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Full Length Research Paper

Do diosgenin ameliorate urinary bladder toxic effect of cyclophosphamide and buthionine sulfoximine in experimental animal models?

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Urotoxicity is a troublesome complication associated with cylophosphamide (CP) and L-buthionine-SR-sulfoximine (BSO) treatment in chemotherapy. With this concern in mind, this present study investigated the potential effects of diosgenin for the first time on urotoxicity induced by acute CP and BSO doses using a Swiss albino mouse model. Toxicity modulation was evaluated through measuring lipid peroxidation (LPO) and anti-oxidants in urinary bladder. The findings reveal that the diosgenin exerted a protective effect not only on LPO but also on enzymatic anti-oxidants. When compared to the controls, the CP-treated animals underwent significant decrease in the glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GP) and catalase (CAT) activities. The level of reduced glutathione (GSH) was also decreased with an increase in LPO in the CP-treated animals. BSO treatment exerted an additive toxic effect in the CP-treated animals. Interestingly, pre-treatment with the diosgenin restored the activities of all enzymes back to normal levels and to exhibit an overall protective effect on the CP and BSO induced toxicities in urinary bladder. The restoration of GSH through the treatment with the diosgenin can play an important role in reversing CP-induced apoptosis and free radical mediated LPO.

Key words: Diosgenin, cyclophosphamide, L-buthionine-SR-sulfoximine, urinary bladder anti-oxidants.

INTRODUCTION

Cyclophosphamide (CP) is one of the most popular alkylating anticancer drugs in spite of its toxic side effects including immunotoxicity, hematotoxicity and mutagenicity. CP is an anti-neoplastic agent with activity against a variety of human tumours. It is commonly used as an anti-neoplastic agent for the treatment of various forms of cancer and as an immunosuppressive drug for certain non-neoplastic conditions before organ transplantation. Several previous studies on murine models have also showed that the administration of CP retards the progression of kidney disease (Hengstler et al., 1997). This agent has similarly been used extensively for the treatment of diffuse proliferative glomerulonephritis in patients with renal lupus (Hengstler et al., 1997).

L-Buthionine-Sulfoximine (BSO) is a sulfoximine used in chemotherapy which irreversibly inhibits gamma-glutamylcysteine synthetase, thereby depleting cells of glutathione, a metabolite that plays a critical role in protecting cells against oxidative stress and resulting in free radical-induced apoptosis.

Despite being effective in the control of various malignant conditions, CP and BSO treatment have been occasionally reported to be associated with serious side

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Abbreviations: **CP**, Cylophosphamide; **BSO**, L-buthionine-SR-sulfoximine; **LPO**, lipid peroxidation; **GR**, glutathione reductase; **GST**, glutathione S-transferase; **GP**, glutathione peroxidase; **CAT**, catalase; **GSH**, reduced glutathione.

#These author contributed equally to this work.

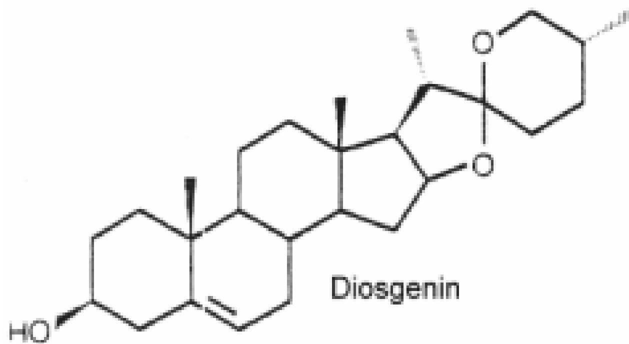

Figure 1. Structure of diosgenin.

effects. The urological side effects of CP are major limiting factor for its use. These side effects include transient irritative voiding symptoms including dypsia, hemorrhagic cystitis, bladder fibrosis, necrosis, contracture and vesicometral flux (Levine and Richie, 1989). Several reports have also linked cases of lung toxicity to this medication (Patel et al., 1984).

