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Effects of the ethanol extract of the inner bark of *Syderoxylum obtusifolium* in the cyclophosphamide-induced cystitis in rats

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In this study, the ethanol extract of the inner bark of the *Syderoxylum obtusifolium* (EESob) was tested in the model of cyclophosphamide-induced hemorrhagic cystitis. Male Wistar rats were submitted to the injection of cyclophosphamide, 1 h after the treatment with EESob (200 or 400 mg/kg) or vehicle. After 24 h, the urinary bladder was excised and the edema, myeloperoxidase activity and malondialdehyde formation were measured. Also, myeloperoxidase activity and malondialdehyde formation in lung, spleen and liver and leukocyte counts in the peripheral blood were evaluated. Injection of cyclophosphamide increased myeloperoxidase activity and edema. The former was inhibited by the EESob at 400 mg/kg, but the latter was not affected. Although cyclophosphamide did not alter the bladder malondialdehyde formation, pre-treatment with EESob at both doses markedly increased this parameter. Lung and liver parameters or leukocyte counts were not altered by EESob. The spleen myeloperoxidase activity and malondialdehyde formation were not affected by cyclophosphamide, but were increased by the treatment with EESob. These results suggests that EESob administration to rats decrease myeloperoxidase activity in bladder tissue, but is accompanied by lipoperoxidation in this tissue, as well as in spleen, which do not support the use of EESob to treat cystitis.

Key words: *Syderoxylum obtusifolium*, cyclophosphamide, inflammation, lipid peroxidation, hemorrhagic cystitis.

INTRODUCTION

Cyclophosphamide is an alkylating agent that belongs to the group of the oxazophorines and is widely used as an antineoplasic drug. Its ability to form crossed bounds to DNA of tumoral cells permits its use to treat many types of neoplasic or non-neoplasic conditions, such as solid tumors, B-cell malignant diseases, thrombocytopenic purpura, rheumatoid arthritis, systemic lupus erythematosis among others (Korkmaz et al., 2007). Hemorrhagic cystitis is the main side effect of the therapy with cyclophosphamide (Philips et al., 1961; Ratliff et al., 1998; Korkmaz et al., 2007). It affects mainly the urinary bladder, the tissue in which the metabolite acrolein (the result from the hepatic metabolism of cyclophosphamide) is able to cause toxicity during its excretion.

The mechanism underlying toxicity of acrolein is complex and includes the depletion of cellular thiols, gene activation (both directly and by affecting transcription factors) and the reaction with cellular

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glutathione or other nucleophiles (Korkmaz et al., 2007; Dobrek and Thor, 2012). These deleterious effects induce ulceration, hemorrhage, edema and necrosis of the urothelium (Zhang et al., 2005; Mukhtar and Woodhouse, 2010) and requires an appropriate treatment in order to avoid withdrawal of cyclophosphamide treatment. Mesna, 2-mercaptoethane sulfonate sodium, is the major option to treat hemorrhagic cystitis, since it binds to acrolein in the urinary bladder or other parts of urinary tract, leading to inactivation of this compound (Kehrer and Biswal, 2000; Korkmaz et al., 2007).

In spite of the efficacy of mesna, novel therapeutic approaches to treat hemorrhagic cystitis are necessary and natural products-based research represents an important source of new isolated molecules or even extracts or essential oils to treat the most diverse diseases (Basso et al., 2005; Alviano and Alviano, 2009; Balbani et al., 2009). In the Northeast of Brasil, the "Caatinga" is a system with a great biodiversity, and many plants of this region are used by the popular medicine (Desmarchelier et al., 1999; Agra et al., 2007; Alburquerque et al., 2007). Between these plants, the *S. obtusifolium* (Humb. ex Roem. & Schult.) T.D. Penn., Sapotaceae, is known as "quixabeira" or "quixaba" and possesses many therapeutic uses. The bark is utilized by the population to treat gastritis, duodenal ulcer, genital damage, ovarian inflammation, chronic inflammation, colic, renal problems, cardiac problems, diabetes, healing and pains in general, among other uses (Albuquerque et al., 2007; Beltrão et al., 2008; Junior et al., 2011).

