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PROTECTIVE EFFECT OF QUERCETIN ON SULPHUR MUSTARD INDUCED OXIDATIVE STRESS IN MICE

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Abstract: ***Sulphur mustard (SM, 2, 2' dichloro diethyl sulphide) is a potent chemical warfare agent for which there is still no effective antidote. SM is known to cause oxidative stress. Therefore, in the present investigation, we studied the protective effect of quercetin, a bioflavonoid following SM toxicity. 2.0 and 4.0 LD_{50} of SM (1 LD_{50} = 9.67 mg/kg; 14 days observation for mortality) was administered to Swiss albino female mice through percutaneous route. SM exposed mice were treated with quercetin (100 and 200 mg/kg) three times by intraperitoneal injection, one immediately following SM exposure, then once each day for 2 days after SM treatment. The effect of quercetin on survival, body weight, markers of oxidative damage (in blood, liver and kidney), WBC counts and purine metabolite were investigated 7 day post exposure. Survival time increased significantly following quercetin treatment. The decrease in body weight due to SM was prevented to a significant extent by quercetin. Significant decrease in reduced glutathione and increase in the level of malondialdehyde indicated oxidative damage to hepatic and renal tissues. Quercetin protected hepatic and renal tissues from oxidative damage caused by SM. Alterations in WBC counts and end product of purine metabolite were also prevented by quercetin. This study shows that quercetin enhanced the survival time, restored the decrease in body weight and protected hepatic and renal tissues from oxidative damage.***

Key Words: Sulphur mustard, Protection, Antioxidant, Quercetin

INTRODUCTION

Sulphur mustard (SM) is a highly reactive bifunctional alkylating agent and produces blisters upon contact with human skin [1]. Several countries have declared possessing SM and are destroying their stock as per the Chemical Weapon Convention's procedure [2]. The distribution, metabolism and elimination of SM have been studied and reported [3]. SM reacts in aqueous phase with compounds containing various nucleophilic functional groups like amino, sulfhydryl, carboxylic and hydroxyl in proteins and nucleic acid. The toxicity of SM is due to interaction with one or more cell constituents [4]. SM has mutagenic and carcinogenic properties [5,6].

The mechanism of SM-induced cell injury is not fully understood and no effective antidote is known. Moreover, there is no effective method for evaluating the efficacy of therapeutic agents in preventing SM-induced injury to human tissues. Several mechanisms have been proposed for SM cytotoxicity including DNA damage, lipidperoxidation, labilization of lysosomes and calcium mediated toxicity [7,8]. On the basis of proposed mechanism number of compounds were tested to attenuate SM toxicity in vitro and in vivo include scavengers of SM and SM induced oxygen radicals [9-11], inhibitor of cell death and promoter of cell survival [7], radio protectors [12,13], decontaminant [14,15], skin ointment [16], and various other pharmacological agents [17].

Quercetin is one of the most common dietary flavonoid with a well characterized in vitro antioxidant activity [18,19]. It is found in a large quantity in fruits, vegetables, tea, wine, nuts, and seeds. Apple and onion are very rich source of quercetin and represents an integral part of the human diet. The daily intake

of quercetin is reported in between 6 to 31 mg per day [20]. Recovery of substantial amount of quercetin form plasma after a quercetin suplimented diet indicates that absorption of these compounds through gastrointestinal tract renders measurable amounts of blood polyphenols which may affect the biochemistry of cells from different tissues particularly in the liver. Flavonids, and specifically flavonols are reported to exhibit a wide range of biological activities, including antioxidant, anticarcinogeic, anti-inflammatory and anti-viral action. In addition, they also inhibit lipid peroxidation, platelet aggregation, capillary permeability and the activity of enzymes including lipoxigenase [21]. The flavonoids exert these effects as chelators of divalent actions and free radical scavengers and thus may be involved in preventing free radical mediated cytotoxicity and lipid peroxidation associated with various diseases and chemical exposure [22,23]. Keeping in view these effects of flavonoid, the present study was design to investigate the potential protective effect of quercetin against oxidative stress mediated sulphur mustard toxicity.

MATERIALS AND METHODS

Chemicals: DTNB (5, 5'-dithiobis (2-nitrobenzoic acid) was obtained from Sigma Chemicals Co. (st. Louis, MO). Quercetin was purchased from Fluka. Sulphur mustard was synthesized in the declared facility of Defence Research and Development Establishment, (DRDE), Gwalior and was found to be 99 % pure by gas chromatography analysis. All other chemicals used were of analytical grade.

