Chapter-5
Prophylactic Effect of THC-Loaded Chitosan Microspheres against Percutaneously Administered Sulphur Mustard

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5.1 **INTRODUCTION**

Bis(2-chloroethyl)sulphide, commonly known as sulphur mustard (SM) or mustard gas is listed in the Schedule 1 of Chemical Weapons Convention (CWC). SM is a compound with potent alkylation, blister-forming, cytotoxic, mutagenic and carcinogenic properties (Wormser, 1991).

SM was one of the first chemical weapons deployed against troops on a battlefield during World War I, almost hundred years ago, over 1 million casualties were caused by SM, earning its title “King of the Battle Gasses” (Paromov et al., 2007). Military use of SM has been documented in a number of situations since then. In 1988, SM was used with devastating results by Iraq military forces against civilian targets and later during the Iran-Iran war (Paromov et al., 2007). Though, the Chemical Weapons Convention is signed and ratified by several countries and the stockpiled chemical warfare agents are being destroyed, still the threat persists from the use of chemical weapons by terrorists as it is a simple chemical compound readily synthesisable without elaborate technology. Still, stockpiles of SM is available with the countries. Fears of an attack may be warranted, but the larger concern may be for the tons of such chemicals that were produced during war and then dumped at sea, buried in landfills or left to decay in storage facilities near populated areas prone to risk of
accidental or occupational exposure due to inappropriate disposal methods (Szinicz et al., 2007; Geraci, 2008).

SM's property to cause harm to multiple organ systems at extremely low doses in virtually any environment make it effective war tool. SM forms sulfonium ion in the body and alkylates DNA leading to DNA strand breaks and cell death (Papirmeister et al., 1991). Due to high electrophilic property of the sulfonium ion, SM binds to a variety of cellular macromolecules (Somani and Babu, 1989). Eyes, skin and respiratory tract are the principal target organs of SM toxicity (Papirmeister et al., 1991; Pechura and Rall, 1993). Extensive exposure may affect the cardiac, reproductive, digestive, hematological and nervous systems (Geraci, 2008).

The first clinical manifestation of SM poisoning in victims occur in the eyes with a sensation of grittiness, lacrimation, photophobia, blepharospasm and corneal ulceration (Balali-Mood and Hefazi, 2006). If exposure to vapor is prolonged, rhinorhea, laryngitis, bronchitis, necrosis of mucous membranes of the respiratory track and bronchopneumonia will occur. Due to high surface area, skin is one of the most vulnerable organs when subjected to SM exposure. It produces severe damage to skin causing lesions and blisters which are extremely slow in healing. Further, it may cause ulceration, vesication and generate secondary infections (Balali-Mood and Hefazi, 2006; Paromov et al., 2007). Due to its hydrophobic nature, SM easily penetrates and accumulates in the lipid components of exposed
tissues. Skin not only accumulates but also distributes SM to other tissues and organs (Paromov et al., 2007).

In the recent years, substantial efforts have been made in developing pharmacological strategies against the toxic effects of SM with a focus on prevention and/or reversing damage on alkylated critical cell targets, improve calcium regulation, protect cell mediated biochemical disruption or prevent cytotoxicity (Papirmeister et al., 1991; Casillas et al., 2000). Though lots of compounds have shown good prophylactic as well as therapeutic protection in vitro, there in vivo efficacy has not been proved (Sawyer et al., 1996; Sawyer, 1998; Sawyer and Risk, 2000).

The lack of correlation between in vivo and in vitro systems is a major drawback in the drug development against SM toxicity. Till date there is no conclusive therapy to counter the systemic toxicity of SM. Cellular mechanism by which SM causes cytotoxicity is studied in past resent years and provide better understanding for therapeutic applications of specific cytoprotectants. After CMC came into force, destruction of SM is being carried out by the declared state parties. An effective prophylactic or therapeutic antidote in addition to the personal protection is required for this purpose (Vijayaraghavan et al., 2002). Taking into consideration the increasing terrorist activities with threat of use of chemical weapons, drug development against SM is required not only for defence personnel but also for civilian usage, as preparedness.
One of the important mechanisms of action of SM cytotoxicity is based on the depletion of reduced glutathione (GSH), and subsequent lipid peroxidation and free radical generation (Somani and Babu 1989; Lakshamana Rao et al., 1999). Development of oxidative stress could be attributed to the propensity of SM to react with sulfhydryls and intracellular reductases, thereby depleting cellular antioxidant (Jurenka, 2009). Recruiting inflammatory cells generate reactive oxygen species that further contribute to oxidative stress.