Desmachelier et al. (1999) determined *in vitro* the antioxidant activity of the aqueous extract of the bark of *S. obtusifolium*. Also, it was shown that methanol extract of the bark of *S. obtusifolium* decreases lipid peroxidation in rat liver tissue (Desmarchelier et al., 2003). A previous study has described that the ethanol extract of the inner bark of *S. obtusifolium* (EESob) is able to decrease the paw edema in rats and peritonitis in mice, both induced by carrageenan, as well as reducing acetic acid-induced abdominal writhes, time reaction in hot plate and the second phase of formalin-induced paw licking/biting in mice (Araújo-Neto et al., 2010), which validates the anti-inflammatory and antinociceptive activities of the inner bark of this plant. In this study, we have evaluated the possibility that EESob may have protective effects on the hemorrhagic cystitis induced by cyclophosphamide in rats, based on the antioxidant and anti-inflammatory activities described for the inner bark of this plant (Desmachelier et al., 1999; Desmarchelier et al., 2003; Araújo-Neto et al., 2010).

MATERIAL AND METHODS

Drugs and reagents

Cyclophosphamide, mesna, hexadecyltrimethylammonium bromide, *o*-dianisidine hydrochloride and Türk solution were purchased from Sigma (USA). Isoflurane (ISOFORINE®) was obtained from Cristália, Itapira, SP, Brazil. Other reagents were obtained from Merck.

Plant material and preparation of the inner bark ethanol extract

The inner bark of *S. obtusifolium* (Humb. ex Roem. & Schult.) T.D. Penn., Sapotaceae, was collected at the Xingó Village, Canindé de São Francisco, Sergipe State, Brazil, in March, 2009 (09° 66' 00" S, 37° 78' 94" W). A specimen was identified by the botanist Dr. Ana Paula Nascimento Prata, Department of Biology at the Federal University of Sergipe, and deposited in the Herbarium of this institution (Marechal Rondon Av., São Cristóvão, Sergipe, 49100-000, Brazil) under the registration number ASE 13,163. The inner bark was dried at 37°C with forced air for 7 days and subsequently powdered (2,800 g) and extracted by maceration at room temperature with 90% ethanol for 5 days. The extract was filtered in vacuum, and the solvent was removed using a rotary evaporator (50°C). The percentage of EESob yield was 16.4% (459.6 g) (Araújo et al., 2010).

Animals

Male Wistar rats (220 to 270 g, n = 8/group) were obtained from the Animal Center of the Federal University of Sergipe. Animals were maintained at 21 ± 2°C, with free access to food (PURINA®) and filtered water under a 12:12 h light/dark cycle. The animals were deprived of food for 8 h before the experiment, but had free access to water. All experimental procedures were conducted in accordance with the guidelines of the Brazilian College of Animal Experimentation and were approved by the Ethics Committee for Animal Use in Research at the Federal University of Sergipe (protocol number 055/09), which was conducted in accordance with the internationally accepted principles for laboratory animal use and care.

Induction of hemorrhagic cystitis

Hemorrhagic cystitis was induced by the injection of cyclophosphamide at a dose of 200 mg/kg in a volume of 5 ml/kg by the intraperitoneal (i.p.) route, according to previous studies (Abraham et al., 2009; Kiuchi et al., 2009). Control animals received saline (0.9%) at a volume of 5 ml/kg (i.p.). After 24 h of cyclophosphamide injection, animals were anesthetized with inhalatory isoflurane (3%). Samples of blood were collected from the abdominal vein; animals were exsanguinated and submitted to transcardiac perfusion with saline 0.9% plus heparin (5 U/L). Urinary bladder tissue and samples of lung, spleen and liver were collected for biochemical dosages.