Similarly, several studies have demonstrated that bladder inflammation induced by CP in rats and mice increased transcript and protein expression in the urinary bladder of several cytokines including IL-6 (Malley and Vizzard, 2002). CP is bioactivated to its reactive electrophilic metabolites through the cellular cytochrome P450 enzyme system, resulting in their ongoing production and slow accumulation in the plasma, causing severe cytotoxic effects in non target tissues (Hamden et al., 2009).

Other studies demonstrated that CP and BSO treatment also results in the production of reactive oxygen species (ROS), cause peroxidative damage to urinary bladder and other vital organs (Patel, 1987). In fact, free radical generation is one of the mechanisms by which CP and its derivatives exert their toxic effects in different tissues. An overall decrease in the GSH content has often been reported in various tissues as a result of CP treatment (Haque et al., 2003; Patel et al., 1984). The role of tissue antioxidants becomes important in the prevention of such peroxidative damage induced by CP treatment. A number of natural products and synthetic compounds have been shown to reduce CP toxicity mainly due to their antioxidant action (Haque, 2003; Abd-Allah, 2005).

The reduction in the GSH (reduced glutathione) levels as a result of CP treatment has also been reported in urinary bladder (Haque et al., 2003). In fact, the reactive metabolites of CP are responsible for its various toxic as well as therapeutic actions (Ahmed and Hombal, 1984). Among these metabolites, phosphoramide mustard is specifically associated with the immunosuppressive action of CP (Ahmed et al., 1984). CP-induced immunosuppression is reported to prompt various types of infection (Angulo et al., 2002). Some of the infectious agents have GSH depleting effects (Hung and Wang, 2004). CP treatment may therefore decrease the GSH content itself but the associated secondary infections are likely to cause an additional decrease in the GSH level. For that reason, a patient undergoing CP chemotherapy needs excessive supply of GSH restoring anti-oxidants or compounds that induce GSH production.

A number of GSH-inducing compounds have been found to be effective in reducing CP toxicity in animals (Manesh and Kuttan, 2005). Several studies reported that many plants extract have shown protective and/or restorative effect on CP-induced decrease in GSH (Haque et al., 2003). *Trigonella foenum graecum* commonly called Fenugreek, is a leguminous plant native to many Asian, middle Eastern and European countries (Chevallier, 2000). It has a long history as both a culinary and to medication. The seeds and leaves of Fenugreek are edible and are used as condiments and as Ayurvedic medicine in the Indian subcontinent to treat diabetes, high cholesterol, wounds, inflammation and gastro-intestinal ailments (Chevallier., 2000).

Among bioactive compounds isolated from Fenugreek seeds are protodioscin, trigoneoside, yamogenin and diosgenin (Murakami et al., 2000; Yoshikawa et al., 1997). Of particular interest, diosgenin [(25R)-5-spirosten-3h-ol], the major steroidal saponin constituent of Fenugreek seeds, is a precursor of steroid hormones such as progesterone, and an anti-inflammatory steroids such as cortisone (Norton. 1998). Diosgenin (such as wild Mexican yam and the genus Agave (*Agave americana* L)) have recently received special attention, for it may inhibit both the initiation and promotion steps of carcinogenesis *in vitro*. It is also the active component responsible for the anti-diabetic and hypocholesterolemic activity of Fenugreek (Xue et al., 2007).

Considering the serious concerns widely voiced about the undesirable side effects associated with CP and BSO treatment, this present study was undertaken to explore the potential gain effects that diosgenin might offer to alleviate this disturbing problem. It aimed to investigate, for the first time, the effects of purified diosgenin in the restoration of anti-oxidants and reduction of lipid peroxidation (LPO) in the urinary bladder of CP-treated animals that are pre-disposed or concomitantly exposed to GSH reducing agent either through infection or use of antibiotics.

MATERIALS AND METHODS

Diosgenin was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Figure 1 shows their chemical structure. Cyclophosphamide monohydrate [2-[bis-(2-chloroethyl) amino] tetrahydro- 2H-1,3,2-oxazaphosphorine 2-oxide monohydrate], CAS 6055-19-2 and BSO [L-buthionine-SR-sulfoximine] and CAS 5072-26-4 were purchased from Sigma–Aldrich Co., St. Louis, MO, USA.