Flavonoids are a group of polyphenolic compounds found ubiquitously in plants. They exhibit a variety of biological activities, such as anti-inflammatory, antioxidant, antiviral and antitumor actions (Formica and Regelson, 1995). Some of these natural antioxidants like gossypin, quercetin, tocopherol acetate and some other flavonoids showed protection against percutaneously administered SM (Vijayaraghavan et al., 1991; Vijayaraghavan et al., 2009; Gautam and Vijayaraghavan, 2007; Gautam et al., 2007). Vijayaraghavan et al., (2006) reported that ethanolic extract of leaf and fruit of *H. rhamnoides* (Sea Buckthorn) provide significant protection against SM induces oxidative stress. Gautam et al (2005) reported that percutaneous administration of SM induce oxidative stress could only partially protected by oral administration of *Aloe vera* gel. Tropical application of *Aloe vera* gel may be beneficial for protecting the skin lesions induced by SM exposure.
Tetrahydrocurcumin (THC) possess strong antioxidant activity due to the presence of hydroxyl and methoxyphenyl groups at 4th and 3rd position and shows identical β-diketone of 3rd and 5th substitution in heptane moiety (Murugan and Pari, 2007; Sugiyama et al., 1996). THC is a phenolic compound, major colourless metabolites of curcumin, which is derived from the plant Curcuma longa (Turmeric), a common spice widely used in India (Patumraj and Yoysungneon, 2007). Holder et al., (1987) have first identified metabolite in intestinal and hepatic cytosol from humans and rats.

Curcumin is the yellow bioactive ingredient of turmeric (powdered rhizomes of Curcuma longa) that has been used as a dietary spice and versatile traditional medicine for thousands of years, in particular in Indian Ayurvedic medicine. Over the past decades, this polyphenolic compound has been successfully applied against a wide variety of disorders, leading to the identification of numerous promising therapeutic benefits such as anticancer, anti-inflammatory, antioxidant, immunomodulatory, cardio/neuro protective activities (Pari et al., 2008; Goel et al., 2008; Epstein et al., 2010).

Many evidences confirm the efficacy of curcumin as an herbal medication on inflammation, antioxidant supply, neoplasm and on the apoptotic pathway (such as NF-KB) (Das and Vinayak, 2012; Schaffer et al., 2011). Curcumin can affect the TGF-beta/Smads signaling pathway and also can regulate inter-cellular adhering molecule such as Laminin and cathepsin that was present as one of
the main mechanisms of SM injury (Li and Chen, 2011; Adelipour et al., 2011; Zhang et al., 2011). The efficacy of curcumin on the response of airway epithelial cell to toxic agents is also reported (Rennolds et al., 2012). Recently, curcumin was effectively used for the treatment of late skin lesions caused by SM exposure (Panahi et al., 2012).

Effective control of severity of SM injury disorder is well established in spite of low systemic availability because of first-pass metabolism and some degree of intestinal metabolism when administered via the oral route (Sharma et al., 2007). Curcumin is slightly absorbed in the gastrointestinal tract because it is practically insoluble in water (Cui et al., 2009). Extensive intestinal and hepatic metabolism and rapid elimination additionally restrain curcumin bioavailability (Shaikh et al., 2009). Whether curcumin or its metabolites, such as THC, are responsible for the reported effects is still under dissolution.

