

Prophylactic Effect of Gossypin Against Percutaneously Administered Sulfur Mustard

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Objective To evaluate the protective efficacy of gossypin (3,3',4',5,7,8-hexahydroxyflavone 8-glucoside) by administering it intraperitoneally, for dose, time, and vehicle dependent effects against sulphur mustard (SM), administered through percutaneous route in mice. **Methods** SM (diluted in PEG-300) was administered percutaneously. The protective efficacy of gossypin was evaluated by administering it intraperitoneally (50, 100, 200, and 400 mg/kg), in various vehicles (water, PEG-300 and DMSO), and time intervals (30 min prior, simultaneous and 2 h post). The time dependent protection of gossypin (200 mg/kg in PEG-300; i.p.) was also evaluated using selected biochemical variables (GSH, GSSG, MDA, total antioxidant status, Hb, WBC count, RBC count, glutathione peroxidase, glutathione reductase, and superoxide dismutase) and liver histology. The protection of gossypin by oral route was also evaluated against percutaneously administered SM. **Results** The protection against systemic toxicity of SM (LD₅₀ 8.1 mg/kg) was better when gossypin was given with PEG-300 (8.0 folds) than DMSO (5.7 folds). No protection was observed when gossypin was administered with water. Good protection (8.0 folds) was observed when gossypin was administered (200 mg/kg in PEG-300; i.p.) at 30 min prior or simultaneous to SM exposure, but no protection was observed when gossypin was administered 2 h post to SM exposure. A significant weight loss was observed 7 days after SM administration (2 LD₅₀), with a significant increase in RBC and Hb. A significant decrease in total antioxidant status of plasma, liver GSH and GSSG levels, and in the activities of glutathione peroxidase, glutathione reductase and superoxide dismutase was also observed 7 days after SM administration. SM treated mouse liver also showed necrosis. A significant protection was observed when gossypin (200 mg/kg in PEG-300; i.p.) was administered either as a pretreatment (30 min before) or simultaneous treatment, and not as a post treatment (2 h). The protective efficacy of gossypin was better through oral route when administered with DMSO (4.8 folds) than with PEG-300 (2.4 folds). No protection was observed when gossypin was administered orally with water. **Conclusion** Percutaneous administration of SM induces oxidative stress and gossypin can protect it as a prophylactic agent by intraperitoneal or oral routes.

Key words: Sulfur mustard; Gossypin; Oxidative stress; Glutathione; Intraperitoneal; Oral

INTRODUCTION

Sulphur mustard (SM) is a blistering agent from which no satisfactory prophylactic or treatment regimen is available. The mechanism and search for a better antidote are being pursued world over. SM, commonly known as a mustard gas, is chemically bis (2-chloroethyl) sulfide and an alkylating agent that causes serious blisters upon contact with human skin^[1-4]. SM forms sulfonium ion in the body and alkylates DNA leading to DNA strand breaks and cell death^[5]. Due to the high electrophilic property of the sulfonium ion, SM binds to a variety of cellular macromolecules^[6]. Eyes, skin, and the respiratory tract are the principal target organs of SM toxicity^[5,7].

Several antidotes have been reported for reducing the systemic toxicity of SM in experimental

animals^[8-14]. Most of the antidotes are in various stages of development and so far none of them is recommended. One of the interesting findings is that percutaneously administered SM is more toxic than subcutaneous and oral routes of administrations^[15]. The most effective way of minimising SM toxicity is either by physical adsorption or by chemical decontamination^[16].

Recently, more emphasis has been given on an *in vitro* screening of compounds against SM toxicity. Importantly, lots of compounds have been found to protect the cell from SM induced cytotoxicity but none of the compounds could protect against *in vivo* toxicity of SM. The lack of correlation between *in vivo* and *in vitro* system is the major draw back in the drug development against SM toxicity.

One of the important mechanisms of action of

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SM cytotoxicity is based on the depletion of reduced glutathione (GSH), and subsequent lipid peroxidation and free radical generation^[6,17]. Flavonoids are a group of polyphenolic compounds found ubiquitously in plants. They exhibit a variety of biological activities, such as antiinflammatory, antioxidant, antiviral and antitumor actions^[18]. Quercetin and other flavonoids have been shown to modify eicosanoid biosynthesis, protect low-density lipoprotein from oxidation, prevent platelet aggregation and promote relaxation of cardiovascular smooth muscle. In addition, flavonoids have been shown to have antiviral and carcinostatic properties. Ethanol-induced oxidative stress is protected by pretreatment with quercetin by directly quenching lipid peroxides and indirectly by enhancing the production of the endogenous antioxidant GSH^[19-20]. The protective effects of quercetin on carbon tetrachloride-induced hepatotoxicity, cyclosporine-induced nephrotoxicity, and cisplatin-induced cytotoxicity in cultured tubular epithelial cells have been reported^[21-23].

