Naunyn-Schmiedeberg's Arch Pharmacol (2011) 384:265–275
DOI 10.1007/s00210-011-0668-0

ORIGINAL ARTICLE

Effects of the hydroalcoholic extract of *Phyllanthus niruri* and its isolated compounds on cyclophosphamide-induced hemorrhagic cystitis in mouse

Vinícios T. Boeira · Carlos E. Leite · André A. Santos Jr · Maria I. Edelweiss · João B. Calixto · Maria M. Campos · Fernanda B. Morrone

Received: 14 March 2011 / Accepted: 6 July 2011 / Published online: 23 July 2011

Abstract The effects of *Phyllanthus niruri* hydroalcoholic extract and the isolated compounds quercetin, rutin, and gallic acid were examined in the mouse model of cyclophosphamide (CYP)-induced hemorrhagic cystitis (HC). HC was induced by a single CYP injection (300 mg/kg, IP), and the animals were evaluated 4 and 6 h after. Some animals were orally treated with the reference compound 2-mercaptoethane sodium sulfonate (Mesna) 80 mg/kg (30 min before CYP) and 160 mg/kg (2 h after CYP). Other groups were treated with *P. niruri* extract (30 and 50 mg/kg), or quercetin, rutin, and gallic acid (10 and 20 mg/kg), given orally, at the same intervals described for Mesna. *P. niruri* extract and its active components produced a significant attenuation of the nociception, edema, and hemorrhage evoked by CYP, which was similar to that seen for Mesna. Gallic acid and rutin displayed greater anti-inflammatory effects, whereas quercetin presented superior antinociceptive activities. Noteworthy is that *P. niruri* extract and compounds significantly reduced CYP-induced liver lipid peroxidation. Our results shed new light on the beneficial effects of *P. niruri* extract and its active compounds in attenuating the collateral effects elicited by the chemotherapeutic agent CYP.

Keywords Quercetin · Gallic acid · Nociception · Lipid peroxidation

V. T. Boeira · F. B. Morrone (✉)
Faculty of Pharmacy, PUCRS,
Av. Ipiranga, 6681-Partenon,
Porto Alegre, RS CEP: 90619-900, Brazil
e-mail: fernanda.morrone@pucrs.br

M. M. Campos
Faculties of Dentistry, PUCRS,
Porto Alegre, Brazil

C. E. Leite · M. M. Campos · F. B. Morrone
Institute of Toxicology, PUCRS,
Porto Alegre, Brazil

A. A. Santos Jr · F. B. Morrone
Programa de Pós-Graduação em Biologia Celular e Molecular, PUCRS,
Porto Alegre, Brazil

M. I. Edelweiss
Faculty of Medicine, UFRGS,
Porto Alegre, RS, Brazil

J. B. Calixto
Department of Pharmacology, Centre of Biological Sciences, UFSC,
Florianopolis, SC, Brazil

Introduction

Hemorrhagic cystitis (HC) is a modification of bladder mucosa, characterized by hematuria and symptoms of irritability, such as dysuria and increase in the frequency and urgency of urination. HC has been commonly associated to radiotherapy near to the pelvic region and to infectious diseases (Cheuk et al. 2007).

Cyclophosphamide (CYP) is an alkylating agent, which acts non-specifically on the cell cycle. It is used for the treatment of many solid tumors, B cell-related malignancies, and some autoimmune diseases. One of the most common adverse effects of CYP is the urotoxicity, which may include HC and bladder fibrosis (Wong et al. 2000). The incidence of CYP urotoxicity is greater with the parenteral administration compared with the oral route.

The urotoxicity of CYP is thought to be due to the formation of acrolein that damages the urothelium. Acrolein

interferes with the tissue antioxidant defense system, produces highly reactive oxygen free radicals, and is mutagenic to mammalian cells (Oboh et al. 2011). Furthermore, the epithelial damage induced by CYP can occur in other organs besides the bladder (Wong et al. 2000).

Oxidative stress is known to be involved in several diseases, representing the cause of toxicity of many drugs. Several studies demonstrated that oxidative stress induced by CYP can damage organs, such as: bladder, brain, and liver (Abraham and Sugumar 2008; Tripathi and Jena 2009; Oboh et al. 2011). The oxidative stress induced by CYP in liver is particularly important because this drug suffers hepatic metabolism by the cytochrome P-450 system to generate active alkylating metabolites, which interfere with cellular DNA synthesis, and ultimately lead to cell death in liver and other tissues (Tripathi and Jena 2009).

Phyllanthus niruri (Euphorbiaceae) is a plant found in the warmer parts of the world; in Brazil, it is popularly known as "break-stone", due to its ability to grow in cracks and crevices of walls or sidewalks. Several chemical constituents have been identified, including flavonoids, alkaloids, terpenoids, lignans, polyphenols, tannins, coumarins, and saponins (Table 1). These substances are found in leaves, stems, and roots of the plant (Calixto et al. 1998; Bagalkotkar et al. 2006).

Research groups in India were the first to investigate the pharmacological properties of *P. niruri* (Chauhan et al. 1977; Thyagarajan et al. 1982; Venkateswaran et al. 1987). *P. niruri* gained worldwide attention in the late 1980s due to its activity against hepatitis B virus (Venkateswaran et al. 1987). The plant also showed potent antispasmodic and analgesic effects (Santos et al. 1994; Martini et al. 2000). In vivo studies revealed that *P. niruri* extract is able to protect against liver damage related to oxidative stress in mice (Bhattacharjee and Sil 2006; Chatterjee et al. 2006). Quercetin is a bioactive flavonoid that displays anti-aggregant, anticancer, anti-fungal (especially anti-dermatophytic), anti-feedant, anti-glaucomic, anti-inflammatory, antioxidant, antiseptic, and antispasmodic activities (Cechinel Filho et al. 1996; Saija et al. 2003). Furthermore, a combination of quercetin and Mesna (the reference compound used to prevent HC) produced a marked inhibition of HC induced by CYP in rats (Ozcan et al. 2005). Rutin is a flavonol glycoside comprised of flavonol quercetin and the disaccharide rutinose. Previous studies have demonstrated the anti-inflammatory effects of rutin in rat paw edema, and neutrophil chemotaxis and degranulation (Selloum et al. 2003). Gallic acid represents a benzenoid compound found in many plants, either in the free-form or as part of tannins. This molecule is known to possess anti-microbial, antioxidant, and cytotoxic properties, and a previous study has shown that gallic acid was able to suppress TNF-α-induced NF–κB activity (Morais et al. 2010). In vitro studies on the mode of action of gallic acid revealed that this compound interferes with the functioning of polymorphonuclear leukocytes, scavenging of superoxide anions, inhibition of myeloperoxidase release, and activity (Kroes et al. 1992). Previous literature evidence shows that all tested compounds are active when dosed by the oral route, according to assessment in different experimental approaches (Arjumand et al. 2011; Kim et al. 2011; Latha and Daisy 2011).

This study analyzed the anti-inflammatory and antinociceptive properties of the hydroalcoholic extract and isolated compounds of *P. niruri*, namely quercetin, rutin, and gallic acid (Fig. 1a–c) in HC induced by CYP in mice. Attempts have also been made to verify the effects of these strategies on the oxidative stress induced by CYP in the mouse liver.