Recently, attention is focused on THC as one of the major metabolites of curcumin due to its enhanced antioxidant activity in both in vitro and in vivo systems (Okada et al., 2001; Pari and Murugan, 2004). Structurally, THC and curcumin have identical bita-diketon structure and phenolic groups, but with a difference that THC lacks the double bonds (Sugiyama et al., 1996; Okada et al., 2001). Sugiyama et al. (1996) demonstrated that THC exhibited similar physiological and pharmacological properties as the active form of
curcumin *in vivo*. Naito et al., (2002) showed clear involvement of THC in biochemical and molecular actions at the cellular level in ameliorating oxidative stress in cholesterol-fed rabbits. Some researchers have also focused on the neuroprotective role of curcumin in amyloid neurotoxicity and amyloid fibrin formation in Alzheimer’s models and other possible neurodegenerative diseases (Lim et al., 2001; Frautschy et al., 2001; Yang et al., 2005). Okada et al. (2001) have reported that THC has more potent antioxidant activity than curcumin.

Curcuminoids induce antioxidant enzymes, such as glutathione peroxide, glutathione S-transferase and NADPH:quinine reductase, but THC was found to be more active than curcumin and scavenged Fe-NAT-induce free radicals more effectively than curcumin *in vitro* (Okada et al., 2001). Venkatesen et al. (2003) reported that THC had higher activity then curcumin in protecting the nitrite induced oxidation of hemoglobin and lysis of erythrocytes. Pari and Murugan (2004) have reported the hepatoprotective effect of THC against erythromycin estolate induced toxicity. THC was reported to show more pronounced protective effect than curcumin against chloroquine (CQ) induced hepatotoxicity and it also exhibits potential protective effect over arsenic-induced hepatotoxicity in rat (Pari and Amali, 2005; Muthumani, 2013). Since, THC shows greater antioxidant activity, among all curcuminoids, and due to their better solubility and stability than curcumin, THC, THC-loaded chitosan microspheres
and placebo chitosan microspheres have been studied for their protective effect against SM toxicity.
5.2 MATERIALS AND METHODS

• Chemicals

Chitosan and THC-loaded chitosan microspheres were prepared by spray-drying technique with mean particle size of 3.2 and 3.8 µm, respectively. Tetrahydrocurcumin (THC) was obtained from Sigma Chemical Co. (St. Louis, MO. USA). SM for the study was synthesised in the Synthetic Chemistry Department of DRDE, Gwalior. 99% purity of SM was observed when analysed using Gas Chromatographer. O-pthalaldehyde (OPT) oxidised and reduced glutathione, 4’6-diamidino-2-phenylindole (DAPI) were from Sigma Chemical Co. (St. Louis, MO. USA). Other chemicals of high purity were from Merck Ltd. (Mumbai, India) or Qualigen Pvt. Ltd. (Mumbai, India).

• Animals

Randomly bred female Swiss albino mice in the weight range of 25 to 30 gm, maintained in Defence Research and Development Establishment’s animal facility were used for the study. The animals were housed in polypropylene cages on steam sterilised paddy husk as the bedding material under controlled environmental conditions with free access to food (standard pellet diet, Amrut Ltd, India) and water allowed until two hours before the experiment. The care and
maintenance of animals were as per approved guideline of the Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA:India). A day before dermal application of SM, hair on the back of the mice were closely clipped using a pair of scissors.

5.2.1 Protective efficacy of Placebo, THC-loaded chitosan microspheres and THC

To evaluate the protective effect of placebo, THC-loaded chitosan microspheres and THC on oxidative stress markers, the following groups (four mice per group) were utilized:

i. Distilled water, p.o. + PEG 300, p.c. (control group)

ii. Distilled water, p.o. + SM, 2 LD$_{50}$ (16.4 mg kg$^{-1}$) in PEG 300, p.c.

iii. Chitosan microspheres (200 mg/kg), p.o., 30 min prior SM + SM, 2 LD$_{50}$ (16.4 mg kg$^{-1}$) in PEG 300, p.c.

iv. THC-loaded chitosan microspheres (200 mg/kg), p.o., 30 min prior SM + SM, 2 LD$_{50}$ (16.4 mg kg$^{-1}$) in PEG 300, p.c.

v. THC (200 mg/kg), p.o., 30 min prior SM + SM, 2 LD$_{50}$ (16.4 mg kg$^{-1}$) in PEG 300, p.c.
SM was diluted in polyethylene glycol (PEG 300) and applied on the back of the mice (percutaneous route). The SM dilutions were made in such a manner that the quantity applied was not more than 100 µl. Placebo, THC-loaded chitosan microspheres and THC was administered orally.