Gossypin (3,3',4',5,7,8-hexahydroxyflavone 8-glucoside; Fig. 1), is a flavonoid known to be present in *Hibiscus species*. It is usually found in the flowers of *Gossypium indicum*, *Hibiscus vitifolius* and *Hibiscus esculentus*. *Hibiscus vitifolius* forms a rich source of gossypin. The presence of glucose moiety in the 8th position of hexahydroxyflavone, makes it soluble in water but sparingly soluble in alcohol and gives a deep yellow color. It has been reported to exhibit anti-inflammatory action through the inhibition of arachidonic acid metabolism by inhibiting the cyclooxygenase and lipoxygenase activity^[24]. It has also been shown to have analgesic activity in mice^[25]. Gossypin has also been reported to protect against beta-amyloid induced toxicity and has a good antioxidant activity^[26-27]. We earlier reported that antioxidants and bioflavonoids are good antidotes to SM toxicity. In the present study the protection of gossypin was evaluated by administering it intraperitoneally for dose, time, and vehicle dependent effects against SM administered through percutaneous route in mice.

MATERIALS AND METHODS

Chemicals

SM synthesized in the Synthetic Chemistry Division was of 99% purity by gas chromatographic analysis. O-phthalaldehyde (OPT) and glutathione were purchased from Sigma Chemicals, USA. Other chemicals were of high purity from Qualigens (India) or Merck (India). Glutathione reductase, superoxide

dismutase, and antioxidant kits were purchased from Cal Biochem (India). Gossypin was purchased from M/s Research Organics (India). The purity of gossypin was more than 95 %. PEG-300 and DMSO were procured from Fluka (USA).

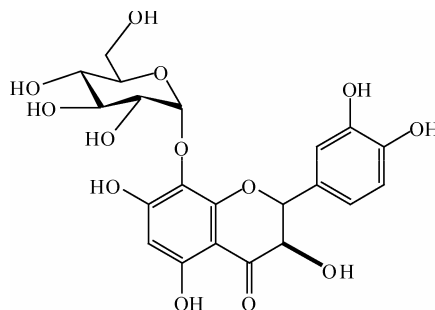


FIG. 1. The structure of gossypin (3,3',4',5,7,8-hexahydroxyflavone 8-glucoside).

Animals

Randomly bred Swiss albino female mice weighing 25-30 g from the institute's animal facility were used in the study. The animals were housed in polypropylene cages under controlled environmental conditions with free access to food (standard pellet diet, Amrut Ltd, India) and water. The care and maintenance of animals were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India). A day before percutaneous exposure to SM, hair on the back of the animals was closely clipped using a pair of scissors. Food and water were withheld two hours prior to the experiment. The Animal Ethical Committee approved this project.

Protective Efficacy of Gossypin Against Acute Toxicity of SM

LD₅₀ of SM diluted in PEG-300 was determined by exposing the animals to logarithmic doses of SM through percutaneous route of administration. The animals were observed for fourteen days and LD₅₀ was determined by the moving average method^[28]. The following experiments were carried out for dose and time response efficacy of gossypin:

- Protective efficacy of gossypin by i.p., in various vehicles i.e., water, PEG300, and DMSO.
- Protective efficacy of gossypin by i.p., at various doses i.e., 50, 100, 200, and 400 mg/kg.
- Protective efficacy of gossypin by i.p., at various time points i.e., 30 min prior,

simultaneous, and 2 hours post treatment.

Four animals per group (vehicle, dose, or time) were used and SM was administered using a microliter (Hamilton) syringe. About 40 to 50 μL SM was applied on the back of the mice and smeared uniformly. Protective index (PI) was determined as a ratio of LD_{50} of SM with treatment to LD_{50} of SM without treatment.

Protective Efficacy of Gossypin by i.p. Route at Various Time Points Against Acute Toxicity of SM Administered Through Percutaneous Route for Biochemical and Histological Markers

In another experiment, gossypin (in PEG 300) at a dose of 200 mg/kg (i.p.) at three time points and SM were administered (2 LD_{50} , p.c.). The following groups were kept for biochemical and histological studies.