The experiments (Figure 2) were performed on male Swiss albino mice (25 ± 2 g) provided by the animal service at the Pasteur Institute of Tunis, Tunisia. The study was approved by the Institutional Animal Ethics Committee (IAEC). The animals were

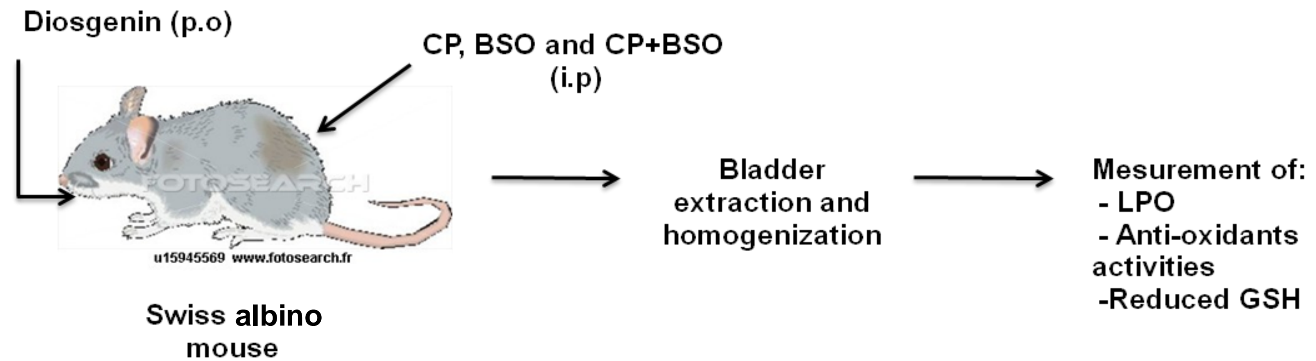

Figure 2. Experimental protocol.

bred and maintained under standard laboratory conditions (temperature 25 ± 2°C, photoperiod of 12 h). Commercial pellet diet and water were given *ad libitum*.

Dosage and experimental groups

BSO, CP and diosgenin were suspended in normal saline. The animals were divided into seven groups, namely Groups I to VII with each group consisting of six animals. Group I (control) were control mice that were administered normal saline p.o. for 10 days and a single i.p (intra-peritoneal injection) injection on the 10th day. Group II (BSO) were mice that received BSO (500 mg/kg body wt) in which a single i.p. injection was administered on the 10th day. Group III (CP) were mice that received CP (50 mg/kg body wt) and a single i.p. dose on the 10th day of treatment. Group IV (BSO + CP) were animals that were administered BSO i.p. 5 h before CP administration. Group V (CP + diosgenin) were animals that were administered diosgenin for 10 days along with a single i.p. injection of CP on the 10th day. Group VI (BSO + diosgenin) were animals that were given diosgenin treatment (100 mg/kg body wt) p.o. for 10 days and a single i.p. injection of BSO on the 10th day along with the extract. Group VII (BSO + CP + diosgenin) were animals that were administered diosgenin for 10 days and CP and BSO on the 10th day. Dosing was performed in such a way that all of the animals could be sacrificed on the same day, that is, day 11. The selection of BSO and CP doses was based on pilot experiments that involved the assay of a wide range of doses and on data provided from previously published reports (Haque et al., 2003; Bin-Hafeez et al., 2003).

Biochemical investigations

Upon the completion of the treatment, the animals were sacrificed under mild anesthesia (methyl isopropyl ether) and their bladders were removed. The bladder tissue was homogenized in chilled phosphate buffer (0.1 M, pH 7.4) using a Potter homogenizer. The homogenate was centrifuged at 10,500g for 30 min at 4°C to obtain the post-mitochondrial supernatant (PMS) which was used for the biochemical measurements as described below.

Lipid peroxidation

LPO was measured using the procedure of Mihara and Asakura, (1978). The assay mixture consisted of 0.67% thiobarbituric acid, TBA (Central Pharmacy, Tunis, Tunisia), 10 mM butylated hydroxy toluene, BHT (Amresco Inc., Solon, OH, USA), 1% ortho-phosphoric acid (Central Pharmacy, Tunis, Tunisia), and tissue homogenate in a total volume of 3 ml. The rate of LPO was expressed as nmol of TBA reactive substances (TBARS) formed/h/g of tissue using molecular extinction coefficient epsilon (є) of 1.56 x 10^5 M^{-1} cm^{-1}.