Experimental design

The following experimental groups were used:

1. Vehicle + saline group: animals were orally pre-treated with vehicle (tween 80, 5%, 10 ml/kg) 1 h prior to injection of saline;
2. Vehicle + cyclophosphamide group: animals were pre-treated with vehicle (tween 80, 5%, 10 ml/kg) by the oral route (p.o.), 1 h prior to injection of cyclophosphamide;
3. Ethanol extract of *S. obtusifolium* (200 mg/kg) + Cyclophosphamide groups: animals were pre-treated with EESob (200 mg/kg, p.o., 10 ml/kg) 1 h prior to injection of cyclophosphamide;

4. Ethanol extract of *S. obtusifolium* (400 mg/kg) + Cyclophosphamide groups: animals were orally pre-treated with EESob (400 mg/kg, p.o., 10 ml/kg) 1 h prior to injection of cyclophosphamide;
5. Mesna + Cyclophosphamide group: animals were treated with i.p. administration of mesna (40 mg/kg, 1 ml/kg), 5 min before and at 4 and 8 h after injection of cyclophosphamide .

The doses of EESob used here were choosen based on a previous study from our group (Araújo-Neto et al., 2010) and the doses of mesna were used as described elsewhere (Morais et al., 1999; Batista et al., 2006).

Determination of urinary bladder edema

Edema of urinary bladder was estimated by weighting this tissue and expressing values as the result of urinary bladder weight (mg) divided by the whole body weight of animals (g).

Determination of myeloperoxidase (MPO) activity

For MPO activity determination, samples of urinary bladder, lung, spleen and kidney were collected, weighed and homogenized with potassium phosphate buffer (50 mM, pH 6.0 containing 0.5% of hexadecyltrimethylammonium bromide), and 1 ml aliquots of the homogenates were incubated at 60°C for 2 h (for inactivation of catalase) and centrifuged (2 min, 8,000 × *g*, 4°C). In a 96-well plate, aliquots of supernatant were incubated with a solution of *o*-dianisidine hydrochloride (0.167 mg/ml containing 0.005% H_2O_2). The MPO activity was measured kinetically in a microliter plate scanner (Labsystem Multiskan) at 460 nm. Results were expressed as units of MPO per mg tissue (UMPO/mg tissue), as previously described (Camargo et al., 2011).

Determination of lipid peroxidation

For determination of thiobarbituric acid reactive substances (TBARS), samples of urinary bladder, lung, spleen and liver were weighted and homogenized in potassium phosphate buffer (50 mM, pH 7.4) containing butylated hydroxytoluene (BHT, 12.6 mM). Then, aliquots of the homogenate (in duplicate) were incubated (90°C, 45 min) with thiobarbituric acid (TBA; 0.37%) in an acidic solution (trichloroacetic acid at 15% and hydrochloric acid at 0.25 N). At the end of incubation, the homogenates were centrifuged (5 min, 8,000 × *g*), and aliquots from the supernatants were extracted with n-butanol, followed by stirring in a vortex for 30 s and further centrifugation (2 min, 8,000 × *g*). The supernatant absorbance was measured at 535 nm in a microplate reader (corrected by the values of absorbance at 572 nm). The results were calculated using a molar extinction coefficient of $1.55 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as pmol of malondialdehyde (MDA) formed per mg of tissue (Bose et al., 1989; Santana et al., 2012).

Leukocyte counts

Total and differential leukocyte counts were performed in aliquots of 20 µl of peripheral blood taken from the tail vein of anesthetized rats immediately before euthanasia. The total leukocyte count was performed in a Neubauer chamber, and the differential count was conducted under a light microscope with immersion oil objective in cytocentrifuged smears colored with GIEMSA NEWPROV®, where 100 cells per slide were counted, based on normal morphological criteria. Results were expressed as number of leukocytes/ml of peripheral blood.

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM) and analyzed by one-way analysis of variance followed by Tukey's *post hoc* test by using GraphPad Prism software (version 4.0). $P < 0.05$ was considered significant.