Group 1 (control)

Group 2 SM+PEG (i.p.)

Group 3 SM+Gossypin, 30 min prior

Group 4 SM+Gossypin, simultaneous administration

Group 5 SM+Gossypin, 2 hours post administration.

Each group consisted of 4 mice who were weighed daily. Seven days after SM administration, the animals were anesthetized with ether and blood was withdrawn from orbital plexus. The animals were then sacrificed by cervical dislocation and liver was dissected out, cleaned and weighed. A portion was used for biochemical estimations and the remaining liver was used for histological examination.

Biochemical Estimations

The liver samples were used for the estimation of reduced glutathione (GSH), oxidized glutathione (GSSG), and malondialdehyde (MDA). Fluorimetric method of Hisin and Hilf^[29] was used for hepatic GSH and GSSG estimation. Briefly, 150 mg of liver tissue was homogenized in phosphate EDTA buffer and the homogenate was centrifuged at 10 000 g. To 0.25 mL of the supernatant 100 μL of 1 mg/mL fluorescent dye OPT was added and after 15 min incubation at room temperature, readings were taken at 420 nm emission and 350 nm excitation. Hepatic lipid peroxidation was determined by measuring the level of MDA according to the method of Buege and Aust^[30]. One hundred milligram of liver was directly homogenized in 5 mL of thiobarbituric acid reagent and boiled for 30 min. The contents in the tubes were cooled, centrifuged and absorbance of the clear supernatant was measured at 535 nm. The amount of MDA formed was calculated using a molar extinction coefficient of $1.58 \times 10^5/\text{M}$ per cm. The blood was used for the estimation of red blood corpuscles (RBC) and haemoglobin (Hb) using a Backman coulter cell

counter (USA).

Estimation of Total Antioxidant Status and Oxidative Stress Enzymes

Total antioxidant status in plasma was estimated in accordance to the kit manual (Cal Biochem, India). Liver samples were also used for the estimation of glutathione peroxidase, glutathione reductase and superoxide dismutase following the kit manual (Cal Biochem, India).

Histological Evaluation

The liver sample was fixed in 10% neutral buffered formalin solution. After proper fixation small pieces were processed by dehydration and embedded in paraffin wax. Sections of 5-6 μm thickness were prepared and stained with hematoxylin and eosin for light microscopy^[31]. The severity of the lesions was characterized using a LEICA-Qwin-500 image analyzer.

Protective Efficacy of Gossypin by Oral Route Against Acute Toxicity of SM Administered Through Percutaneous Route

Gossypin was dissolved in water, PEG 300 or DMSO and given orally at a dose of 200 mg/kg. SM was diluted in PEG 300 and administered at logarithmic doses by percutaneous route, simultaneously with gossypin. The animals were observed for fourteen days and LD_{50} was determined by the moving average method^[28].

Statistical Analysis

All the variables were analyzed by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. A probability of less than 0.05 was taken as statistically significant. SigmaStat (SPSS Inc., USA) was used for statistical calculations.

RESULTS

The LD_{50} of SM observed in this study was 8.1 mg/kg through percutaneous route. Protective efficacy of gossypin in different vehicles (i.p.) compared with vehicle alone against SM is given in Table 1. The protection against systemic toxicity was maximum when gossypin was given with PEG 300 (8.0 fold) and DMSO (5.7 fold) through intraperitoneal route but no protection was observed when gossypin was administered with water. PEG-300 and DMSO *per se* gave a protection of 2.9 and 2.8 folds respectively. Intraperitoneal administration of gossypin gave a dose dependent

protection (Table 2). Good protection was observed in 200 and 400 mg/kg but less protection was observed in 50 and 100 mg/kg body weight. Gossypin also showed time dependent protection against percutaneously administered SM (Table 3). Very good protection (8.0 folds) was observed when gossypin was administered in PEG-300 (200 mg/kg, i.p. route) at 30 min prior and simultaneous to SM exposure, but no protection was observed when

gossypin was administered in PEG-300 2 hours after SM exposure (protection index 0.9).

A significant weight loss was observed 3 and 7 days after SM administration. Pretreatment at 30 min before and simultaneous treatment of gossypin, but not 2 h post treatment, significantly protected against the body weight loss (Table 4). The relative weight of liver was not significantly changed compared to controls after SM administration.