Measurement of GSH

GSH content was measured in the PMS of urinary bladder using the method of Haque et al. (2003). PMS (1ml) was precipitated with 1ml of 4% sulfosalicylic acid (Amresco). The samples were incubated at 4°C for 1 h and then centrifuged for 15 min at 1200 g and 4°C. The assay mixture consisted of 0.2 ml of filtered aliquot, 2.6 ml of sodium phosphate buffer (0.1 M, pH 7.4) and 0.2 ml 100 mM DTNB (dithio-bis-2-nitrobenzoic acid, Sigma–Aldrich) in a total volume of 3 ml. The absorbance of reaction product was measured at 412 nm and the results expressed as nmol GSH/g tissue.

Anti-oxidant enzyme measurements

Glutathione-S-transferase (GST) activity was assayed using the method of Haque et al. (2003). The reaction mixture consisted of 1.675 ml sodium phosphate buffer, 0.2 ml of 1mM GSH (Sigma–Aldrich), 0.025 ml of 1mM CDNB (1-chloro-2,4-dinitrobenzene, Amresco) and 0.1ml of PMS in a total volume of 2ml. The change in absorbance was recorded at 340 nm and the enzyme activity calculated as nmol CDNB conjugates formed/min/mg protein using є of 9.6x10^3 M^{-1} cm^{-1}. GR (glutathione reductase) activity was assayed by the method of Sharma et al. (2001). The assay mixture consisted of 1.6 ml sodium phosphate buffer, 0.1ml of 1mM ethylenediamine tetra acetic acid disodium salt (EDTA, Amresco), 0.1 ml NADPH (nicotinamide adenine dinucleotide phosphate reduced, Sigma-Aldrich) and 0.1ml oxidized glutathione (Sigma-Aldrich) as well as PMS (0.1ml) in total volume of 2ml. The enzyme activity measured at 340 nm was calculated as nmol NADPH oxidized/min/mg of protein, using є of 6.22 x10^3 M^{-1} cm^{-1}. Glutathione peroxidase (GP) activity was assayed using the method of Sharma et al. (2001). The assay mixture consisted of 1.49 ml sodium phosphate buffer, 0.1ml EDTA (1mM), 0.1 ml sodium azide (1mM) (Central Pharmacy, Tunis, Tunisia), 0.1 ml of 1 mM GSH (Sigma-Aldrich), 0.1 ml NADPH (0.02 mM), 0.01 ml of 0.25 mM hydrogen peroxide (H_2O_2, CDH Chemicals) and 0.1 ml PMS in a total volume of 2ml. The oxidation of NADPH was recorded spectrophotometrically at 340 nm. The enzyme activity was calculated as nmol NADPH oxidized/min/mg of protein using є of 6.22 x10^3 M^{-1} cm^{-1}. CAT (catalase) activity was assayed using the method of Haque et al. (2003). The assay mixture consisted of 1.95