Table 1 Chemical constituents isolated from *P. niruri*

Class	Compounds
Alkaloid	4-Methoxy-nor-securinine, nirurine, ent-norsecurinine
Benzenoid	Gallic acid, corilagin
Coumarin	Ellagic acid, ethyl brevifolin carboxylate
Flavonoid	Quercetin, rutin, astragalin, quercitrin, isoquercitrin, kaempferol-49-rhamnopyranoside, eridictyol-7-rhamnopyranoside, fisetin-4-*O*-glucoside, nirurin
Lignan	Phyllanthin, hypophyllanthin, niranthin, nirtetralin, phyltetralin, hinokinin, isolintetralin
Lipid	Ricinoleic acid
Phytallate	Phyllester
Sterol	Estradiol, *b*-sitosterol, isopropyl-24-cholesterol
Tannin	Geraniin
Triterpene	Lupeol acetate, lupeol, 3,7,11,15,19,23-hexamethyl-2Z,6Z , 10Z,14E,18E,22E-tetracoshenen-1-ol, phyllanthenol, phyllanthenone, phyllantheol

Adapted from Calixto et al. 1998

Materials and methods

Animals

Non-fasted male Swiss mice (eight per group, 25–30 g) were used. The animals were maintained in controlled temperature (22±2°C) and humidity (60–70%) under a 12 h light/dark cycle. Food and water were available ad libitum. The studies reported in this manuscript followed the "Principles of Laboratory Animal Care" from NIH publication No. 85–23 and ethical guidelines for investigation of experimental pain in conscious animals and were approved by the Institutional Animal Ethics Committee (CEUA 09/00103).

Drugs and reagents

The following drugs and reagents were used: cyclophosphamide, Mesna (Mitexan, Baxter Oncology Gmbh, Frank-

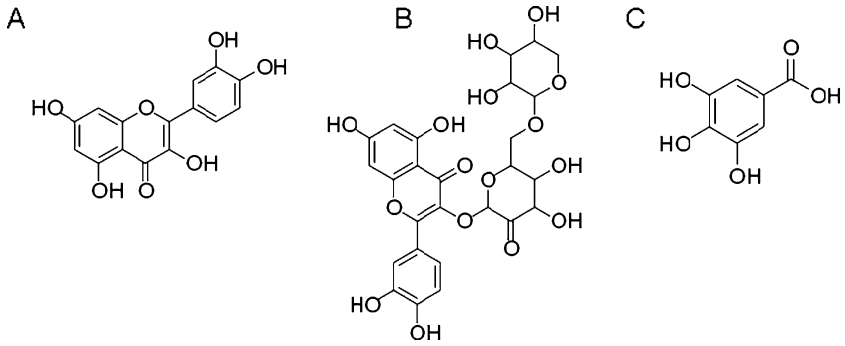

Fig. 1 Chemical structures of *P. niruri* isolated compounds. **a** quercetin, **b** rutin, and **c** gallic acid

furt, Germany). The compounds quercetin, rutin, and gallic acid were obtained from Sigma Chemical Company, USA (purity grade >95%). For the hydroalcoholic extract, the botanical material was collected in Santa Catarina, Brazil, and was classified by Dr. Leila da França Amaral. The extract preparation was carried out according to Santos et al. (1994). All the substances were prepared in NaCl 0.9% solution (saline) containing 1% Tween 80. The doses of the extract and compounds were chosen on the basis of literature data (Santos et al. 1994; Martini et al. 2000).

General protocols

HC was induced by a single administration of CYP (300 mg/kg, IP), as described before (Olivar and Laird 1999). The hydroalcoholic extract obtained from the leaves of *P. niruri* (30 and 50 mg/kg, PO) was administered 30 min before CYP, and an additional treatment was performed 2 h later. Separate groups of animals were treated with quercetin, rutin, or gallic acid (10 and 20 mg/kg, PO), at the same time intervals. The positive control drug Mesna was also administered in two doses: The first dose (80 mg/kg, PO) was administered 30 min before, and the second one (160 mg/kg, PO) was given 2 h after CYP.

Assessment of nociception

The mice were placed individually in observation boxes and were acclimatized for 30 min prior to behavioral testing. The following behavioral changes were evaluated: (1) activity (walking, rearing, climbing, grooming, etc.); (2) immobility; and (3) behaviors indicative of visceral pain ("crises"). In addition, the behavioral alterations were scored according to the following scale: 0=normal; 1=piloerection; 2=strong piloerection; 3=labored breathing; 4=licking of the abdomen; and 5=stretching and contractions of the abdomen (Olivar and Laird 1999; Wantuch et al. 2007). The mice were observed for 2 min, every 30 min, in a total period of 4 h after CYP, and the nociceptive responses were provided as the sum of total scores. At the end of the 4-h observation period, an open-field test was carried out. The animals were placed individually in a box divided in nine squares, for 5 min, and the number of squares crossed with the four paws was taken as an index of locomotor activity.

The animals were killed 6 h following CYP administration, and the bladders were collected for macroscopic and histological analyses, as described in the next sections.

Gross evaluation

The gross evaluation followed criteria established by Gray et al. (1986). All bladders were dissected free from connecting tissues and transected at the bladder neck. Each bladder was macroscopically evaluated by an examiner blind to the experimental groups. The edema was categorized as severe (3+), moderate (2+), mild (1+), or absent (0). The edema was considered severe when fluid was seen externally in the walls of the bladder, as well as internally. When the edema was confined to the internal mucosa, it was reported as moderate; when slight edematogenic signals were observed, the edema was defined as mild. The bladders were also surveyed for bleeding and categorized into four designations, considering the presence of intravesical clots (3+), mucosal hematomas (2+), telangiectasia or dilatation of the bladder vessels (1+), or normal aspect (0).

Histological analysis

Following the gross evaluation, the bladders were fixed in buffered formalin solution (10%) for 24 h. Subsequently, the samples were embedded in paraffin. Five-micrometer slices were obtained and stained with hematoxylin and eosin. A pathologist who was blinded to the treatment reviewed each specimen, considering the presence and the intensity of edema, hemorrhage, and tissue damage.

Liver lipid peroxidation

Liver lipid peroxidation was measured as described by Tuzmen et al. (2008). The liver homogenate (10% *w/v*) was

prepared in saline. The sample was centrifuged and, the supernatant was used for the analysis. Alkaline hydrolysis of protein-bound malondialdehyde (MDA) was achieved by incubating this mixture in a 60°C water bath for 30 min, followed by thiobarbituric acid (39.9 mM, 250 μl) and phosphoric acid (440 mM, 750 μl) addition and incubation in a 95°C water bath for 60 min. The samples were injected into a high-performance liquid chromatograph equipped with ultraviolet detector (Agilent Technologies® Inc., USA). The protein content in the supernatant was determined with a commercial kit (Labtest®). Lipid peroxidation was calculated from the standard curve using the 1,1,3,3-tetraethoxy propane (97%) and expressed as nanomoles per milligram protein.

Statistical analysis

The results are presented as the mean±standard error mean of eight animals. The percentages of inhibition were calculated as the mean of inhibitions obtained for each individual experiment. Statistical comparison of the data was performed by one-way ANOVA followed by Bonferroni test. Values less than 0.05 ($P<0.05$) were considered significant.