Animals: Swiss albino female mice randomly bred in Institute's animal facility, weighing between 24 to 30 g were used in this study. The animals were maintained on dust free and sterilized rice husk bedding in polypropylene cages and kept at room temperature with 12 h light/dark cycles. The animals were fed standard pellet diet (Ashirwad Brand, Chandigarh, India), Food and water were given ad libitum. This study was approved by the Institute's Ethical Committee on Animal Experiments.

SM exposure: SM was diluted freshly in polyethylene glycol 300 (PEG 300) and was applied uniformly, using a micro syringe on to the back of the mice on a circular area of about 1.5 cm diameter, after closely clipping the hair (the hair on the application site was clipped 24 h before SM application). The animals were held for 1-2 minutes and then left in the cage. The SM was applied as a single dose of 1.0 and 2.0 LD50 (1 LD_{50} = 9.67 mg/kg; 14 days observation for mortality). The animals were observed for 14 days.

S**urvival study:** For survival study two doses (2.0 and 4.0 LD_{50}) of SM were administered. The animals from each group were divided into three sub-groups of five animals each and one of the treatment administered through ip route in the groups as follows: (i) Group-I, SM-H2O; (ii) Group-II, SM + Quercetin (100 mg/kg); (iii) Group-III, SM + Quercetin (100 mg/kg). Control animals were given water only. Quercetin was given three times by intraperitoneal injection, once immediately following SM administration, then once each day for two days after SM administration. Weight of all the animals were recorded daily and abnormality in gross behavioral change or food and water intake were also registered. The mortality of the animals was recorded up to 14 days of post administration and data used for percent survival.

Biochemical study: Biochemical study two doses (2.0 and 4.0 LD_{50}) of SM were administered. The animals from each dose group were divided into four sub-groups of five animals each, and one of the treatment administered through ip route in the groups as follows: (i) Group-I, Control; (ii) Group-II, SM-Control; (iii) Group-III, SM + Quercetin (100 mg/kg); (vi) Group-VI, SM + Quercetin (200 mg/kg). Control animals were administered PEG-300 and given water only. The treatment was given three times by intraperitoneal injection, once immediately following SM administration, then once each day for two days after SM administration.

The body weights of the animals were recorded daily. The food and water intake was monitored. The animals were sacrificed for biochemical studies 7 days after SM administration. The animals were anaesthetized with ether and blood was collected from orbital sinus in heparinized vials. Then the animals were sacrificed by cervical dislocation. Liver and kidney were excised quickly, rinsed in 0.9% saline, blotted and weighed to determine the OBI and used for biochemical studies.

The level of GSH in blood, hepatic and renal tissue was analyzed by colorimetric assay of non-protein sulfhydryl content using standard procedure [24].

Hepatic and renal lipid peroxidation was determined by measuring the level of MDA according to the method of Buege and Aust [25]. Blood uric acid was measured by phosphotungstic acid method using commercial diagnostic kits (Merck Limited, India). White blood corpuscle (WBC) counts was done by using Backman Coulter Cell counter (Model Coulter AcT Diff).

Statistical analysis: For the survival study, the data were analyzed by Friedman repeated measures ANOVA on ranks and compared with the SM treated group by Dunnett's multiple comparison method. The biochemical data were analyzed by one way ANOVA followed by Dunnett's multiple comparison method for comparing control and various treatments groups. A probability of less than 0.05 was taken as significant. SigmaStat (Jandel Scientific Corporation Inc., San Rafael, CA, USA) was used for the statistical analysis.

RESULTS

SM administered through percutaneous route caused decrease in body weight (Figure 1). The body weight started decreasing after 24 h post exposure and decrease was significant on 3rd day post exposure in all the doses of SM. Quercetin was able to protect body weight loss at a dose of 100 and 200 mg/kg against 2.0 and 4.0 LD_{50} of SM. Quercetin is a safe drug and LD_{50} is more than 5.0 g through oral and intraperitoneal route (data not shown). The effect of quercetin on survival time following 2.0 and 4.0 LD_{50} SM exposure is presented in Table 1. Survival time was calculated on the basis of 14 days observation period for all the SM exposures. Median represents percent of mice dying at 7 days of post exposure. The median (50th percentile) and 25th and 75th percentile were obtained from SigmaStat. Quercetin treatment at the dose of 100 and 200 mg/kg was able to increase the survival time. SM (2.0 LD_{50}), Q-100 and Q-200 mg/kg showed 50. 0.0, and 0.0 percent mortality on 7th day, respectively. The percent survival following 4.0 LD_{50} SM administration, and treatment with Q-100 and Q-200 mg/kg showed 100.0, 25.0 and 0.0 percent mortality on 7th day, respectively (Table 1). A significant increase in survival time was recorded in quercetin treated groups. Quercetin (100 and 200 mg/kg) treated groups did not show any mortality upto 7 day post exposure in 2.0 LD_{50} SM exposed group.