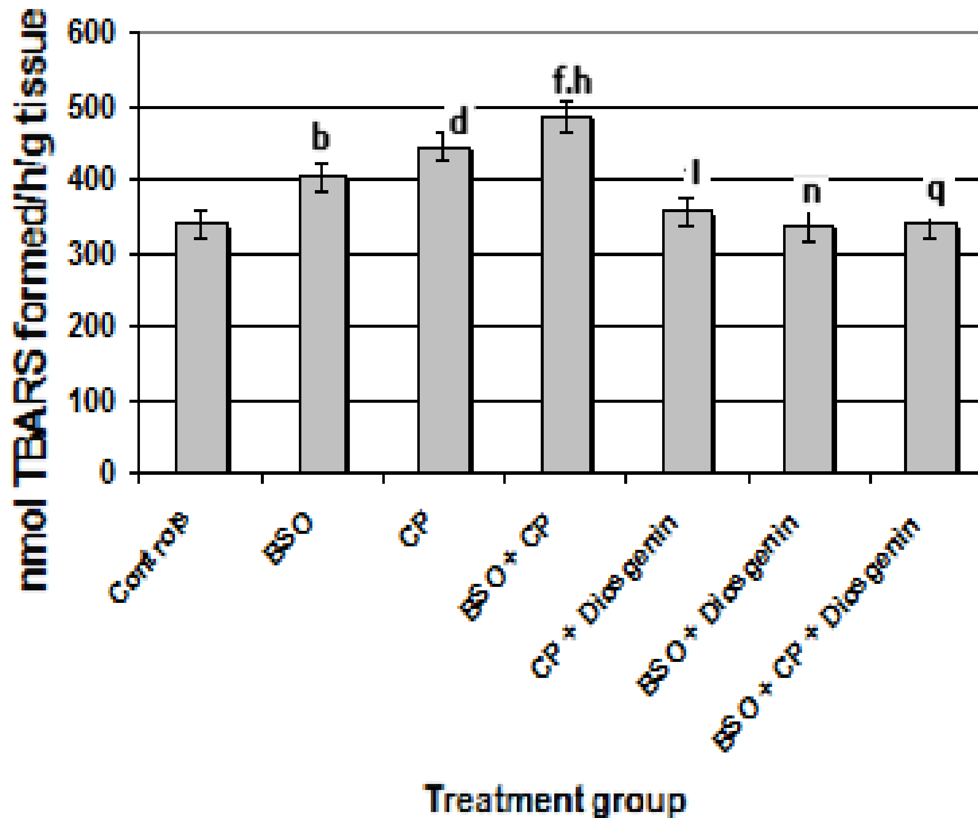

Figure 3. Effect of diosgenin, BSO and CP on the lipid peroxidation (LPO) in urinary bladder of mice. Significant differences are indicated by [b]$P < 0.01$ and [d]$P < 0.01$ in group II (BSO) and group III (CP) treated animals, respectively and [f]$P < 0.01$ in group IV (BSO + CP) when compared with the control animals (group I). [h]$P < 0.01$ indicates significant levels of BSO + CP group when compared to CP group. [l]$P < 0.01$ and [n]$P < 0.05$ indicate significant difference of data of group V (CP + Diosgenin) and group VI (BSO + Diosgenin) when compared with group III and group II, respectively. [q]$P < 0.01$ when group IV data was compared with group VII. Values are means ± SE (n = 6).

ml phosphate buffer, 1ml H_2O_2 (0.09 M) and 0.05 ml of PMS in a final volume of 3 ml. The change in absorbance was recorded kinetically at 240 nm. CAT activity was calculated in terms of nmol H_2O_2 consumed/min/mg protein.

Protein measurement

Protein was measured by the method of Lowry et al. (1951).

Statistical analysis

Single factor one-way analysis of variance (ANOVA) was performed to determine significant differences in results of various groups. The statistical significance level was set at P values < 0.05. A Student–Newman-Keuls test was then carried out to analyzed and compare the significance of the treatment groups. The values were expressed as mean ± SE.

RESULTS

No mortality and significant change in the body weight were observed in different groups of animals

Lipid peroxidation

The findings reveal that BSO treatment brought about a significant increase (P < 0.01) in the LPO levels in the bladder over the control values (Figure 3). The administration of CP also induced LPO in a significant way. Likewise, the cumulative effect of BSO + CP resulted in a significant increase in LPO as compared to Group I (control). The BSO + CP group (Group IV) also underwent a significant (P < 0.01) increase in terms of LPO when compared to the CP group (Group III) alone (Figure 3). The animals pre-treated with diosgenin and subsequently exposed to BSO (BSO + diosgenin) on the other hand were noted to undergo a significant (P < 0.01) reduction in terms of LPO levels in the bladder. When the values obtained for the CP + diosgenin group (Group V) were compared to those of the CP group (Group II), diosgenin treatment was also observed to significantly (P