TABLE 1
Protective Efficacy of Gossypin (200 mg/kg, i.p.) in Three Different Vehicles Against SM (in PEG 300) Applied Percutaneously

Group	Vehicle Alone LD ₅₀ (mg/kg)	Gossypin in Vehicle		PI*
		PI*	LD ₅₀ (mg/kg)	
SM + Distilled Water	8.1 (5.3-12.3)	-	8.1 (5.3-12.3)	-
SM + PEG 300	19.3 (7.3-51.5)	2.9	64.9 (35.7-118.1)	8.1
SM + DMSO	22.8 (2.4-34.8)	2.8	45.9 (30.0-70.2)	5.7

Note. *Protective index (PI) = Ratio of LD₅₀ with treatment to LD₅₀ without treatment. Figures in parentheses are confidence limits. Vehicle or gossypin was administered simultaneously with SM. The dose of vehicle was 4.0 g/kg equal to 0.01 mL for a 25 g mouse.

TABLE 2
Protective Efficacy of Gossypin (i.p.) at Different Doses Against SM (in PEG-300) Applied Percutaneously

Group	Dose of Gossypin	LD ₅₀ (mg/kg)	Confidence Limit (mg/kg)	PI*
SM Only	-	8.1	5.3-12.3	--
SM + Gossypin	50 mg/kg	19.3	7.3-51.4	2.4
SM + Gossypin	100 mg/kg	30.7	21.6-43.5	3.8
SM + Gossypin	200 mg/kg	64.9	35.7-118.1	8.1
SM + Gossypin	400 mg/kg	45.9	30.0-70.2	5.7

Note. *Protective index (PI) = Ratio of LD₅₀ with treatment to LD₅₀ without treatment. Gossypin (in PEG 300) was administered simultaneously with SM.

TABLE 3
Protective Efficacy of Gossypin (In PEG-300, 200 mg/kg, i.p.) Administered at Various Time Points Against SM (in PEG 300) Applied Percutaneously

Group	Time of Antidote	LD ₅₀ (mg/kg)	Confidence Limit (mg/kg)	PI*
SM Only	-	8.1	5.3-12.3	-
SM + Gossypin	-30 min	64.9	35.7-118.1	8.1
SM + Gossypin	0 min	64.9	35.7-118.1	8.1
SM + Gossypin	+2 h	6.8	4.8-6.9	0.9

Note. *Protective index (PI) = Ratio of LD₅₀ with treatment to LD₅₀ without treatment.

TABLE 4
Protective Efficacy of Gossypin (in PEG 300; 200 mg/kg, i.p.) at Three Different Time Points on Body Weight Change Against SM (in PEG 300) Applied Percutaneously ($\bar{x} \pm s$)

Group	1 Day After	3 Days After	7 Days After
Control	100.9 ± 1.2	101.8 ± 1.4 ^b	101.8 ± 1.3 ^b
SM Only (2 LD ₅₀)	100.9 ± 1.2	74.8 ± 1.6 ^a	68.6 ± 5.9 ^a
SM + Gossypin (-30 min)	96.7 ± 0.8	100.1 ± 2.4 ^b	100.1 ± 2.4 ^b
SM + Gossypin (0 min)	95.2 ± 0.9	98.1 ± 2.6 ^b	98.1 ± 2.6 ^b
SM + Gossypin (+2 h)	94.0 ± 1.7	63.9 ± 3.9 ^a	63.9 ± 3.9 ^a

Note. n=4, Significance P <0.05; ^aControl vs treatment; ^bSM vs treatment.

The biochemical changes with and without treatment of gossypin against SM are summarized in Table 5. The liver GSH and GSSG levels after SM administration were significantly decreased compared to controls. The decrease was found to be 44.5% and 60.6% in GSH and GSSG respectively. A significant protection was observed at 30 min pre and simultaneous treatment of gossypin, but not at 2 h after treatment. An increase in MDA level was observed in SM group, but it was not statistically

significant. No such increase in the level was observed at 30 min pre and simultaneous treatment of gossypin. But 2 h post treatment a slight increase in the level was observed at 2 h after treatment compared to controls (not significant). A significant increase in RBC count and Hb content (144.6% and 157.8% respectively) was observed. This increase was significantly protected by gossypin by 30 min pre and simultaneous treatment but not by 2 h post treatment (Table 6).