Treatments	SM dose (mg/kg)	
	2.0 LD_{50}	2.0 LD_{50}
SM	50 (0,100)	100 (6,100)
SM-Q-100 mg/kg	0 [a] (0,75)	25 [a] (0,50)
SM-Q-200 mg/kg	0 [a] (0, 0)	0 [a] (0, 75)
Chi-square	26.6	35.5
P	< 0.001	< 0.001

Table 1: Effect of quercetin on percent survival of mice following sulphur mustard administration through percutaneous route (50^{th} Percentile mortality [b]).[a] Statistically significant compared to SM group by Friedman's repeated measures ANOVA on ranks with Dunnett's multiple comparison. n=4. [b] The 50^{th} percentile is the percentage of mice dying within 7 days of post exposure. Values in parentheses are 25^{th} and 75^{th} percentiles.

Treatments	Blood GSH	Liver GSH	Kidney GSH
SM 2.0 LD_{50}			
Control	0.73 ± 0.10	7.28 ± 0.40	4.24 ± 0.18
SM	0.52 ± 0.08	3.10 ± 0.38 [a]	2.36 ± 0.19 [a]
SM-Q-100 mg/kg	0.63 ± 0.06	4.64 ± 0.54 [a]	3.20 ± 0.09 [a, b]
SM-Q-200 mg/kg	0.62 ± 0.05	6.28 ± 0.62 [b]	3.60 ± 0.15 [a, b]
SM 4.0 LD_{50}			
SM	0.56 ± 0.13	3.48 ± 0.34 [a]	2.50 ± 0.10 [a]
SM-Q-100 mg/kg	0.69 ± 0.15	5.25 ± 0.38 [a,b]	3.14 ± 0.17 [a, b]
SM-Q-200 mg/kg	60 ± 0.04	5.16 ± 0.29 [a,b]	3.68 ± 0.21 [b]

Table 2: Effect of quercetin on reduced glutathione content of blood, liver and Kidney following percutaneous administration of sulphur mustard in female mice. Values are Mean ± SE; n=5. [a] $P < 0.05$ compared to control group. [b] $P < 0.05$ compared to SM group. GSH, µ mol/l of blood and µ mol/g of tissue.

Treatments	Liver MDA	Kidney MDA
SM 2.0 LD_{50}		
Control	43.1 ± 2.31	50.6 ± 2.90
SM	70.0 ± 3.87 [a]	72.6 ± 2.16 [a]
SM-Q-100 mg/kg	44.4 ± 1.18 [b]	50.2 ± 2.42 [b]
SM-Q-200 mg/kg	42.2 ± 2.2 [b]	49.8 ± 1.82 [b]
SM 4.0 LD_{50}		
SM	77.8 ± 3.23 [a]	81.4 ± 2.92 [a]
SM-Q-100 mg/kg	57.6 ± 4.92 [a, b]	78.2 ± 3.90 [a]
SM-Q-200 mg/kg	35.4 ± 2.61 [b]	59.2 ± 4.42 [b]

Table 3: Effect of quercetin on malondialdehyde (MDA) level in liver and kidney following percutaneous administration of sulphur mustard in female mice. Values are Mean ± SE; n=5. [a] $P < 0.05$ compared to control group. [b] $P < 0.05$ compared to SM group. *MDA = malondialdehyde as nmol/g of tissue.*

There was significant decrease in the OBI of liver following both the exposed doses of SM. There was no significant alterations in the OBI of kidney following lower dose of SM. However, at higher dose OBI of kidney altered significantly (Fig. 2 and 3). The effect of different doses of quercetin following 2.0 and 4.0 LD_{50} SM on GSH content of blood, hepatic and renal tissues is shown in Table 2. The hepatic GSH decreased significantly in SM exposed animals. Quercetin (100 and 200 mg/kg) was able to protect hepatic and renal GSH. However, the level of GSH was not altered in the blood following percutaneous exposure of SM. The effect of SM

