is similar to the values obtained for the synthetic antioxidants BHT, TBHQ and quercetin, which are compounds with known antioxidant activity (Silva, Jorge, 2011; Wang *et al.,* 2008; Scherer, Godoy, 2009).

Quantification of total polyphenols and anthocyanins

The antioxidant activity obtained in this study might be related to the high content of total polyphenols found in lyophilized *E. edulis* pulp (811 ± 16.7 mg EAG/g). Borges *et al.* (2011b) reported values ranging from 303.63 to 806.37 mg EAG/g for fresh *E. edulis* pulp obtained after extraction using different variables. Bicudo et al. (2014) performed experiments with *E. edulis* fruit collected at different stages of maturation and reported total polyphenol concentrations ranging from 49.09 to 81.69 mg EAG/g, which are substantially lower than those obtained in this study. It is known that the polyphenol content in *E. edulis* pulp is highly influenced by environmental, climatic and fruit maturation factors (Borges *et al.,* 2011a; Bicudo, Ribani, Beta, 2014) as well as the extraction time and solvents used (Borges *et al.*, 2011b).

Other studies have also reported levels of anthocyanins near the concentration range observed in the fruit of the *E. edulis* palm tree. Ribeiro *et al.* (2011) reported an anthocyanin content of 148.67 mg/100 g in fruits collected in the state of Rio de Janeiro (Brazil), and Borges *et al.* (2011a) reported anthocyanin levels ranging from 14,84 to 409,85 mg/100 g in fruits collected from different regions in the state of Santa Catarina (Brazil) with different climatic conditions. Brito *et al.* (2007) obtained a content of 290 mg/100 g from fruits collected in the state of São Paulo (Brazil). In addition, Borges *et al.* (2011a) noted that the fruits of palms grown in the summer months, during a common fruiting period and at medium altitude have a higher anthocyanin content than fruits obtained from other regions; thus, these researchers concluded that a higher incidence of solar radiation on the fruits has a positive effect on anthocyanin synthesis and that the solar intensity affects the metabolism and synthesis of flavonoids in general and promotes a greater accumulation of anthocyanins. The fruits of *E. edulis* used in this research were cultivated at an altitude of 70 m and collected in the Brazilian winter (August), which could explain the anthocyanin content obtained in the study.

In vivo evaluation of the nephroprotective activity of *E. edulis* pulp based on the creatinine and urea levels

In the present study, the mice were administered

contrast medium at a dose similar to that used clinically (1.5 g/kg iodine in routine angiographic practice). Prostaglandin and NO syntheses were also inhibited to simulate the clinical situations that predispose patients to NIC, which result in increases in oxidative stress, reductions in the renal blood flow, and thereby vasoconstriction and hypoxia (Selistre *et al*., 2015; Kongkham, Sriwong, Tasanarong, 2013). However, analysis of the creatinine and urea dosage of the NIC and CN groups verified the presence of azotemia with a significant elevation of these nitrogen compounds (Figure 1 and 2). Other investigations have also reported an experimental model of NIC characterized by an elevation of the serum creatinine level of ≥25% in rats and mice (Billings *et al*., 2008; Lee *et al*., 2006). No difference in the creatinine and serum urea levels were found between the CN and EE400WT groups, indicating that under nonpathological conditions, *E. edulis* pulp does not induce nephropathy.

The animals of the EE100, EE200 and EE400 groups presented serum creatinine levels equal to those of the CN and NAC groups (Figure 1). Because the induction of NIC elevated the creatinine level by more than 25%, as determined through a comparison of the NIC and CN groups, these data show that although the groups treated with *E. edulis* pulp cannot be differentiated from the NIC group based on the creatinine level, this treatment tended to provide renal protection with a possible reduction of damage due to glomerular filtration. However, under these experimental conditions, the group treated with N-acetylcysteine, which forms part of the prophylactic regimen that is currently used, showed better results (Pattharanitima, Tasanarong, 2014; McCullough, Akrawinthawong, 2013).

The urea level was also analyzed (Figure 2), and the serum values of urea in the animals that received 100 mg/kg *E. edulis* pulp suggest that *E. edulis* provided renal protection against some deleterious mechanisms associated with glomerular filtration after the induction of NIC. The animals treated with 100 mg/kg *E. edulis* pulp had lower urea levels than the NAC group, which curiously showed values similar to those found in the NIC group. Selistre *et al*. (2015) studied the importance of urea in the differential diagnosis of acute renal failure and stated that one of the criteria for diagnosing acute kidney injury is the absolute increase in the serum creatinine level. The EE200 and EE400 groups did not differ from the NIC group, and thus, a possible protective effect of the pulp at high doses can be ruled out.

Cross-linking protein products containing dityrosine (AOPP)

The AOPP analysis revealed oxidative stress in the renal tissue of animals after the induction of contrast nephropathy (Figure 3). AOPPs are a group of protein products generated by proteins with hypochlorous acid and chloramines in response to oxidative stress (Colombo *et al*., 2015). These oxidants are produced by the myeloperoxidase released by activated neutrophils in response to an imbalance in the antioxidant mechanisms, and as a consequence, these free radicals act on proteins to produce dityrosine (Piowowar, 2010).