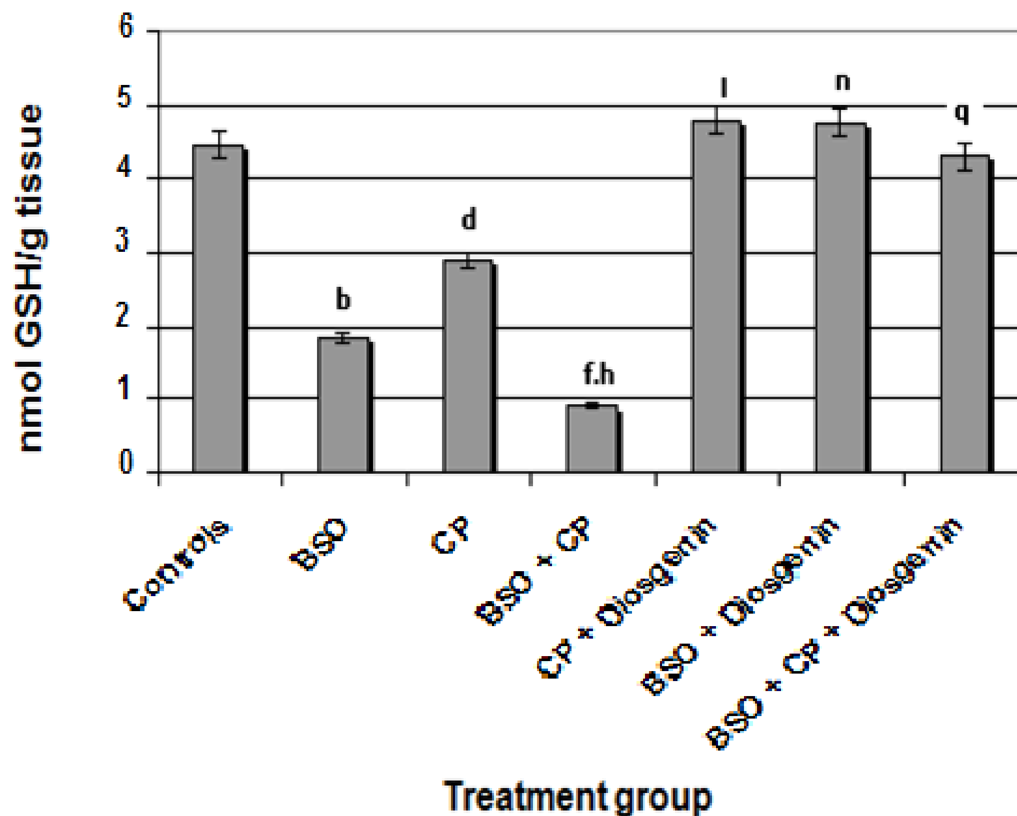

Figure 4. Effect of diosgenin, BSO and CP on GSH content in urinary bladder of mice. Significant differences are indicated by $^{b}P < 0.01$and $^{d}P < 0.01$ in group II (BSO) and group III (CP) animals, respectively and $^{f}P < 0.01$ in Group IV (BSO + CP) when compared with control animals (Group I). $^{h}P < 0.01$ indicates significant change in BSO + CP group when compared with CP group. $^{l}P < 0.01$ and $^{n}P < 0.01$ indicate significant differences in observations in group IV (CP + diosgenin) and group VI (BSO + Diosgenin) when compared with GSH data of groups III and II, respectively. $^{q}P < 0.01$ when GSH data of group IV was compared with group VII. Values are means ± SE (n = 6).

< 0.05) reduce the levels of LPO in the bladder. As shown in Figure 3, the comparison between the LPO values obtained for the BSO + CP group (Group IV) and the BSO + CP + diosgenin treatment group clearly shows a significant decrease (P < 0.01) in the case of the latter (Group VII).

Reduced glutathione

The findings reveal that when compared to the control values (4.5 nmol GSH/g tissue), the BSO, CP and BSO + CP-treated groups underwent significant (P < 0.01) decrease of 1.9, 2.9 and 0.9 nmol GSH/g tissue in GSH, respectively (data generated for the cellular GSH of urinary bladder are shown in Figure 4). Likewise and when compared to the CP group (Group III), the BSO + CP group (Group IV) showed a significant (P < 0.01) decrease in terms of GSH levels. As shown in Figure 4. When the BSO and BSO + Diosgenin groups were compared, the GSH content of latter was noted to undergo a significant (P < 0.01) increase. Similarly, the GSH content in the bladder of the CP + Diosgenin group (Group V) showed a significant increase (P < 0.01) when compared to the group given only CP (Group III). When the GSH values obtained for the BSO + CP group (Group IV) were compared to those of the BSO + CP + Diosgenin group (Group VII), a significant (P < 0.01) restoration in GSH was recorded (Figure 4).