Results

The oral administration of the reference compound Mesna (80 mg/kg, 30 min prior, and 160 mg/kg, 2 h after CYP) produced a significant reduction of CYP-induced nociceptive behavior, with an inhibition of 83±8% (Fig. 2a–d). Interestingly, the oral treatment of animals with the hydroalcoholic extract of *P. niruri* (30 and 50 mg/kg, 30 min prior, and 2 h after CYP) also displayed a marked inhibition of nociception evoked by CYP. The percentages of inhibition were 79±6% and 85±9%, respectively (Fig. 2a). The isolated compound quercetin (10 and 20 mg/kg, PO), dosed at the same schedules of treatment as described for the extract, was able to inhibit the nociception induced by CYP in 62±16% and 84±10%, respectively (Fig. 2b). A similar effect was observed for gallic acid (10 and 20 mg/kg, PO), with inhibition percentages of 51±14% and 71±13%, correspondingly

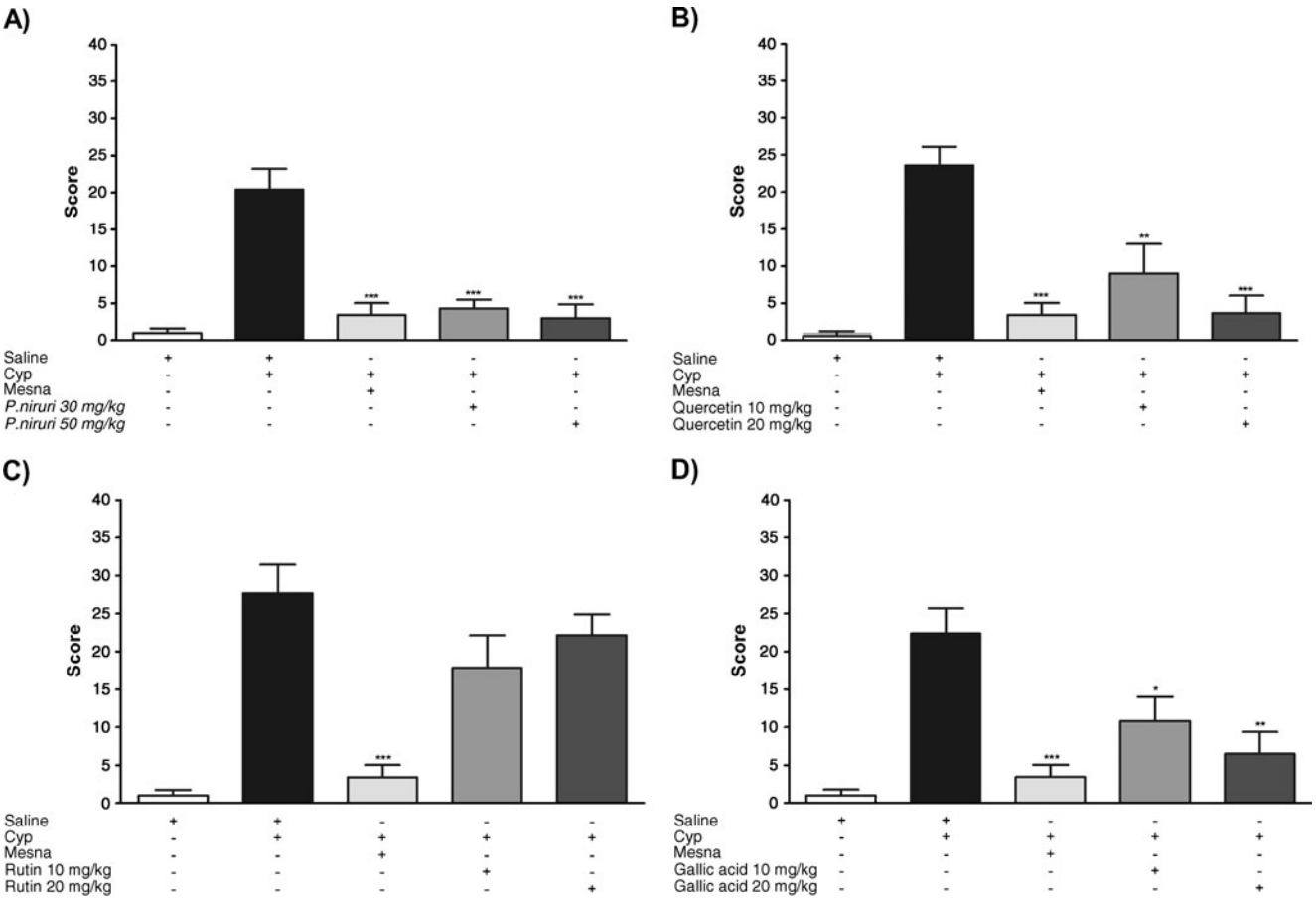

Fig. 2 Effect of treatment with Mesna, *P. niruri* extract (**a**), quercetin (**b**), rutin (**c**), or gallic acid (**d**) on nociceptive responses induced by CYP (control). Each *column* represents the mean of eight animals and the *vertical lines* show the SEM. *Asterisks* denote the significance levels in comparison to control values: *$P<0.05$; **$P<0.01$; ***$P<0.001$

(Fig. 2d). The inhibitory effects observed following treatment with the hydroalcoholic extract or the compounds quercetin and gallic acid were not significantly different in comparison to that obtained with Mesna. In contrast, rutin (10 and 20 mg/kg, PO) failed to significantly alter the nociception scores (Fig. 2c). The extract and all the isolated compounds did not significantly change total locomotor activity in the open-field paradigm (results not shown).

To assess the edema and the hemorrhage formation in CYP-injected mice, we have carried out a macroscopic analysis of the bladders. Confirming the literature data (Gray et al. 1986), the score of edema and hemorrhage in the CYP group was significantly higher than the score of 0 in the saline group (Figs. 3 and 4). The oral treatment with Mesna (80 mg/kg, 30 min prior, and 160 mg/kg, 2 h after CYP injection) caused a significant inhibition of edema induced by this chemotherapeutic agent (88±11%; Fig. 3a–d). Oral treatment with the hydroalcoholic extract of *P. niruri* (30 and 50 mg/kg, 30 min prior and 2 h after CYP), significantly reduced the edema scores in 49±12% and 62±16%, respectively (Fig. 3a). The isolated compound quercetin (10 and 20 mg/kg, PO), given at the same intervals of time, also induced a significant reduction of edema in 55±18% and 62±13%, respectively (Fig. 3b). Furthermore, the chemical constituents rutin and gallic acid (10 and 20 mg/kg, PO), dosed at the same schedules of treatment described for the extract, were capable of inhibiting CYP-induced edema formation. The percentages of inhibition were 62±10% and 62±14% for rutin (Fig. 3c), and 48±19% and 99±1% (Fig. 3d) for gallic acid, correspondingly. Again, there was no significant difference between the inhibitory effects reached after treating animals with *P. niruri* extract or isolated compounds, in relation to Mesna treatment.

Regarding the hemorrhage, data on Fig. 4a–d reveal that oral administration of Mesna (80 mg/kg, 30 min prior, and 160 mg/kg, 2 h after CYP) resulted in a marked reduction of this parameter (68±5%). The oral treatment with *P. niruri* extract (30 and 50 mg/kg) or the isolated compound rutin (10 and 20 mg/kg), both dosed 30 min prior, and 2 h after CYP, also produced a prominent reduction of hemorrhage, an effect that was not significantly different when compared