TABLE 5

Protective Efficacy of Gossypin (in PEG 300; 200 mg/kg, i.p.) at Three Different Time Points on Various Biochemical Variables Against SM (in PEG 300) Applied Percutaneously ($\bar{x} \pm s$)

Group	Liver GSH (%)	Liver GSSG (%)	Liver MDA (%)
Control	100.7 \pm 4.5 ^b	93.7 \pm 5.3 ^b	100.1 \pm 1.3
SM Only (2 LD ₅₀)	44.5 \pm 10.6 ^a	60.6 \pm 3.5 ^a	117.1 \pm 2.3
SM + Gossypin (-30 min)	108.8 \pm 13.4 ^b	93.6 \pm 5.5 ^b	106.2 \pm 3.5
SM + Gossypin (0 min)	93.3 \pm 9.1 ^b	114.3 \pm 7.8 ^{ab}	101.9 \pm 5.2
SM + Gossypin (+2 h)	47.6 \pm 5.9 ^a	70.3 \pm 4.0 ^a	124.2 \pm 15.1

Note. $n=4$, Significance $P < 0.05$; ^aControl vs treatment; ^bSM vs treatment control values. GSH = 4.61 \pm 0.08 μ moles/gm of tissue. GSSG = 1.82 \pm 0.10 μ moles/gm of tissue. MDA = 4.47 \pm 0.22 nmoles/gm of tissue.

TABLE 6

Protective Efficacy of Gossypin Through Intraperitoneal Route at Three Different Times Points on Hematological Change Against SM Applied Percutaneously ($\bar{x} \pm s$)

Group	RBC (%)	Hb (%)
Control	100.0 \pm 1.3 ^b	100.1 \pm 2.1 ^b
SM Only (2 LD ₅₀)	144.6 \pm 5.0 ^a	157.8 \pm 8.7 ^a
SM + Gossypin (-30 min)	105.8 \pm 7.5 ^b	123.2 \pm 1.7 ^{ab}
SM + Gossypin (0 min)	98.0 \pm 3.4 ^b	112.4 \pm 5.7 ^b
SM + Gossypin (+2 h)	137.0 \pm 5.6 ^a	152.8 \pm 9.5 ^a

Note. $n = 4$, Significance $P < 0.05$; ^aControl vs treatment; ^bSM vs treatment control values. RBC = 8.85 \pm 0.28 $\times 10^6$ cells/ μ L. Hb = 10.8 \pm 0.34 g/dL.

The effect of SM and its protection by gossypin on total antioxidant status, glutathione peroxidase, glutathione reductase and superoxide dismutase are shown in Fig. 2. A significant decrease was observed in the total antioxidant status, glutathione peroxidase, glutathione reductase, and superoxide dismutase in SM group compared to control group. Pre treatment with gossypin, 30 min prior or simultaneous treatment significantly protected against the levels of total antioxidant status and glutathione reductase. A superoxide dismutase level was not protected by gossypin treatment.

Control mice showed normal liver histological features with normal hepatic cord and hepatocytes (Fig. 3A). SM treated liver showed severe degeneration and clumping of cytoplasm. Severe necrosis was also observed in SM treated liver (Fig.

3B). The lesions were minimal in pre and simultaneous treatments of gossypin (Fig. 3C and 3D). Two hours post treatment of gossypin did not reduce the severity of hepatic lesions (Fig. 3E).

Since intraperitoneal administration of gossypin protected against SM when administered as a prophylactic agent, the oral efficacy was carried in the three vehicles. The protective efficacy of gossypin was very good through oral route when administered with dimethylsulfoxide (DMSO) with a protection of 3.2 folds, compared to PEG-300 with a protection of only 1.4 folds. No protection was observed when gossypin was dissolved in water and administered orally (PI=1.0). Administration of the vehicle alone gave a protection of 1.4 and 1.2 folds for DMSO and PEG-300, respectively (Table 7).