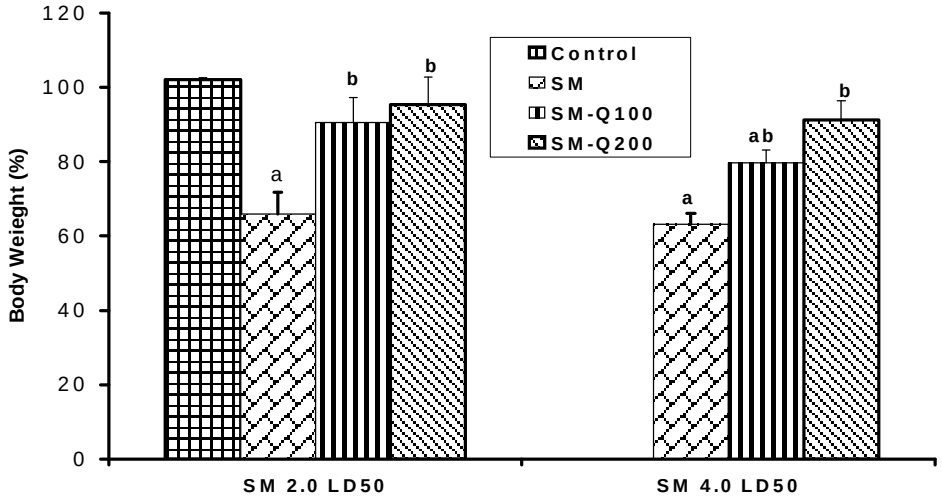

Fig. 1: Effect of quercetin on percent change in body weight on 7th day day following 2.0 and 4.0 LD_{50} SM post exposure through percutaneous route in female mice. Values are mean ± SE (n=4). [a] Statistically significant compared to control group. [b] Statistically significant compared to SM control group.

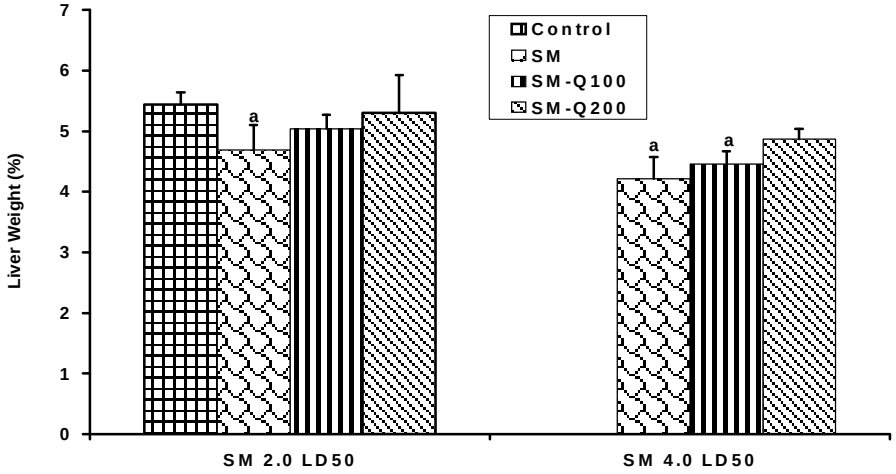

Fig. 2: Effect of quercetin on organ to body weight ratio of liver on 7th day following 2.0 and 4.0 LD_{50} SM post exposure through percutaneous route in female mice. Values are mean ± SE (n=5).[a] Statistically significant compared to control group.