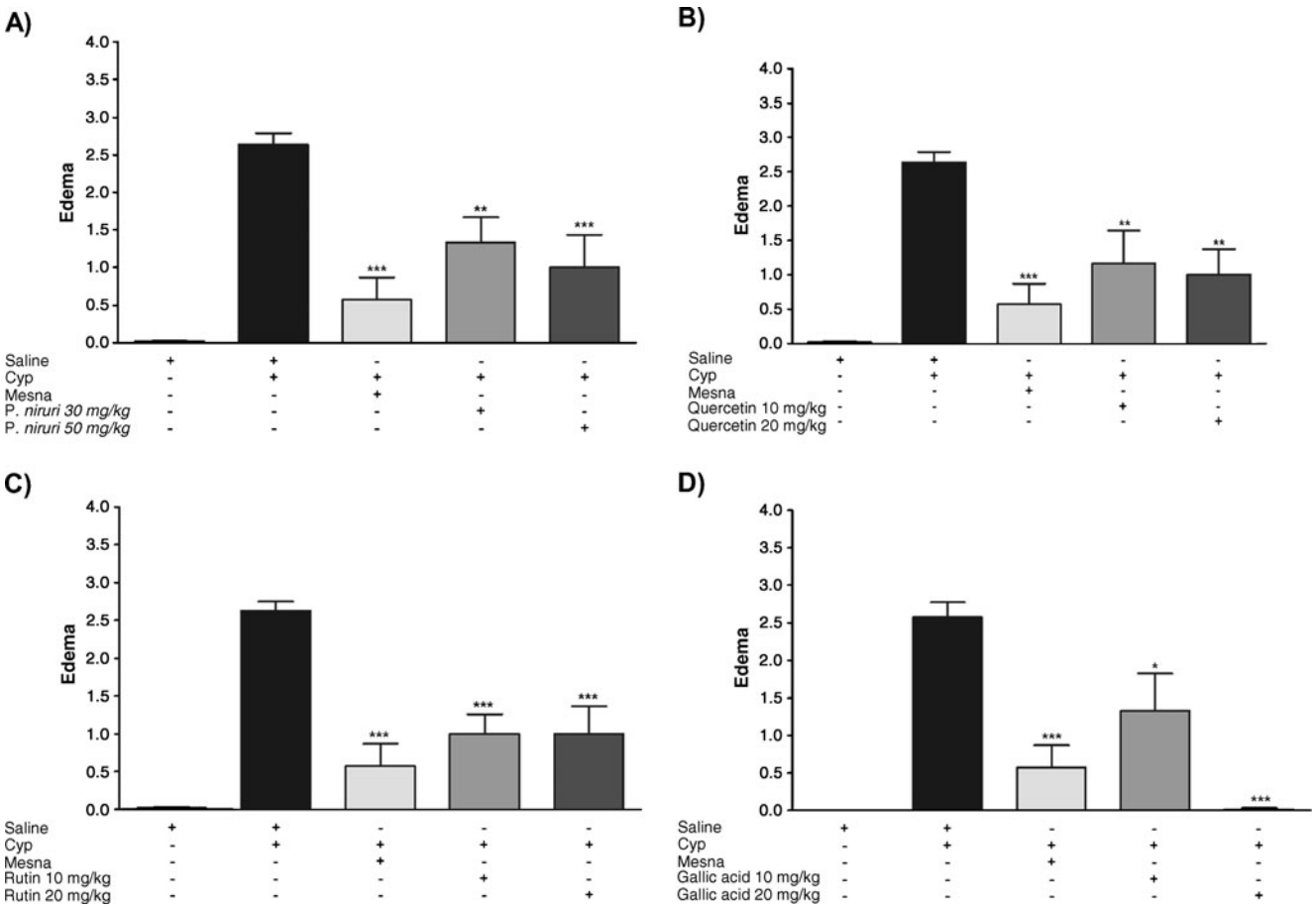

Fig. 3 Effect of treatment with Mesna, *P. niruri* extract (**a**), quercetin (**b**), rutin (**c**), or gallic acid (**d**) on edema scores. Each *column* represents the mean of eight animals and the *vertical lines* show the SEM. *Asterisks* denote the significance levels in comparison to control values: *$P<0.05$; **$P<0.01$; ***$P<0.001$

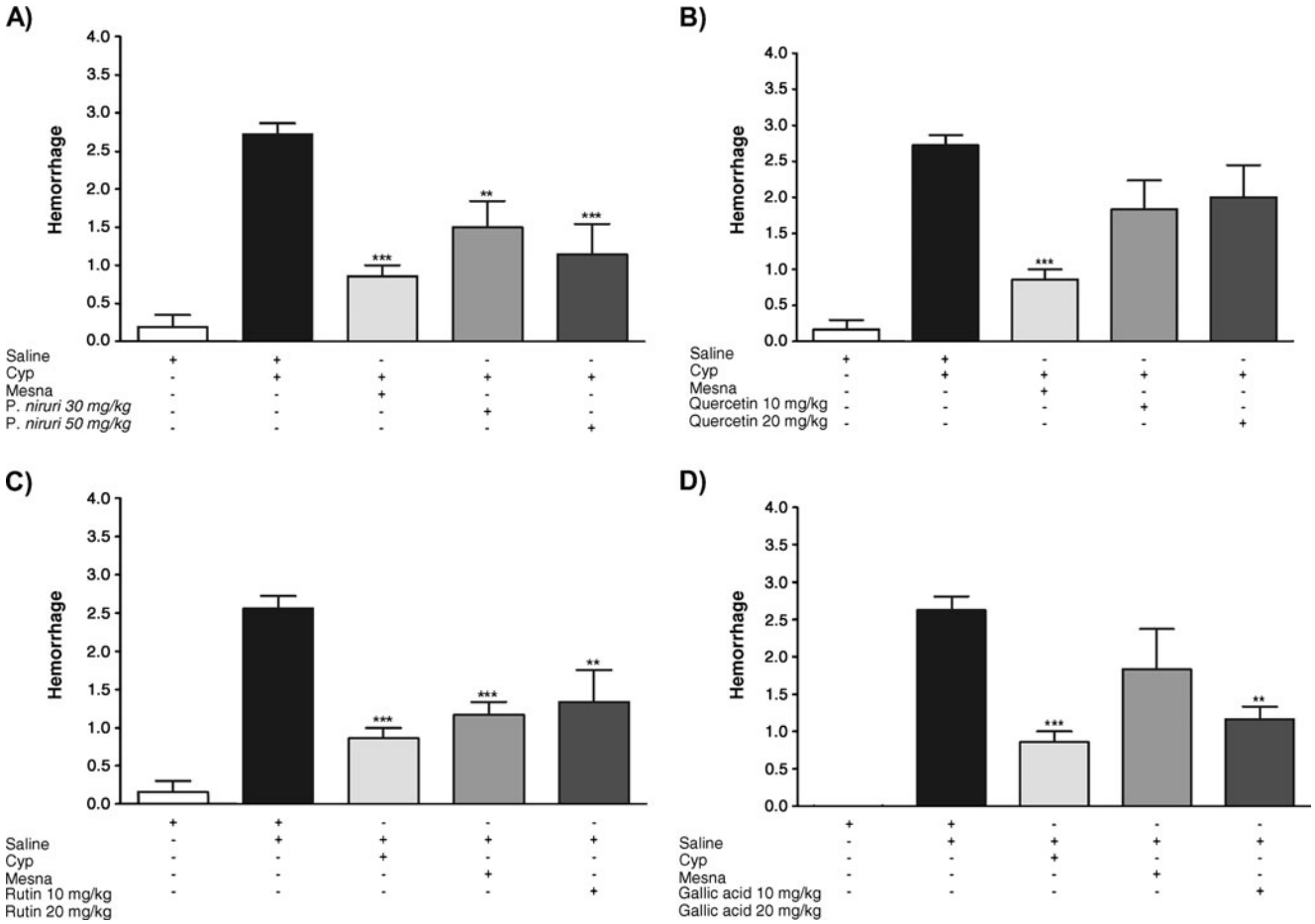

Fig. 4 Effect of treatment with Mesna, *P. niruri* extract (**a**), quercetin (**b**), rutin (**c**), or gallic acid (**d**) on hemorrhage. Each *column* represents the mean of six to eight animals and the *vertical lines* show the SEM. *Asterisks* denote the significance levels in comparison to control values: $^{**}P<0.01$; $^{***}P<0.001$

with Mesna. The percentages of inhibition were 45±12% and 58±14% (*P. niruri* extract, 30 and 50 mg/kg; Fig. 4a), and 54±6% and 48±16% (rutin, 10 and 20 mg/kg; Fig. 4c), respectively. On the other hand, only the 20-mg/kg dose of gallic acid significantly inhibited the bladder hemorrhage caused by CYP (55±6%; Fig. 4d), and no significant difference was observed when compared with Mesna administration. Nevertheless, the oral treatment with quercetin (either 10 or 20 mg/kg; Fig. 4b) did not significantly affect this parameter.