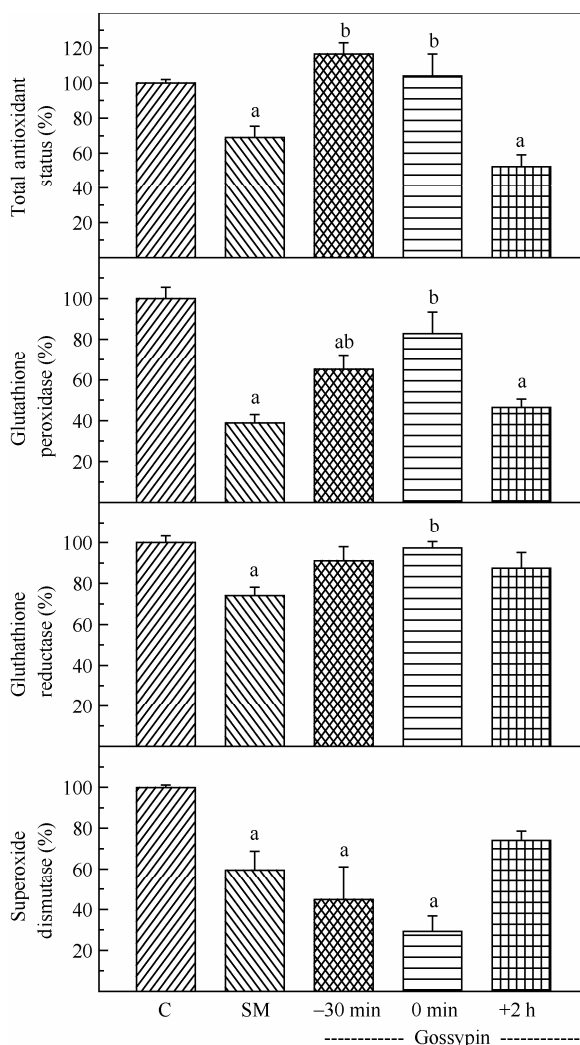


FIG. 2. Effect of gossypin (200 mg/kg, i.p.) at different time intervals against percutaneously administered SM (2 LD₅₀). n=4, $\bar{x} \pm s$, Significance $P < 0.05$; ^aControl vs treatment; ^bSM vs treatment Control values-Antioxidant status (plasma)=139.1±5.6 μmol/L Glutathione Peroxidase (liver)=768.0±42.1 nanomoles NADPH/min/mg protein Superoxide Dismutase (liver)=4.65±0.79 U/mg protein Glutathione Reductase (liver)=54.2±5.1 nanomoles NADPH/min/mg protein.

TABLE 7

Protective Efficacy of Gossypin (200 mg/kg, p.o.) in Three Different Vehicles Against SM (in PEG 300) Applied Percutaneously

Group	Vehicle Alone LD ₅₀ (mg/kg)	Gossypin in Vehicle		PI*
		PI*	LD ₅₀ (mg/kg)	
SM + Distilled Water	5.7 (2.6-12.7)	-	5.7 (2.6-12.7)	1.0
SM + PEG 300	6.8 (4.5-10.4)	1.2	8.1 (2.6-25.5)	1.4
SM + DMSO	8.1 (2.4-34.8)	1.4	18.2 (10.0-32.9)	3.2

Note. *Protective index (PI) = Ratio of LD₅₀ with treatment to LD₅₀ without treatment. Figures in parentheses are confidence limits. Vehicle or gossypin was fed orally, simultaneously with SM. The dose of vehicle was 4.0 g/kg equal to 0.01 mL for a 25 g mouse.