RESULTS

Effect of EESob on the urinary bladder edema, myeloperoxidase activity and lipoperoxidation

Administration of cyclophosphamide to rats increased significantly ($P < 0.05$) the urinary bladder weight when compared with saline-injected group, which is a sign of tissue edema. This was diminished significantly ($P < 0.05$) by the treatment with mesna but not by the pretreatment with the EESob (200 or 400 mg/kg) (Figure 1). Associated with edema, cyclophosphamide induced a significant increase of the MPO activity ($P < 0.05$) in the urinary bladder when compared with saline group. The EESob decreased this activity ($P < 0.05$) at the dose of 400 mg/kg when compared with the group injected with vehicle, which was also achieved with the treatment with mesna ($P < 0.05$). The dose of 200 mg/kg did not alter significantly this parameter, although there was a clear tendency to lower values in this group when compared with vehicle-treated group (Figure 2). The determination of lipid peroxidation in urinary bladder did not demonstrate any alteration of the MDA formation in this tissue after 24 h of the injection of cyclophosphamide when compared with saline-administrated group. This was also not altered by the treatment with mesna, however, the oral pretreatment of rats with EESob increased the MDA in urinary bladder tissue when compared with the cyclophosphamide-injected and vehicle-treated group ($P < 0.05$ or $P < 0.01$ for 200 or 400 mg/kg, respectively) or saline-injected and vehicle-treated ($P < 0.05$ for both 200 and 400 mg/kg) (Figure 3).

Effect of EESob on the other tissues and blood leukocyte counts

The MPO activity and lipid peroxidation were also evaluated in other tissues such as lung, spleen and liver. Lung MPO activity was not significantly affected by the cyclophosphamide injection, although there was a tendency for higher values in this group when compared with saline-injected animals. The treatment with mesna or EESob did not change significantly the lung MPO activity (Table 1). Also, in the lungs of animals injected with cyclophosphamide or treated with mesna or EESob, there was no alteration of MDA formation (Table 1).

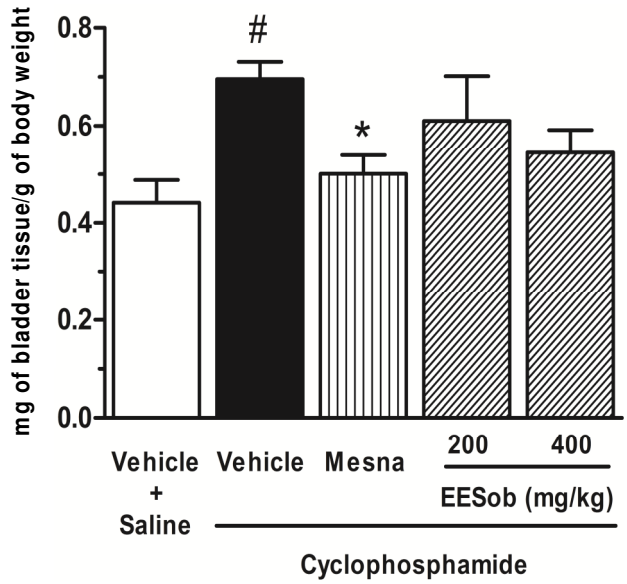

Figure 1. Lack of effect of the ethanol extract of *Syderolxylum obtusifolium* (EESob) on the urinary bladder weight of rats submitted to cyclophosphamide-induced cystitis. Animals were treated with EESob (200 or 400 mg/kg, p.o.), vehicle or mesna (40 mg/kg, i.p.) and injected with cyclophosphamide (200 mg/kg, i.p.). After 24 h, the urinary bladder weight was measured as an indication of edema and expressed as mg of urinary bladder/g of whole body weight of animals. $^{\#}P < 0.05$ versus vehicle + saline; *P < 0.05 versus vehicle + cyclophosphamide.