Histological analysis revealed that treatment with Mesna and *P. niruni* extract produced a general reduction of HC characteristics. In the CYP group, there were marked changes in all layers of the bladder. The epithelium was thin and sometimes denuded. The submucosa was inflamed and enlarged and presented severe hemorrhagic cystitis with edema, transmural hemorrhage, and fibrin deposits. Mesna-treated animals had characteristics of the normal bladder congestion. The epithelium was found to be thick with normal-size nuclei and with mild abnormal attenuation. The submucosa presented mild edema and no inflammation. The muscularis had normal appearance. The animals treated with gallic acid presented mucosal erosion, submucosal mild edema, hemorrhage with neutrophil infiltration, and fibrin deposits. Alternatively, the animals treated with *P. niruri* extract showed mild submucosal edema, minimal hemorrhage, few ulcerations, and minimal inflammation. Finally, rutin and quercetin groups presented submucosal mild edema, transmural hemorrhage, multiple ulcerations, fibrin deposition, and neutrophil infiltration (Fig. 5a–g).

The administration of CYP (300 mg/kg, IP) also resulted in increased lipid peroxidation, as indicated by increased liver MDA levels. The oral administration of Mesna (80 mg/kg, 30 min prior and 160 mg/kg, 2 h after CYP) produced a significant reduction of MDA levels (52±3%; Fig. 6a–d). Increased liver MDA levels were also diminished by *P. niruri* hydroalcoholic extract (30 and 50 mg/kg, PO, 30 min prior and 2 h after CYP), in 28±6% and 31±4%, respectively (Fig. 6a). In this case, the grade of inhibition was significantly different in relation to the Mesna group. The isolated compound quercetin (10 and

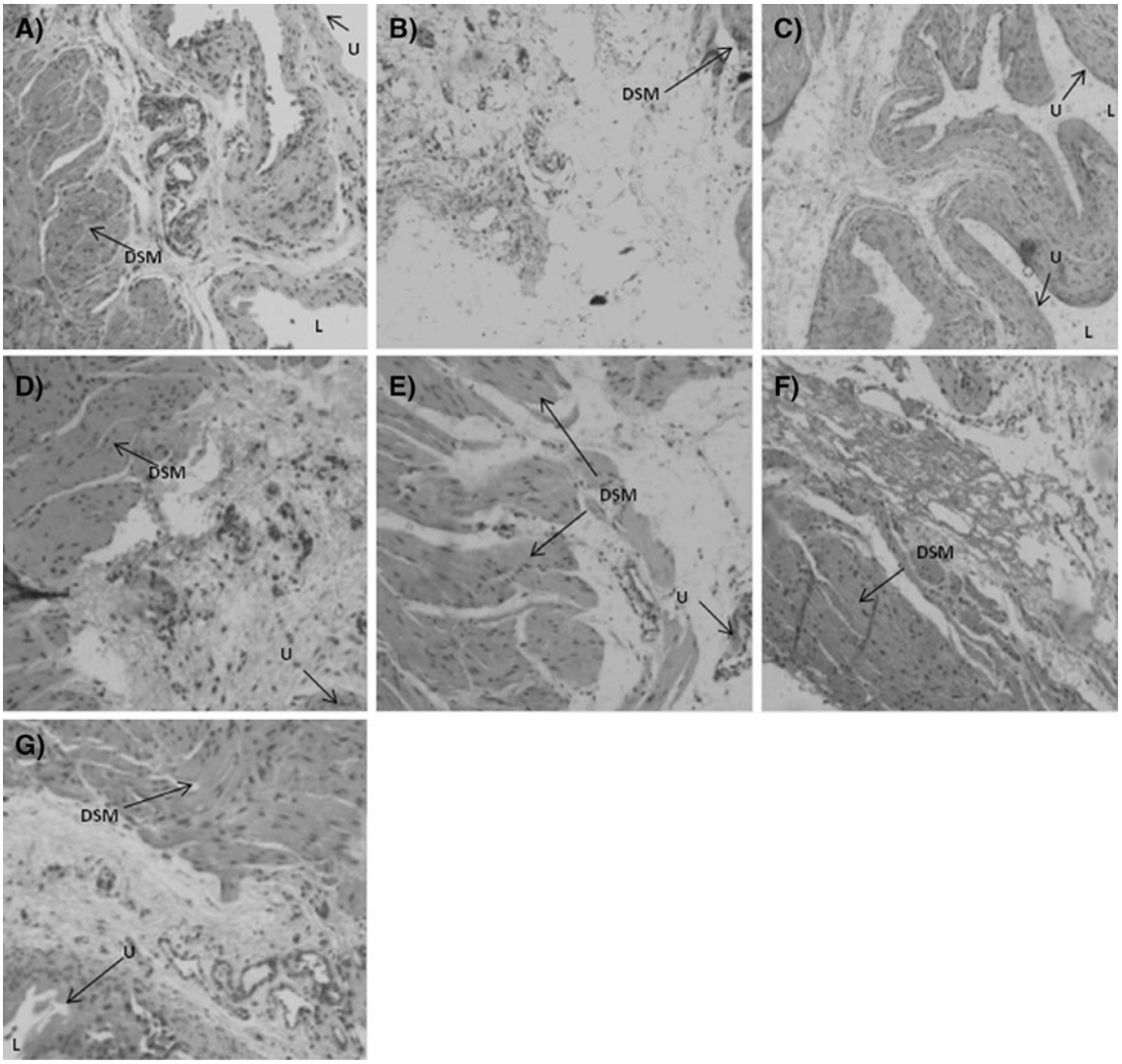

Fig. 5 Representative bladder walls images in histological cross-section. **a** Saline: bladder with normal appearance, with absence of edema, hemorrhage, or inflammation. **b** CYP: severe hemorrhagic cystitis with edema, transmural hemorrhage, mucosal ulceration, and inflammation. **c** Mesna group: near-normal appearance. **d** Gallic acid: mucosal erosion, submucosal edema, and hemorrhage. **e** *P. niruri*: mild submucosal edema, minimal hemorrhage, few ulcerations, and no inflammation. **f** Rutin: submucosal edema, hemorrhage, and erosions. **g** Quercetin: edema, transmural hemorrhage, mucosal erosion, and fibrin deposition. The *arrows* indicate: urothelium (*U*); detrusor smooth muscle (*DSM*), and lumen (*L*). All *panels*, hematoxylin–eosin stain. Original magnification ×40 in all panels

20 mg/kg) also significantly reduced the levels of MDA in CYP-treated mice (32±6% and 25±5%, respectively; Fig. 6b), presenting a significant difference when compared with Mesna. Furthermore, the liver lipid peroxidation induced by CYP was significantly reduced by rutin treatment (20 mg/kg), with an inhibition of 34±7%, whereas the dose of 10 mg/kg was found ineffective (Fig. 6c). There was no significant difference between the effects of Mesna and that seen for the dose of 20 mg/kg. The administration of gallic acid (10 and 20 mg/kg) also largely prevented CYP-induced increase of MDA levels, with inhibitions of 43±4% and 47±4%, respectively (Fig. 6d), showing, in these two doses, no significant difference when compared with effect of treatment with Mesna.