Anti-oxidant enzymes

BSO and CP treatments were observed to induce significant (P < 0.01) decrease in terms of GST, GR, GP and CAT activities in the bladder when compared to the control group (Table 1). The BSO + CP group also showed an additive significant (P < 0.01) decrease in the activities of GST, GR and GP when compared to the CP and the control groups. However, no significant difference was observed in terms of CAT activity between the animals of group IV (BSO + CP) and group III (CP). The activity of those anti-oxidant enzymes increased significantly (P < 0.01) in both the BSO + Diosgenin

Table 1. Activities of anti-oxidant enzymes in the urinary bladder of mice in different treatment groups.

Group	Activity of anti-oxidant enzyme			
	GST	**GR**	**GP**	**CAT**
I (Controls)	144 ± 3	143 ± 4	155 ± 5	104 ± 3
II (BSO)	100 ± 2 [b]	92 ± 4 [b]	122 ± 3 [b]	97 ± 2 [b]
III (CP)	115 ± 3 [d]	122 ± 5 [d]	139 ± 4 [c]	84 ± 3 [d]
IV (BSO + CP)	79 ± 3 [f,h]	86 ± 2 [f,h]	118 ± 3 [f,h]	82 ± 3 [f]
V (CP + Diosgenin)	153 ± 5 [l]	152 ± 5 [l]	162 ± 2 [l]	107 ± 5 [l]
VI (BSO + Diosgenin)	146 ± 3 [n]	149 ± 6 [n]	156 ± 4 [n]	113 ± 7 [n]
VII (BSO + CP + Diosgenin)	145 ± 4 [q]	148 ± 6 [q]	154 ± 4 [q]	104 ± 5 [q]

Values are means ± SE (n = 6). GST expressed as nmol CDNB conjugates/min/mg protein, GR as nmol NADPH oxidized/min/mg of protein and GP as nmol NADPH oxidized/min/mg protein. CAT activity is expressed as nmol H_2O_2 consumed/min/mg protein. Significant differences are indicated by [b]P < 0.01, [d]P < 0.01, [c]P < 0.05 and [f]P < 0.01 when compared with control animals. (Group I) [n]P < 0.01 when compared with Group II, [h]P < 0.01 and [l]P < 0.01 when compared with CP-treated animals (Group III) and [q]P < 0.01 when compared with Group IV.

(Group VI) and CP + Diosgenin (Group V) groups when compared to their respective controls, the BSO (Group II) and CP (Group III) (Table 1) groups. The animals treated with diosgenin and subsequently exposed to BSO + CP (Group VII, BSO + CP + Diosgenin) showed a significant increase (P < 0.01) in the activities of all the anti-oxidant enzymes when compared to the BSO + CP group (Group IV).

DISCUSSION

The main objective of this study was to explore the potential gain efficacy that diosgenin might offer in preventing and reducing undesirable urotoxicity induced by acute CP and BSO doses *in vivo*. To our knowledge, this is the first study demonstrating that diosgenin has the potential to reduce urotoxicity. In this context, several studies have suggested that oxidative stress could play an important role in CP and BSO induced toxicity. Since the bladder is the site where urine is stored, the concentration of CP toxic metabolites is higher in the bladder than in other organs, which are likely to increase the toxic load of CP in the bladder to manifolds (Beyer-Boon et al., 1978).

As far as the present study is concerned, the findings indicated that CP caused a significant reduction in the levels of all the antioxidants in urinary bladder. It also increased LPO in the bladder. The oxidative products of the CP responsible for the induction of LPO and the generation of ROS resulted in inflammation, thus disturbing the overall redox cycling of the bladder (Cooper et al., 1986). It is worth noting in this context that BSO, a known depletor of glutathione, inhibits c-glutamylcysteine synthetase (c-GCS) (Griffith et al., 1982).