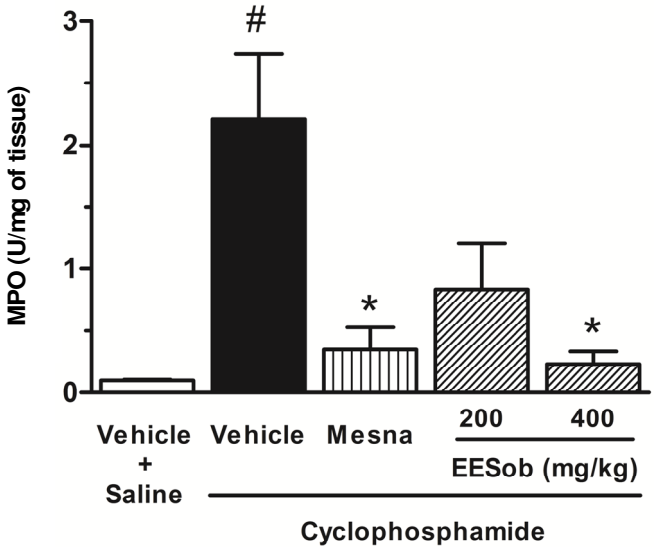

Figure 2. The ethanol extract of *Syderolxylum obtusifolium* (EESob) reduced the myeloperoxidase (MPO) activity in urinary bladder of rats during cyclophosphamide-induced cystitis. Animals were treated with EESob (200 to 400 mg/kg, p.o.), vehicle or mesna (40 mg/kg, i.p.) and injected with cyclophosphamide (200 mg/kg, i.p.). After 24 h, the MPO activity was determined in urinary bladder homogenate and was expressed as units (U) of MPO/mg of tissue. $^{\#}P < 0.05$ versus vehicle + saline; *P < 0.05 versus vehicle + cyclophosphamide.

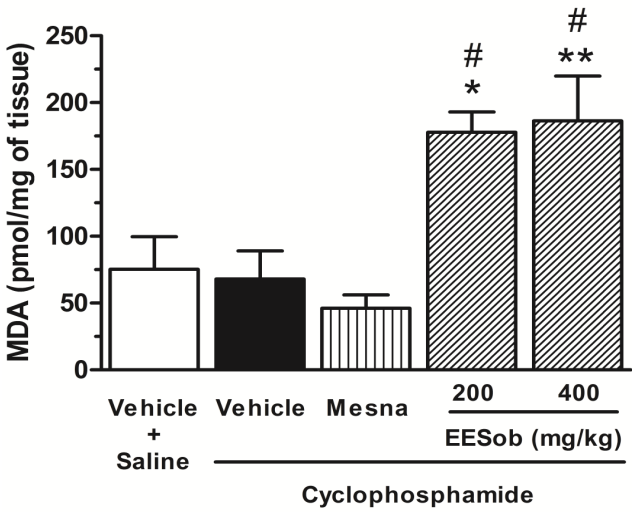

Figure 3. The ethanol extract of *Syderolxylum obtusifolium* (EESob) increases lipid peroxidation in urinary bladder of rats during cyclophosphamide-induced cystitis. Animals were treated with EESob (100 to 400 mg/kg, p.o.), vehicle or mesna (40 mg/kg, i.p.) and injected with cyclophosphamide (200 mg/kg, i.p.). After 24 h, the malondialdehyde (MDA) formation in urinary bladder was measured and expressed as pmol of MDA/mg of tissue. $^{\#}P < 0.05$ versus vehicle + saline; *P < 0.05 or **P < 0.01 versus vehicle + cyclophosphamide.

Spleen MPO activity was not modified by the injection of cyclophosphamide and by the treatment with mesna, however, the pretreatment with EESob enhanced spleenMPO activity at the doses of 200 mg/kg ($P < 0.05$, when compared with vehicle + saline group) or 400 mg/kg ($P < 0.001$, when compared with either vehicle + saline or vehicle + cyclophosphamide groups) (Table 1). Besides, a significant increase in the MDA formation of EESob (400 mg/kg)-pretreated group ($P < 0.05$, when compared with vehicle + saline group) was found. Treatment with mesna did not change the MDA formation in spleen (Table 1). In the liver of the groups studied, there was no alteration of the basal MPO activity or MDA formation (data not shown).

Table 2 demonstrates that cyclophosphamide treatment decreased significantly the total peripheral blood leukocyte counts, as well as the mononuclear cell counts ($P < 0.001$ each). Polymorphonuclear cell counts were not affected by the cyclophosphamide administration. Treatment with mesna or EESob (200 or 400 mg/kg) did not modify these counts (Table 2).

DISCUSSION

In the present study, we tested the possibility that the EESob could reduce hemorrhagic cystitis in rats, by administrating this extract previous to the injection of cyclophosphamide. The EESob recently have its anti-

Table 1. Effect of the ethanol extract of *Syderolxylum obtusifolium* (EESob) on the myeloperoxidase activity and lipid peroxidation of lung and spleen of rats during cyclophosphamide (CYP)-induced cystitis.