Discussion

The use of CYP is frequently associated with serious side effects, although the most clinically relevant event is the urological toxicity, especially manifested as HC. This is not a direct effect of CYP but is related to the formation of 4-hydroxy metabolites, mainly acrolein (Topal et al. 2005; Lawson et al. 2008). Despite that the prophylactic administration of Mesna has been shown to be a useful measure

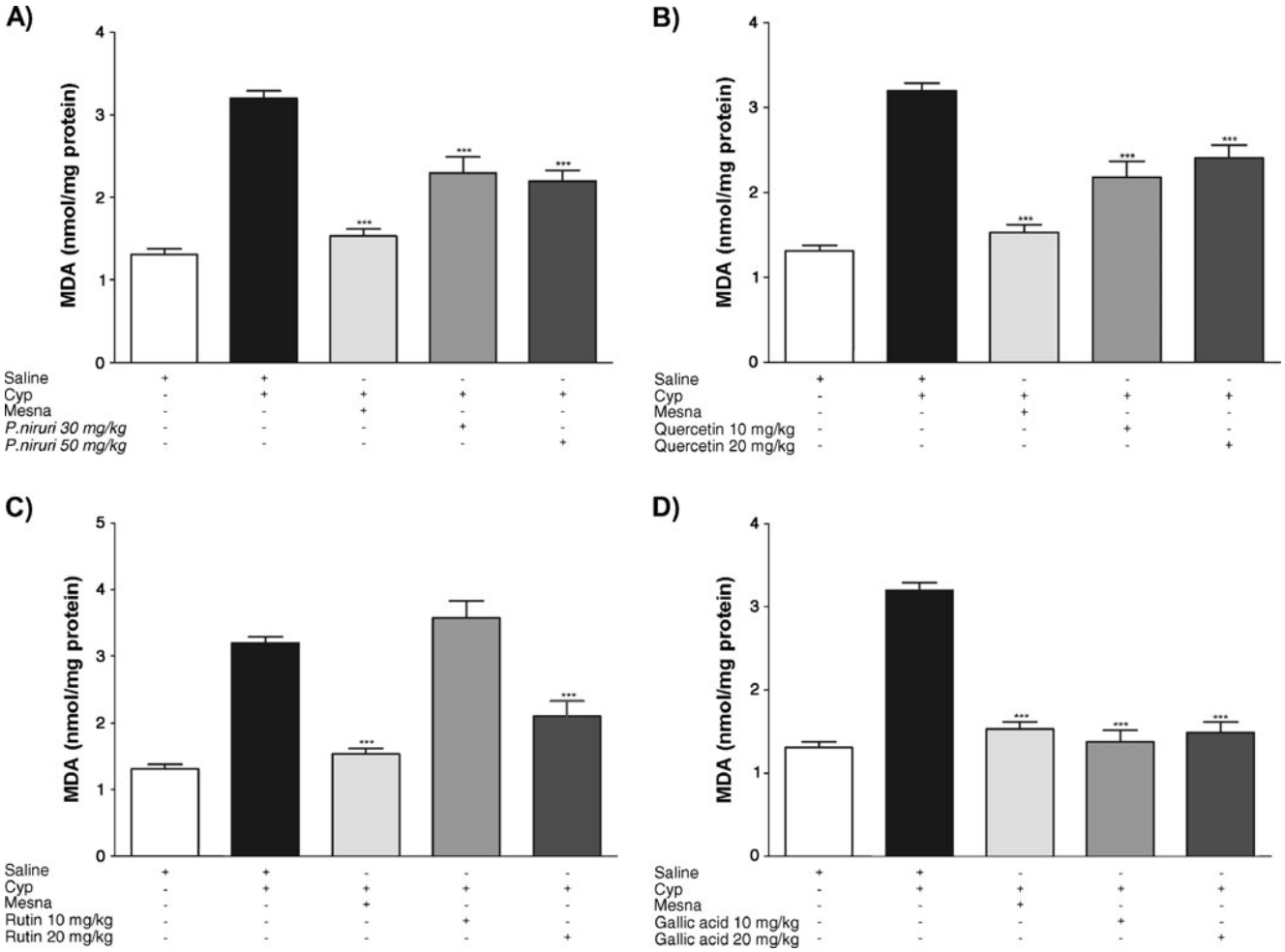

Fig. 6 Effect of treatment with Mesna, *P. niruri* extract (**a**), quercetin (**b**), rutin (**c**), or gallic acid (**d**) on liver MDA levels. Each *column* represents the mean of eight animals and the *vertical lines* show the SEM. *Asterisks* denote the significance levels in comparison to CYP group values: ***$P<0.001$

for preventing HC, a great percentage of patients under treatment with CYP will develop HC (Chow et al. 2006; Monach et al. 2010). Furthermore, the treatment with Mesna, especially in children and young adults, might be associated with remarkable side effects, such as hypersensitivity-like cutaneous and systemic reactions (Khaw et al. 2007). Therefore, new and safer strategies are required for managing this condition.

P. niruri has been used for a long time in folk medicine for the treatment of several illness conditions, being recognized for its beneficial effects on the genitourinary system. The phytochemical studies on this plant revealed the presence of several compounds, such as flavonoids, alkaloids, lignans, and terpenes, which are recognized for their anti-inflammatory and analgesic properties (Calixto et al. 1998; Bagalkotkar et al. 2006). This study evaluated the effects of treatment with the hydroalcoholic extract from *P. niruri* and some of the purified compounds, namely quercetin, rutin, and gallic acid on the nociceptive and inflammatory changes evoked by CYP in mice. We have also investigated to what extent these agents might prevent hepatic free radical generation associated to CYP.

The model of CYP-induced HC was firstly developed in rats by Botta et al. (1973). Later, this model was validated in mice by several research groups as a useful experimental approach for evaluating inflammatory and visceral nociceptive changes associated to CYP (Olivar and Laird 1999; Lima-Junior et al. 2007; Wantuch et al. 2007; Tripathi and Jena 2009). Our study revealed the beneficial effects for *P. niruri* extract and the isolated compounds quercetin, gallic acid, and rutin in preventing the inflammatory and nociceptive alterations evoked by CYP administration in mice, when compared with those seen with Mesna.

Urotoxic effects of CYP are largely associated with marked painful alterations in clinics. Acrolein is known for its ability to activate sensory neurons, causing severe acute pain and neuro-inflammation, probably by stimulating glutamate *N*-methyl-D-aspartate and tachykinin NK_1 recep-

tors (Meen et al. 2002; Lima-Junior et al. 2007). The pro-algesic actions of acrolein have been recently associated with TRPA1 receptor activation, which belongs to the transient receptor ion channel superfamily (Bautista et al. 2006). Noteworthy, TRPA1 is largely responsive to chemical noxious agents, inducing the activation of several intracellular pathways implicated in pain (Basbaum et al. 2009). Confirming previous literature data (Olivar and Laird 1999; Wantuch et al. 2007), we demonstrate that single IP injection of CYP resulted in a marked spontaneous visceral pain-like behavior in mice. As expected, the detoxifying agent Mesna was effective to prevent the spontaneous nociception evoked by CYP. Of high interest, pain-like crises induced by CYP were consistently prevented by oral treatment with the hydroalcoholic extract from *P. niruri*. In fact, previous studies revealed marked analgesic effects for this plant extract in several models of nociception in mice (Santos et al. 1994; Santos et al. 1995; Martini et al. 2000). We might suggest that antinociceptive actions of *P. niruri* extract are mainly related to the presence of compounds quercetin and gallic acid. This proposition is based on the findings showing that both compounds displayed marked reductions of visceral pain-like alterations evoked by CYP, whereas rutin was devoid of analgesic actions. Remarkably, neither Mesna or *P. niruri* extract, nor isolated compounds, induced any significant alteration of locomotor activity when assessed in the open-field test, discarding possible unspecific side effects. Outstandingly, quercetin was able to inhibit nociception in a host of rodent pain models, including the visceral nociception elicited by acetic acid (Calixto et al. 1998; Comalada et al. 2005).