The animals with induced NIC that were previously treated with the lowest doses of *E. edulis* pulp presented AOPP levels similar to those of the control animals. These data reinforce the fact that the treatment provided some protection to the animals because contrast nephropathy caused a significant elevation of the level of AOPPs in the NIC group. However, the EE400 group presented a marked increase in AOPPs, suggesting that this dosage does not provide any renal protection and raising the possibility of a possible synergetic pro-oxidant effect of *E. edulis* at a high dose.

The AOPP evaluation performed in this experiment confirms that although the animals treated with the lowest doses of *E. edulis* could not be differentiated from the NIC group, *E. edulis* pulp tended to provide renal protection in animals with induced contrast nephropathy through the antioxidant activity of the polyphenols in the pulp.

Histological analysis

In the representative micrographs showing the renal histology of the different groups of mice (Figure 4), it is possible to observe that the animals of the NIC group presented typical lesions of contrast nephropathy in proximal tubular cells. Pathological changes, such as vacuolar degeneration, intense vacuolization of proximal tubular cells and balloonization, were also described by Eduardo-Carraro *et al*. (2016) and Yokomaku *et al*. (2008), who studied rats with induced nephropathy. Therefore, in addition to the above-described biochemical parameters, our histopathological data indicate a significant disturbance of the renal structure in the NIC group in comparison with the control animals, which is in agreement with other studies (Billings *et al*. 2008; Yokomaku *et al.,* 2008; Khan *et al*., 2013).

In this experiment, tubular necrosis, which was described by Yokomaku *et al.* (2008) and Billings *et al.* (2008), was not observed. However, nephropathy can

occur within a period of 24-72 hours (Andreucci *et al*., 2014), and in the present research, the animals were euthanized within 24 hours. Therefore, there was no time for the ischemia to cause irreversible lesions, such as acute tubular necrosis, and thus, only vacuolar degeneration was detected.

Two mechanisms for the induction of nephropathy by iodinated contrast have been proposed: ischemia injury and direct tubular damage. Both mechanisms lead to a decrease in the glomerular filtration rate, indicating the initiation of acute renal injury (LRA) (Caiazza *et al*., 2013). In ischemic lesions, the generated vasoconstriction leads to subsequent hypoxia, which causes endothelial damage through the production of reactive oxygen species (EROS), and this damage, in turn, directly injures the renal tubules and the vascular endothelium. EROS can also react with the nitric oxide present in the endothelium to form peroxynitrite, a highly harmful oxidant. The deposition of contrast in the renal tubules directly causes tubular damage, leading to cytotoxicity (Andreucci *et al*., 2014). According to Bucher *et al*. (2014), an increase in contrast-induced osmolarity is a secondary event to the occurrence of cytoplasmic vacuolation in proximal tubular cells, and Martín *et al.* (2014) assessed the direct toxic effect of contrasting media on tubular epithelial cells, which results in disturbances to the hemodynamics of renal blood flow.

The histological analysis of the kidneys collected from the EE100 and NAC groups demonstrated better preserved cellular structures, which suggests that similar to treatment with *N*-acetylcysteine, 100 mg/kg *E. edulis* pulp, which is rich in polyphenols, provides protection to renal tissue and is capable of combating free radicals generated by oxidative stress in contrast nephropathy. It is known that juçara is rich in polyphenols (Paredes-López *et al*., 2010; Borges *et al*., 2011a; Inácio *et al*., 2013; Bicudo, Ribani, Beta, 2014) and that these antioxidant compounds are able to stabilize or deactivate free radicals before they attack their biological targets in cells (Ribeiro *et al*.,2010). The increased toxicity caused by iodate contrast detected in the animals that received the higher doses of *E. edulis* pulp suggests that juçara pulp loses its protective effect on renal tissue at a dose higher than 200 mg/kg.

Determination of acute toxicity (LD_{50})

Acute toxicity was estimated to be induced by doses higher than 2000 mg/kg, and this fruit was classified as category 5 according to protocol OECD 423 (2011), which indicates that it is a relatively low acute toxicity hazard. These results indicate that there is a safety margin for the use of *E. edulis* pulp as a therapeutic agent, although additional toxicological studies are still required. Acute toxicity is defined as adverse effects occurring within a short time (24 hours) after administration of a single or multiple doses (Oga, Camargo, Batistuzzo, 2008).

As shown in Table I, no differences in biochemical parameters were found between the control group and the 2000 mg/kg *E. edulis*-treated group. The creatinine levels revealed a difference between the treated and controls, reinforcing the hypothesis that at doses higher than 400 mg/kg, *E. edulis* pulp can exert a possible pro-oxidant effect.

CONCLUSION

Under the conditions used in this experiment, we conclude that at low doses, *E. edulis* pulp tends to provide protection against the development of renal dysfunction in mice with induced contrast nephropathy. However, *N*-acetylcysteine showed better results. In addition, *E. edulis* pulp, which is enriched in polyphenols, particularly anthocyanins, markedly reduced the incidence of glomerular and renal tubular lesions. Further studies are needed to better elucidate the efficacy of *E. edulis* pulp in the prevention of NIC.

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