When the data pertaining to GSH reduction was compared, BSO was found to exert a more significant effect than CP. In fact, a difference of 42% was observed between the two items involved, confirming that BSO is a more potent depletor of GSH than CP, a result which is in good agreement with the findings previously reported by Ishikawa et al. (1989). The CP-induced depletion of GSH is primarily mediated by the interaction of its reactive metabolite, acrolein with GSH (Kehrer and Biswal, 2000). Acrolein interacts not only with GSH but also with cysteine, which is one of the constituent amino acids of GSH (Kehrer and Biswal, 2000). It is for this reason that a number of sulfhydryl (–SH) compounds and cysteine itself, have been observed to protect the animals from toxic effects of CP (Ishikawa et al., 1989).

Furthermore, the i.p administration of CP was observed to induce LPO in bladder in a significant way. In fact, CP-induced LPO has been described in different tissues of exposed animals (Haque et al., 2003; Patel et al., 1984). The role of acrolein in the CP-induction of LPO also has been implied (Adams and Klaidrnan, 1993). It has been suggested that by binding to nucleophilic amino acids, acrolein could directly affect transcription and modulate this process through its ability to deplete GSH (Kehrer and Biswal, 2000).

Moreover, BSO treatment was noted to result in the depletion of GSH and the increase of LPO in urinary bladder. The depletion of GSH was in fact previously reported to increase the susceptibility of cells to apoptosis (Zucker et al., 1997). When BSO and CP were administered together, an additive effect was observed in the cases of GSH, LPO and other parameters. The purpose of using BSO together with CP was to study the likely scenario where a host is exposed to a combination of GSH depleting agents, including pathogens, and to assess whether an herbal extract treatment of Fenugreek like diosgenin can have any modulatory impacts on their commutative/additive effects. Not only did diosgenin pre-treatment show protective effects on CP urotoxicity but was also effective in protecting the animals treated with the CP + BSO combination (Group VII). The diosgenin pre-treatment was observed to restore the depleted GSH

and other anti-oxidants, and to simultaneously reduce LPO in the bladder. Immunomodulatory herbal extracts such as diosgenin that has valuable GSH restoring effects presented in the current work hold great promise in reducing the adverse effects of CP in patients with cancer.

The results of this present study in agreement with previously reported findings demonstrated that treatment with CP and BSO significantly enhances ROS formation in the bladder as evidenced by the increased LPO and decreased GSH (Ishikawa et al., 1989). Evidence for the involvement of oxidative stress, resulting in increased LPO from the onset of several diseases is accumulating.

The findings of this work revealed that diosgenin completely counteracts CP-induced oxidative stress, which is evidenced by the increased GSH and the decreased LPO levels. The molecular mechanism of diosgenin is directly related to its notable ability to scavenge most of the radical species. These results are in agreement with other previous reports indicating that *in vitro*, diosgenin was able to completely prevent CP-induced ROS generation (son et al., 2007). As recently reviewed in previous reports, Fenugreek seeds have antioxidant activity and have been shown to produce beneficial effects such as neutralization of free radicals and enhancement of antioxidant apparatus (Anuradha and Ravikumar, 1998, 2001). Furthermore, the poly-phenolic fraction of the seeds was found to inhibit peroxide-induced oxidative damage and prevent haemolysis of erythrocytes *in vitro* (Kaviarasan et al., 2004). In a previous study, researchers found that the administration of Fenugreek seeds protects rat liver from ethanol induced oxidative stress (Thirunavukkarasu et al., 2003).

In the same way, it was also reported that diosgenin reduce the plasma level of total cholesterol and elevate HDL and conferred more resistant to lymphocyte DNA damage induced by oxidative stress (Son et al., 2007).

Finally, diosgenin was found to exert a suppressed action on the NF-kB gene, which has been identified in the regulatory of regions of the entire antioxidative enzyme gene (Shishodia et al., 2006). In short, this present study investigated, for the first time, the potential antitoxic activities of diosgenin focusing on the reduced LPO effect and increased anti-oxidant activities. The findings demonstrate that diosgenin is endowed with promising properties and attributes that make it a potential attractive candidate for application as anti-toxic and therapeutic agent in the medical industry. For this reason, further studies some of which are currently under way are needed to determine other pharmacological activities pertaining to this natural compound.

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