CYP-induced HC has been associated with the activation of transcriptional factors, such as NF–κB and AP-1, and the production of various inflammatory mediators, including cytokines, free radicals, prostanoids, and nitric oxide (Kiuchi et al. 2009; Tripathi and Jena 2009). The activation of the aforesaid pathways leads to the development of marked inflammatory signals such as edema and hemorrhage. A previous study demonstrated that a combination of quercetin and Mesna produced a pronounced inhibition of HC induced by CYP in rats (Ozcan et al. 2005). In the present study, we reported that oral administration of *P. niruri* extract or the compounds rutin and gallic acid strikingly prevented the inflammatory signs associated to CYP in mice. Of note, gallic acid practically abolished the edema when given orally at the dose of 20 mg/kg, being even more effective than the reference drug Mesna. Conversely, the compound quercetin failed to significantly affect the hemorrhage in CYP-injected mice, although it reduced the bladder edema. It is tempting to suggest that increased doses of quercetin might produce significant alleviation of CYP-induced hemorrhage. Noteworthy, 4-*O*-methylgallic acid, the major metabolite of gallic acid, consistently reduced the production of nitric oxide, prostaglandin E_2, and inflammatory cytokines and prevented the activation of NF–κB in lipopolysaccharide-stimulated macrophages in vitro (Na et al. 2006). Furthermore, Park et al. (2008) demonstrated that a series of flavonoids, including quercetin and rutin, decreased the expression of pro-inflammatory cytokines and suppressed NF–κB activation in mast cells stimulated by phorbol ester plus A23187 (a calcium ionophore). One might suggest that interference with these inflammatory pathways might mediate the beneficial effects of *P. niruri* extract and isolated compound in CYP mouse model of cystitis.

The toxic aldehyde metabolite of CYP acrolein is formed by means of hepatic microsomal enzymatic hydroxylation (Oter et al. 2004; Topal et al. 2005). Therefore, the biotransformation of CYP can also be associated to liver damage. In fact, acrolein induces DNA damage of normal cells, via oxidative stress, leading to toxicological effects on several target organs, besides the urinary bladder (Tripathi and Jena 2009). Considering the potential antioxidant effects of *P. niruri* and its active compounds (Manjrekar et al. 2008), we decided to investigate the effects of these strategies on the liver lipid peroxidation following CYP administration in mice. Our data revealed that *P. niruri* hydroalcoholic extract or the isolated compounds quercetin, rutin, and gallic acid produced a significant inhibition of the liver peroxidation by product MDA. Outstandingly, the administration of 20 mg/kg of gallic acid inhibited this toxicological parameter, in a manner similar to that observed for Mesna. Therefore, *P. niruri* and its isolated compounds not only prevented urinary bladder damage but were also able to reduce liver damage associated to CYP administration.

Although quercetin displayed carcinogenic activity in the kidney of male rats when administered in the feed for 2-years (Dunnick and Hailey 1992), it is well known that this compound has protective effects in several in vivo models of oxidative stress (Hapner et al. 2010; Li et al. 2010; Mi et al. 2010). Furthermore, a recent paper has shown that quercetin reduced cisplatin-induced nephrotoxicity in rats, without compromising its anti-tumor activity (Sanchez-Gonzalez et al. 2011). It is worthy to note that we have not observed any signs of nephropathy after the treatments with *P. niruri* or with the isolated compounds.

Altogether, the pre-clinical findings presented herein point out *P. niruri* extract and the purified compounds quercetin, rutin, and gallic acid, as promising strategies for controlling the toxic effects caused by CYP, especially in the eminency of Mesna side effects. Among all the evaluated strategies, gallic acid sounds to be the most encouraging molecule, as it was highly effective against nociceptive, inflammatory, and oxidative effects elicited by CYP.

Acknowledgments V.T.B. is an undergraduate pharmacy student receiving grants from Ministério da Agricultura, Pesca e Abastecimento, Brazil.

References

Abraham P, Sugumar E (2008) Enhanced PON1 activity in the kidneys of cyclophosphamide treated rats may play a protective role as an antioxidant against cyclophosphamide induced oxidative stress. Arch Toxicol 82:237–238

Arjumand W, Seth A, Sultana S (2011) Rutin attenuates cisplatin induced renal inflammation and apoptosis by reducing NFkappaB, TNF-alpha and caspase-3 expression in Wistar rats. Food Chem Toxicol

Bagalkotkar G, Sagineedu SR, Saad MS, Stanslas J (2006) Phytochemicals from *Phyllanthus niruri* Linn. and their pharmacological properties: a review. J Pharm Pharmacol 58:1559–1570

Basbaum AI, Bautista DM, Scherrer G, Julius D (2009) Cellular and molecular mechanisms of pain. Cell 139:267–284

Bautista DM, Jordt SE, Nikai T, Tsuruda PR, Read AJ, Poblete J, Yamoah EN, Basbaum AI, Julius D (2006) TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. Cell 124:1269–1282

Bhattacharjee R, Sil PC (2006) The protein fraction of *Phyllanthus niruri* plays a protective role against acetaminophen induced hepatic disorder via its antioxidant properties. Phytother Res 20:595–601

Botta JA Jr, Nelson LW, Weikel JH Jr (1973) Acetylcysteine in the prevention of cyclophosphamide-induced cystitis in rats. J Natl Cancer Inst 51:1051–1058

Calixto JB, Santos AR, Cechinel Filho V, Yunes RA (1998) A review of the plants of the genus *Phyllanthus*: their chemistry, pharmacology, and therapeutic potential. Med Res Rev 18:225–258

Cechinel Filho V, Santos AR, De Campos RO, Miguel OG, Yunes RA, Ferrari F, Messana I, Calixto JB (1996) Chemical and pharmacological studies of *Phyllanthus caroliniensis* in mice. J Pharm Pharmacol 48:1231–1236

Chatterjee M, Sarkar K, Sil PC (2006) Herbal (*Phyllanthus niruri*) protein isolate protects liver from nimesulide induced oxidative stress. Pathophysiology 13:95–102

Chauhan JS, Sultan M, Srivastava SK (1977) Two new glycoflavones from the roots of *Phyllanthus niruri*. Plant Med 32:217–222

Cheuk DK, Lee TL, Chiang AK, Ha SY, Lau YL, Chan GC (2007) Risk factors and treatment of hemorrhagic cystitis in children who underwent hematopoietic stem cell transplantation. Transpl Int 20:73–81

Chow YC, Yang S, Huang CJ, Tzen CY, Huang PL, Su YH, Wang PS (2006) Epinephrine promotes hemostasis in rats with cyclophosphamide-induced hemorrhagic cystitis. Urology 67:636–641

Comalada M, Camuesco D, Sierra S, Ballester I, Xaus J, Galvez J, Zarzuelo A (2005) In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF–kappaB pathway. Eur J Immunol 35:584–592

Dunnick JK, Hailey JR (1992) Toxicity and carcinogenicity studies of quercetin, a natural component of foods. Fundam Appl Toxicol 19:423–431

Gray KJ, Engelmann UH, Johnson EH, Fishman IJ (1986) Evaluation of misoprostol cytoprotection of the bladder with cyclophosphamide (Cytoxan) therapy. J Urol 136:497–500

Hapner CD, Deuster P, Chen Y (2010) Inhibition of oxidative hemolysis by quercetin, but not other antioxidants. Chem Biol Interact 186:275–279

Khaw SL, Downie PA, Waters KD, Ashley DM, Heath JA (2007) Adverse hypersensitivity reactions to Mesna as adjunctive therapy for cyclophosphamide. Pediatr Blood Cancer 49:341–343

Kim JH, Kang MJ, Choi HN, Jeong SM, Lee YM, Kim JI (2011) Quercetin attenuates fasting and postprandial hyperglycemia in animal models of diabetes mellitus. Nutr Res Pract 5:107–111