Tissue	Group	MPO (UMPO/mg of tissue)	MDA (pmol of MDA/mg of tissue)
Lung	Vehicle+Saline	10.1±1.1	27.8±7.2
	Vehicle+CYP	15.3±2.3	31.8±2.0
	Mesna+CYP	10.3±2.3	13.2±4.8
	EESob (200 mg/kg)+CYP	12.4±2.8	32.3±7.7
	EESob (400 mg/kg)+CYP	12.1±2.8	35.7±6.9
Spleen	Vehicle+Saline	17.9±2.1	35.5±7.5
	Vehicle+CYP	20.6±1.3	39.1±4.7
	Mesna+CYP	14.1±1.8	18.9±6.7
	EESob (200 mg/kg)+CYP	26.1±1.8$^{\#}$	53.0±6.4
	EESob (400 mg/kg)+CYP	30.7±1.4$^{\#\#\#.\ ***}$	60.3±4.4$^{\#}$

Rats were treated with EESob (200 to 400 mg/kg), mesna (40 mg/kg) or vehicle and injected with saline or CYP (200 mg/kg). After 24 h of induction, the MPO activity and malondialdehyde (MDA) formation were determined in the lung or spleen tissues. Data are expressed as mean ± SEM for n = 6 rats. $^{\#}P < 0.05$ or $^{\#\#\#}P < 0.001$ versus respective vehicle + saline group; $^{***}P < 0.001$ versus respective vehicle + CYP group.

Table 2. Lack of effect of the ethanol extract of *Syderolxylum obtusifolium* (EESob) on the blood leukocyte counts of cyclophosphamide (CYP)-induced cystitis.

Group	Total count (cells/ml × 10^6)	Mononuclear cell (cells/ml × 10^6)	Polymorphonuclear cells (cells/ml × 10^6)
Vehicle+Saline	13.7±0.6	10.4±0.8	3.2±0.9
Vehicle+CYP	6.4±0.5$^{\#\#\#}$	2.5±0.7$^{\#\#\#}$	3.9±0.7
Mesna+CYP	5.8±1.6$^{\#\#\#}$	1.9±0.6$^{\#\#\#}$	3.8±1.1
EESob (200 mg/kg)+CYP	6.4±1.9$^{\#\#\#}$	3.1±0.2$^{\#\#\#}$	3.3±1.8
EESob (400 mg/kg)+CYP	5.0±0.9$^{\#\#\#}$	3.1±0.7$^{\#\#\#}$	2.1±0.4

Rats were treated with EESob (200 to 400 mg/kg), mesna (40 mg/kg) or vehicle and injected with saline or CYP (200 mg/kg). After 24 h of induction, the total and differential counts were performed in the peripheral blood. Data are expressed as mean ± SEM for n = 6 rats. $^{\#}P < 0.05$ or $^{\#\#\#}P < 0.001$ versus vehicle + saline.

inflammatory effect described, as well as its antinociceptive and antioxidant activities (Araújo-Neto et al., 2010). In this study, the authors used traditional models of inflammation, and showed that EESob reduced the rat paw edema (antiedematogenic effect) and leukocyte migration to peritoneal cavity (anti-chemotactic effect), both of which are induced by carrageenan, a widely used phlogistic agent. Besides, Desmarchelier et al. (2003) demonstrated that the methanol extract of the inner bark of *S. obtusifolium* has antioxidant activity, which is reasonable as well for the EESob, since Araújo et al. (2010) have detected the presence of flavonoids in the EESob.

As popularly known, flavonoids possess various biological properties such as antioxidant and anti-inflammatory activities (Ghasemzadeh and Ghasemzadeh, 2011). Altogether, these previous data encouraged us to investigate the activity of EESob in the model of hemorrhagic cystitis in rat. The results obtained showed that although EESob did not modify the urinary bladder edema, it reduced the MPO activity in this tissue. As MPO activity is an indicator of the presence of neutrophils in the tissue (Bradley et al., 1982; Camargo et al., 2011) and the neutrophil migration resulting from cyclophosphamide injection was reduced by EESob, this anti-chemotactic effect of EESob in the urinary bladder confirmed the findings by Araújo-Neto et al. (2010) in the peritonitis model. Nevertheless, the antiedematogenic property of EESob was not achieved in the bladders of animals injected with cyclophosphamide.