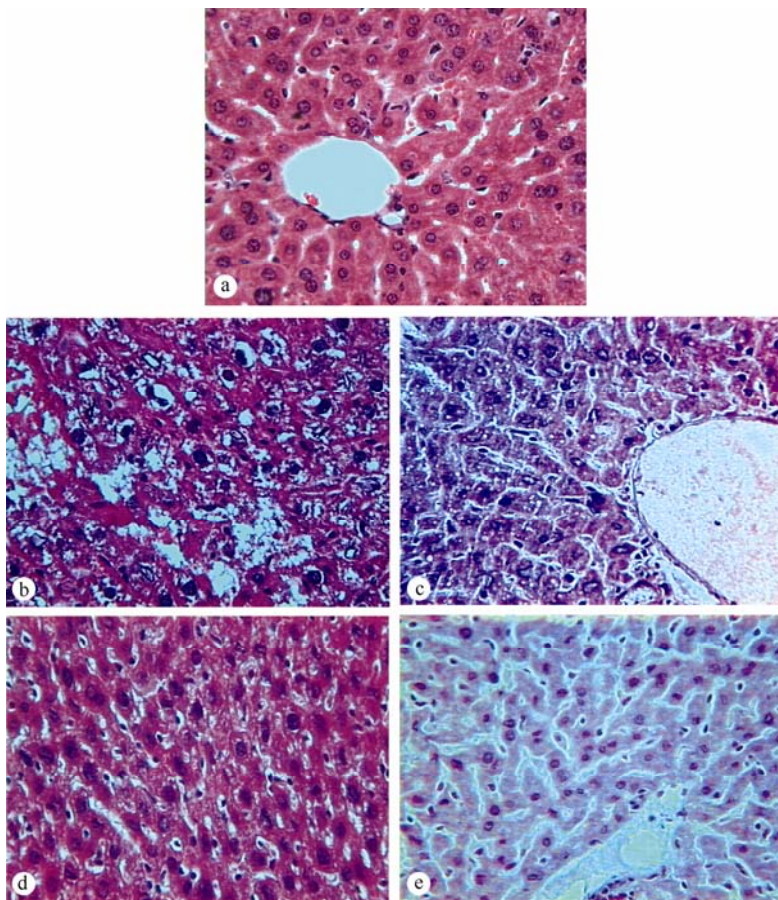


FIG. 3. Photomicrographs of mice liver; H & E, 100 \times . (a) Control liver showing normal hepatic parenchyma, hepatic lobules and hepatocytes, (b) SM administered mice liver (2 LD₅₀) showing granulovacuolar degeneration and perinuclear clumping of cytoplasm, (c and d) – 30 min and simultaneous treatment respectively of 200 mg/kg of gossypin with SM, showing reduced hepatic lesions, (e) 2 hours post treatment of gossypin 200 mg/kg with SM, showing occasional hepatic parenchymal degeneration.

DISCUSSION

Despite enormous research efforts during the last several decades, no specific antidote for SM has been identified. One important reason is that though several antidotes have been shown to be effective in various *in vitro* systems, their efficacy *in vivo* has not been proved. The reason may be that SM has been administered parentally and not percutaneously, or SM simulants have been used for the antidote screening. It has been observed that the toxicity pattern of SM is different from a SM simulant, chloroethylethyl sulfide (CEES) and percutaneously administered SM is more toxic than subcutaneously administered SM^[15,32]. Among the antidotes that have been reported to give satisfactory protection against SM are anti-inflammatory agents^[10], anti-oxidants^[11,33] and SM scavengers^[8,10]. Gossypin has been reported to have anti-oxidant and anticancer activities^[26].

Viswanathan *et al.*^[25] reported that gossypin inhibits acetic acid-induced writhing in a dose dependent manner by the involvement of opiate receptors showing analgesic action of gossypin. Gabor^[34] has reported its anti-inflammatory activity. Ferrandiz *et al.*^[35] reported that flavonoids inhibits arachidonic acid metabolism in sonicated sheep platelets, due to the presence of catechol group, which inhibits lipoxygenase activity. The inhibition of arachidonic acid metabolism is one of the mechanisms by which gossypin exerts its anti-inflammatory effect. Hepatoprotective action of gossypin in isolated rat hepatocytes has also been demonstrated^[36]. Gossypin is also reported to protect primary culture of rat cortical cells against oxidative stress and beta amyloid-induced toxicity^[27]. Since gossypin is water soluble glycoside with a wide pharmacological action, the present study was initiated to evaluate its efficacy against SM. We found that gossypin had very good protective efficacy

when given by intraperitoneal administration.

In the present study, the LD₅₀ of SM varied from 5.7 mg/kg to 8.1 mg/kg, by the percutaneous route. Gossypin (200 mg/kg), administered in different solvents, showed maximum protection in DMSO than in PEG, but no protection was found when it was administered with water. Gossypin has one glucose moiety in its 8th position that makes it water soluble. In spite of this there was no protection when gossypin was administered with water. DMSO readily crosses most of the membranes of the body without affecting the integrity of the membrane and also facilitates the absorption of a number of compounds increasing their bioavailability. Polyethylene glycol also increases the absorption of the compounds by increasing the membrane permeability. Various reports are available that DMSO *per se* is a pharmacologically active compound^[37]. It has potent antioxidant^[38] and anticarcinogenic^[39] activity. It was also reported that it protects against SM-induced toxicity *in vitro* models^[40]. Intraperitoneal route of gossypin gives better protection than oral because of its better absorption. The flavonoids get in the metabolic pathway of sulfation or methylation in the body, which reduces its activity^[41]. Through intraperitoneal route, the compound containing active hydroxyl group is probably transported without metabolism to interact with prooxidants. Polyethylene glycol or dimethyl sulfoxide has a role in increasing the permeability through the membrane. DMSO is an amphipathic molecule with a high polar domain and two apolar methyl groups, making it soluble in aqueous as well as organic media. It is one of the most common solvents for the *in vivo* administration of several water insoluble substances. Despite being frequently used, as solvents in biological studies and a vehicle for the drug therapy, DMSO has few undesirable effects and is not usually used as a drug. Hence it PEG is used as a solvent because of its inert nature and no side effect. Our study showed that gossypin in PEG had a much better protection effect than PEG alone. A similar result was also observed in DMSO when administered intraperitoneally. This experiment shows that protection of gossypin in PEG is not due to solvent alone but due to gossypin also. Solubility of gossypin is comparatively less in PEG and better in DMSO due to its amphipathic nature. The protection given by gossypin by the oral route is less than the intraperitoneal route. Better protection was observed when gossypin was dissolved in DMSO. This is due to the poor absorption of flavonoids by the oral route.