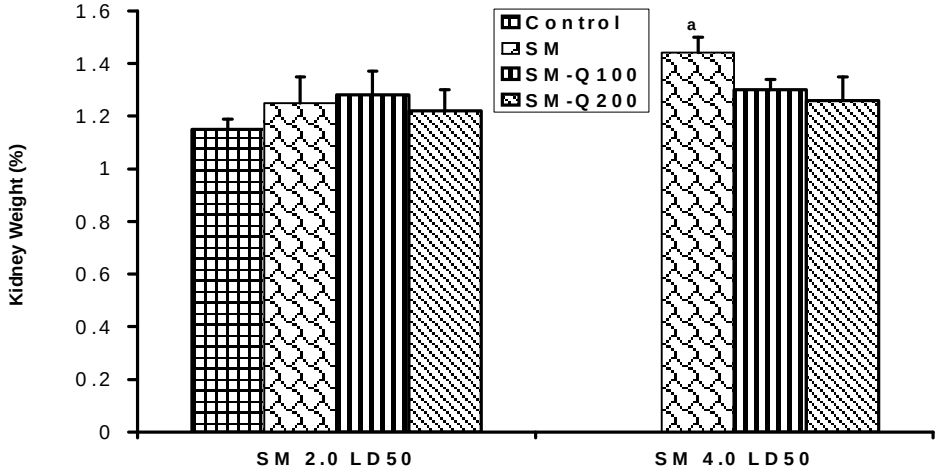

Fig. 3: Effect of quercetin on organ to body weight ratio of kidney on 7th day following 2.0 and 4.0 LD_{50} SM post exposure through percutaneous route in female mice. Values are mean ± SE (n=5).[a] Statistically significant compared to control group.

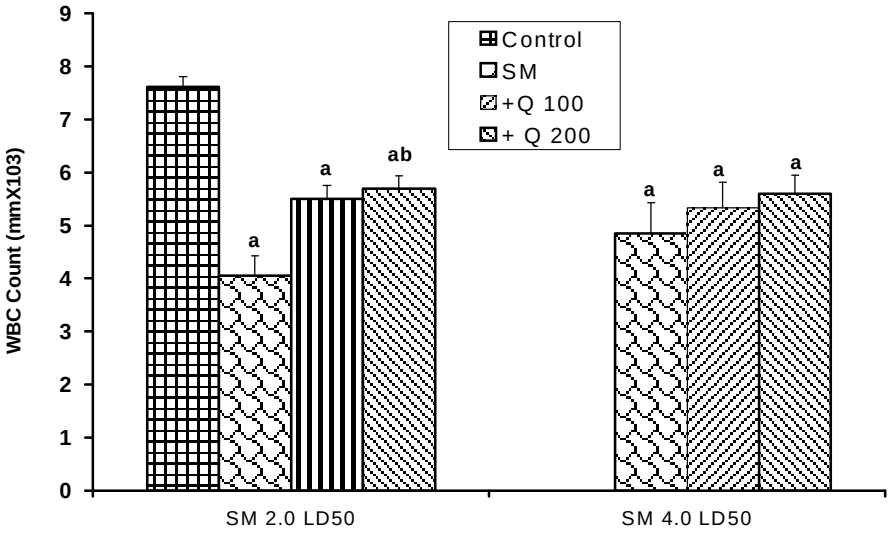

Fig. 4: Effect of quercetin on WBC counts on 7th day day following 2.0 and 4.0 LD_{50} SM post exposure through percutaneous route in female mice. Values are mean ± SE (n=5). [a]Statistically significant compared to control group. [b] Stat- istically significant compared to SM control group.

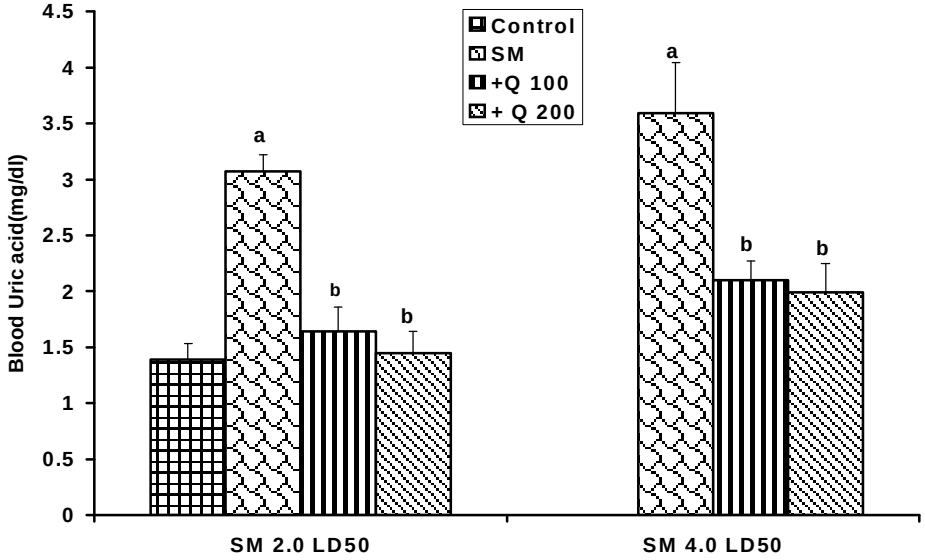

Figure 5: Effect of quercetin on blood uric acid on 7th day following 2.0 and 4.0 LD_{50} SM administration through percutaneous route in female mice. Values are mean ± SE (n=5). [a] Statistically significant compared to control group. [b] Statistically significant compared to SM control group