Kiuchi H, Takao T, Yamamoto K, Nakayama J, Miyagawa Y, Tsujimura A, Nonomura N, Okuyama A (2009) Sesquiterpene lactone parthenolide ameliorates bladder inflammation and bladder overactivity in cyclophosphamide induced rat cystitis model by inhibiting nuclear factor-kappaB phosphorylation. J Urol 181:2339–2348

Kroes BH, van den Berg AJ, Quarles van Ufford HC, van Dijk H, Labadie RP (1992) Anti-inflammatory activity of gallic acid. Plant Med 58:499–504

Latha RC, Daisy P (2011) Insulin-secretagogue, antihyperlipidemic and other protective effects of gallic acid isolated from *Terminalia bellerica* Roxb. in streptozotocin-induced diabetic rats. Chem Biol Interact 189:112–118

Lawson M, Vasilaras A, De Vries A, Mactaggart P, Nicol D (2008) Urological implications of cyclophosphamide and ifosfamide. Scand J Urol Nephrol 42:309–317

Li G, Ma A, Shi W, Zhong X (2010) Quercetin protects hamster spermatogenic cells from oxidative damage induced by diethylstilboestrol. Andrologia 42:285–290

Lima-Junior RC, Sousa DI, Brito GA, Cunha GM, Chaves MH, Rao VS, Santos FA (2007) Modulation of acute visceral nociception and bladder inflammation by plant triterpene, alpha, beta-amyrin in a mouse model of cystitis: role of tachykinin NK(1)-receptors, and K(+)(ATP) channels. Inflamm Res 56:487–494

Manjrekar AP, Jisha V, Bag PP, Adhikary B, Pai MM, Hegde A, Nandini M (2008) Effect of *Phyllanthus niruri* Linn. treatment on liver, kidney and testes in CCl4 induced hepatotoxic rats. Indian J Exp Biol 46:514–520

Martini LH, Souza CR, Marques PB, Calixto JB, Yunes RA, Souza DO (2000) Compounds extracted from *Phyllantus* and *Jatropha elliptica* inhibit the binding of [3H]glutamate and [3H]GMP-PNP in rat cerebral cortex membrane. Neurochem Res 25:211–215

Meen M, Coudore-Civiale MA, Parry L, Eschalier A, Boucher M (2002) Involvement of *N*-methyl-D-aspartate receptors in nociception in the cyclophosphamide-induced vesical pain model in the conscious rat. Eur J Pain 6:307–314

Mi Y, Zhang C, Li C, Taneda S, Watanabe G, Suzuki AK, Taya K (2010) Quercetin attenuates oxidative damage induced by treatment of embryonic chicken spermatogonial cells with 4-nitro-3-phenylphenol in diesel exhaust particles. Biosci Biotechnol Biochem 74:934–938

Monach PA, Arnold LM, Merkel PA (2010) Incidence and prevention of bladder toxicity from cyclophosphamide in the treatment of rheumatic diseases: a data-driven review. Arthritis Rheum 62:9–21

Morais MC, Luqman S, Kondratyuk TP, Petronio MS, Regasini LO, Silva DH, Bolzani VS, Soares CP, Pezzuto JM (2010) Suppression of TNF-alpha induced NFkappaB activity by gallic acid and its semi-synthetic esters: possible role in cancer chemoprevention. Nat Prod Res 24:1758–1765

Na HJ, Lee G, Oh HY, Jeon KS, Kwon HJ, Ha KS, Lee H, Kwon YG, Kim YM (2006) 4-O-Methylgallic acid suppresses inflammation-associated gene expression by inhibition of redox-based NF-kappaB activation. Int Immunopharmacol 6:1597–1608

Oboh G, Akomolafe TL, Adefegha SA, Adetuyi AO (2011) Inhibition of cyclophosphamide-induced oxidative stress in rat brain by polar and non-polar extracts of Annatto (*Bixa orellana*) seeds. Exp Toxicol Pathol 63:257–262

Olivar T, Laird JM (1999) Cyclophosphamide cystitis in mice: behavioural characterisation and correlation with bladder inflammation. Eur J Pain 3:141–149

Oter S, Korkmaz A, Oztas E, Yildirim I, Topal T, Bilgic H (2004) Inducible nitric oxide synthase inhibition in cyclophosphamide induced hemorrhagic cystitis in rats. Urol Res 32:185–189

Ozcan A, Korkmaz A, Oter S, Coskun O (2005) Contribution of flavonoid antioxidants to the preventive effect of Mesna in cyclophosphamide-induced cystitis in rats. Arch Toxicol 79:461–465

Park HH, Lee S, Son HY, Park SB, Kim MS, Choi EJ, Singh TS, Ha JH, Lee MG, Kim JE, Hyun MC, Kwon TK, Kim YH, Kim SH (2008) Flavonoids inhibit histamine release and expression of proinflammatory cytokines in mast cells. Arch Pharm Res 31:1303–1311

Saija A, Tomaino A, Trombetta D, Pellegrino ML, Tita B, Messina C, Bonina FP, Rocco C, Nicolosi G, Castelli F (2003) 'In vitro' antioxidant and photoprotective properties and interaction with model membranes of three new quercetin esters. Eur J Pharm Biopharm 56:167–174

Sanchez-Gonzalez PD, Lopez-Hernandez FJ, Perez-Barriocanal F, Morales AI, Lopez-Novoa JM (2011) Quercetin reduces cisplatin nephrotoxicity in rats without compromising its anti-tumour activity. Nephrol Dial Transplant

Santos AR, Filho VC, Niero R, Viana AM, Moreno FN, Campos MM, Yunes RA, Calixto JB (1994) Analgesic effects of callus culture extracts from selected species of *Phyllanthus* in mice. J Pharm Pharmacol 46:755–759

Santos ASR, Cechinel Filho V, Yunes RA, Calixto JB (1995) Further studies on the antinociceptive action of the hydroalcoholic extracts from plants of the genus *Phyllanthus*. J Pharm Pharmacol 47:66–71

Selloum L, Bouriche H, Tigrine C, Boudoukha C (2003) Anti-inflammatory effect of rutin on rat paw oedema, and on neutrophils chemotaxis and degranulation. Exp Toxicol Pathol 54:313–318

Thyagarajan SP, Thiruneelakantan K, Subramanian S, Sundaravelu T (1982) In vitro inactivation of HBsAg by Eclipta alba Hassk and *Phyllanthus niruri* Linn. Indian J Med Res 76(Suppl):124–130

Topal T, Oztas Y, Korkmaz A, Sadir S, Oter S, Coskun O, Bilgic H (2005) Melatonin ameliorates bladder damage induced by cyclophosphamide in rats. J Pineal Res 38:272–277

Tripathi DN, Jena GB (2009) Intervention of astaxanthin against cyclophosphamide-induced oxidative stress and DNA damage: a study in mice. Chem Biol Interact 180:398–406

Tuzmen N, Candan N, Kaya E, Demiryas N (2008) Biochemical effects of chlorpyrifos and deltamethrin on altered antioxidative defense mechanisms and lipid peroxidation in rat liver. Cell Biochem Funct 26:119–124

Venkateswaran PS, Millman I, Blumberg BS (1987) Effects of an extract from *Phyllanthus niruri* on hepatitis B and woodchuck hepatitis viruses: in vitro and in vivo studies. Proc Natl Acad Sci USA 84:274–278

Wantuch C, Piesla M, Leventhal L (2007) Pharmacological validation of a model of cystitis pain in the mouse. Neurosci Lett 421:250–252

Wong TM, Yeo W, Chan LW, Mok TS (2000) Hemorrhagic pyelitis, ureteritis, and cystitis secondary to cyclophosphamide: case report and review of the literature. Gynecol Oncol 76:223–225