A dose dependent protection was observed in gossypin treatment. Maximum protection was observed at 200 mg/kg dose. A time dependent

protection was also observed in the case of gossypin. Maximum protection was found when gossypin was administered 30 min prior or simultaneous treatment, but no protection was observed when it was administered 2 h post treatment. This shows that gossypin can protect against SM toxicity only when it is administered as a prophylactic agent to interact with SM metabolite and not as a post treatment. SM releases arachidonic acid, which is the first event of inflammatory response in the body^[42]. Gossypin is reported to affect arachidonic acid metabolism and inhibition of arachidonic acid metabolism is one of the mechanisms by which flavonoids exert their anti inflammatory effect^[24]. This is one possibility that gossypin is able to protect against SM toxicity as a prophylactic agent.

Body weight loss is a constant observation after percutaneous administration of SM. This is partially due to decreased food and water intake as a result of SM-induced injury to epithelial cells of the esophagus. SM is an alkylating agent, mostly affecting the maturing cells. The pre and simultaneous treatment of gossypin is capable of protecting the cells against SM. In the case of 2 h post treatment, gossypin has no role in reviving the affected cells.

Several studies have reported a significant decrease in GSH and GSSG after SM administration. Glutathione is a tripeptide that accounts for 90% of cellular non-protein thiol^[43]. It plays an important role in protecting cells against reactive oxygen species. Significant reduction of GSH due to SM is indicative of oxidative stress and cellular damage. There is also the possibility of interaction of GSH with SM. Being an electrophile, SM has high affinity towards sulfhydryl groups and depletes GSH in the body^[44]. In this study the decrease was significantly prevented by 30 min pre and simultaneous treatment of gossypin but not by two hours post treatment of gossypin. This clearly indicates that SM-induced changes are immediate and irreversible. Histological study on the liver also supports that the effect is immediate and prophylaxis is the better way of minimising SM toxicity. It was reported that flavonoids act mostly when it is administered before treatment^[45]. It is expected that flavonoids donate its hydroxyl group to the free radicals, sparing GSH to interact with other free radicals. Elevation of MDA, which is prominent after SM administration, shows recovery when gossypin is administered. Usually lipid peroxidation takes place when reactive oxygen species are formed in the presence of iron molecule. Flavonoids are known to chelate iron, thereby removing the causal factor for the development of free radicals. Due to the generation of reactive

oxygen species by SM, the membrane loses its integrity and fluidity. So it may be possible that endothelial cells fail to retain plasma in blood, and blood increases its viscosity and density, thus increasing the RBC count and haemoglobin concentration. Pre as well as simultaneous treatments of gossypin protect against the effects, but not the post treatment. Many reports are available that RBC count and haemoglobin concentration are increased after SM exposure^[46-47].

Total antioxidant status decreased by SM administration is protected by pre and simultaneous treatment of gossypin not by 2 h post treatment. In this study we also observed a decrease in GSH and GSSG, which is not usually found in ROS generation. SM causes multiorgan failure and its effect is not necessarily on gene level. These findings show that ROS is not the only mechanism underlying SM toxicity, some other factors are also responsible for its toxicity.

A number of reports demonstrate that inflammation plays a vital role in SM toxicity^[5,48] and arachidonic acid pathway is one of the key pathways involved in inflammation. Lefkowitz^[42] reported that arachidonic acid is released in response to SM. Since gossypin inhibits the breakdown of arachidonic acid, it protects against SM toxicity as a prophylactic agent. In conclusion, gossypin in a lipophilic solvent is a good antidote for SM when it is administered prophylactically. Though flavonoids are good prophylactic agents for SM, they may also be used as a cytoprotectant.

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