was more prominent in animals exposed to higher dose of SM. Effect of quercetin on hepatic and renal MDA content following SM (2.0 and 4.0 LD_{50}) is shown in Table 3. The level of MDA was significantly increased in hepatic and renal tissues following SM exposure. Quercetin treatment at the dose of 100 and 200 mg/kg was able to decrease hepatic as well renal lipid peroxidation. (Table 3). SM significantly decrease the WBC count in mice as compared to control animals. Only in lower dose of SM (2.0 LD_{50}) quercetin (200 mg/kg) was able to restore WBC count. Quercetin (100 mg/kg) failed to protect the decrease in WBC counts due to SM (Fig. 4).

Effect of quercetin on purine catabolism following SM administration through percutaneous route in mice is shown in Fig. 5. The level of uric acid in the blood increased following SM administration. Quercetin has shown a beneficial effect on restoring blood uric acid level in both the doses of SM.

DISCUSSION

The mechanism of SM induced injury is not fully understood. The primary target organs of SM induced toxicity are eyes, respiratory tract, and skin. SM is also capable of exerting systemic toxicity, leading to death particularly in laboratory animals [2]. SM is highly lipophilic and absorbed very quickly through the skin. After a latent period of 6-24 h erythema and blisters appear on the skin. Generally in the animal's skin blisters do not appear but otherwise, the lesions are similar to those observed in humans. Pulmonary complications, mainly on the upper respiratory tract, such as haemorrhagic inflammation, sore throat, hoarseness, cough, bronchitis and bronchopneumonia are observed in SM exposed victims [4].

In the present investigation various doses of quercetin protected the body weight reduction and increased survival time following percutaneous administration of SM. Dose dependent decrease in body weight following SM administration in mice have been reported [9]. Our earlier studies showed that flavonoids (gossypin, hydroxyethyl rutoside and quercetin) and vitamin E increased survival time, protected loss of body weight and decreased lipid peroxidation following percutaneous exposure of SM [9,11]. The protection may be due to the antioxidant and free radical scavenging properties of quercetin [26].

The reduced glutathione (GSH) is the main nonenzymatic antioxidant defence with in the cell, reducing different peroxides, hydroperoxides and radicals [19]. It is usually assumed that GSH depletion reflect intracellular oxidation. On the contrary, an increase in GSH concentration could be expected to prepare the cell against a potential oxidative insult. [27]. Percutaneous exposure of SM induced remarkable decrease in the concentration of GSH in blood, hepatic and renal tissue. The GSH content of hepatic and renal tissues was restore to a significant extent by quercetin. Quercetin has been previously found to increase intracellular GSH concentration in various cell lines [27-29]. These studies reports that increase in GSH is preceded by quercetin by stimulation of y-glutamylcysteine synthetase, the enzyme involved in glutathione synthesis [28,29]. The hepatic lipid peroxidation was also protected by quercetin. In our previous study, we found that percutaneous and inhalation exposure of SM depleted GSH in blood, lung and hepatic tissue and induced hepatic and lung lipid peroxidation as evidenced by enhanced level of MDA [9,11]. SM cause significant depletion WBC counts. In this study we observed that WBC counts were improved by quercetin in lower dose of SM. However, at higher dose of SM quercetin was failed to restore WBC counts.

Percutaneous exposure of SM increases uric acid accumulation in the blood. SM enters the systemic circulation and alkylates the DNA, leading to DNA strand breaks and apurination. Apurinated bases are catabolized to hypoxanthine, xanthine, and finally to uric acid, resulting in the increased level of blood uric acid. This effect was antagonised by the antioxidant (quercetin). Protection of blood uric acid may be due to the inhibition of ADP degradation which is the end product of ATP loss after DNA alkylation. Decrease in uric acid level is normally associated with starvation, but in the present study though there was decline in food intake after SM administration, increased uric acid level was observed [30]. SM induced skin lesions are similar to thermal injury. Increased uric acid level is also reported in thermal injury due to increased xanthine oxidase activity [31]. Increased excretion of uric acid has been reported following percutaneous administration of SM [10]. In the present study we have used two doses of quercetin as protective agents against SM toxicity through percutaneous route. Quercetin was able to restore loss in body weight, increased survival time and reduced oxidative stress produced by SM in a dose dependent manner. Quercetin's ability to reduce SM induced toxicity may be due to its antioxidant property.

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