Although these results suggest that EESob could be used to treat bladder inflammation, the EESob markedly

increased the lipid peroxidation in this tissue. Lipid peroxidation is a catalytic degradation process caused by free radicals that distress the integrity of the poly-unsaturated fat acid of the cellular membranes and generate aldehydes such as MDA (Uruñuela et al., 2002). The findings that EESob enhanced lipid peroxidation is important and makes impracticable the use of EESob at the doses employed in animals in this study. Of interest, the ethanol extract of inner bark of *Caesalpinia pyramidalis* (Tull.), used in this same model of cystitis, in the same doses in our laboratory, did not increase but rather decreased the lipid peroxidation in urinary bladder (unpublished data), indicating that the dose is not the only reason for this damaging effect to urinary bladder. Moreover, this result implies that EESob cannot be used to treat bladder inflammation and does not agree with the antioxidant effect of EESob described previously by Desmarchelier et al. (2003) that determined a half maximum inhibitory concentration (IC_{50})% of 918 mg/kg for the methanol extract of the inner bark of *S. obtusifolium* in the spontaneous lipid peroxidation in rat liver tissues. Whether there are significant differences between the methanol extract used by Desmanchelier et al. (2003) and the EESob prepared in the present study remains to be elucidated.

Besides the deleterious effect of EESob on the urinary bladder lipid peroxidation, we also detected that spleen MPO activity and lipid peroxidation were also increased by the treatment with EESob, which may contribute in some extent to the injury in this tissue. The EESob produced no lipid peroxidation or MPO activity in lung or liver of rats. In this regard, it is important to note that the inner bark of this plant is used by the population in the Northeast of Brazil (Desmarchelier et al., 2003; Alburquerque et al., 2007, Araújo-Neto et al., 2010) and the present results may indicate that care should be taken when using the inner bark of this species. It is worthwhile mentioning that the bark of this plant is used in many parts of Brazil, but no toxic effects were still described. In spite of the popular belief that traditional medicines are safe (Jordan et al., 2010), it is largely known that many plants have potential to cause toxicity, which could also be the case of EESob. However, further toxicologic studies are necessary to clarify this issue.

The components present in the EESob are still unknown. In the leaves of *S. obtusifolium*, there were identified some compounds like triterpene, quercetin and kaempferol derivatives, among others (Passos-Oliveira et al., 2012), but the compounds presented in EESob remains to be elucidated. Several phytochemicals are described by their ability to inhibit the hemorraghic cystitis induced by cyclophosphamide. This is the case of the flavonoids ternatin, isolated from *Egletes viscosa* Less. (Vieira et al., 2004), quercetin, rutin and gallic acid, isolated from *Phyllanthus niruri* (Boeira et al., 2007) or the sesquiterpene lactone parthenolide, isolated from *Tanacetum partenium* (Kiuchi et al., 2009) which are able to reduce bladder inflammation and oxidative stress.

In our study, we also detected a decrease in the total leukocyte and mononuclear cell numbers in peripheral blood, but not polymorphonuclear counts, which were not changed by EESob, indicating that this extract may not have any effect in the hematopoietic effects induced by cyclophosphamide. These effects of cyclophosphamide are well described (DeWys et al., 1970; Kusano et al., 2004) as damaging activities to the hematopoietic stem cells and precursor cells and to the hematopoietic microenvironment, leading to myelosuppression.

Taken together, these results demonstrate that EESob administration to rats may decrease MPO activity in bladder tissue but is accompanied by lipoperoxidation in this tissue, as well as in spleen. These findings do not support the use of EESob for the treatment of hemorrhagic cystitis and indicates the need for toxicological studies involving EESob.

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ABBREVIATIONS

EESob, Ethanol extract of the inner bark of the *Syderoxylum obtusifolium*; **MPO,** myeloperoxidase; **MDA,** malondialdehyde.

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