The animals were weighed daily and on 7th day blood was drawn from ocular plexus under ether anesthesia. Then animals were sacrificed by cervical dislocation and liver and spleen were removed for further analysis. Pieces of liver were removed, blotted, weighed and used for the estimation of GSH, GSSG and malondialdehyde (MDA). Pieces of liver and spleen were also removed for histological studies. The collected blood samples were used for the measurement of RBC, WBC count and hemoglobin estimation.

5.2.2 Organ to Body Weight Indices

After sacrificing the animals, liver, spleen and kidney were dissected out, freed from adhering tissues and weighed. The organ to body weight indices (OBWI) were calculated following the formula:

\[
OBWI = 100 \times \left( \frac{\text{organ weight}}{\text{body weight}} \right)
\]

5.2.3 Biochemical and hematological evaluation

The liver samples were used for the estimation of reduced glutathione (GSH), oxidised glutathione (GSSG), and malondialdehyde
(MDA). Fluorimetric method of Hisin and Hilf (1976) was used for the determination of hepatic GSH and GSSG estimation. For this, approx.
150 mg of liver tissue was homogenized in 4 ml of phosphate EDTA buffer (pH 8.0) using a teflon coated homogenizer. Metaphosphoric acid (25% w/v) was added to precipitate the proteins present in the homogenate. It was centrifuged at 10,000 g at 4°C for 15 min and the supernatant was used for the estimation of GSH and GSSG. 0.25 ml of supernatant was added to 100 µl of 1mg/ml fluorescent dye OPT. After 15 min of incubation at room temperature, readings were taken at 420 nm emission and 350nm excitation.

Hepatic lipid peroxidation was determined by measuring the level of MDA according to the method of Buege and Aust (1978). One hundred milligram of liver was directly homogenised in 0.15 M KCL (10% homogenate), 0.2 ml of 30% trichloroacetic acid (TCA) and 0.5ml of 0.8% thiobarbituric acid (TBA) was added and homogenate was boiled for 30 min. The content of the tubes were cooled up to room temperature, centrifuged and absorbance of the clear supernatant was measured at 535 nm. The amount of MDA was calculated using molar extinction coefficient of 1.58 x 10^5/M per cm. The blood was used for the estimation of RBC content, WBC content and blood hemoglobin using Backman Coulter Cell Counter (USA).
5.2.4 **Histological Studies**

Sample of liver and spleen were fixed in 10% neutral buffered formalin solution. After proper fixation, small pieces were processed, dehydrated and embedded in paraffin wax. Multiple sections of 5 to 6 µm thickness were prepared and stained with haematoxylin and eosin for light microscopic observation (Mc Manus and Mowry, 1965). Lesions were marked and compared with that of control. The severities of lesions were characterised using LEICA-Qwin-500 Image Analyser (Leica Orthoplan, Germany).

5.2.5 **Statistical Analysis**

All the variables were analyzed by one-way ANOVA followed by Bonferroni multiple comparisons test. A probability of <0.05 and less is taken as statistically significant. Sigma State (SPSS Inc., USA) was used for statistical analysis.
5.3 RESULT AND DISCUSSION

5.3.1 Effect on % Body Weight Change and Organ to Body Weight Ratio after SM Application

The effect of pretreatment of chitosan microspheres, THC-loaded chitosan microspheres and THC on body weight change is given in Table 5.1. One of the major symptoms of SM toxicity was decrease in body weight. The animal body weight decreased progressively after 2LD\(_{50}\) administration of SM. After 24 hour of SM exposure no significant change in animal body weight was observed. The change in animal body weight was observed after 3\(^{rd}\) day, it’s reduced nearly 26% while after 7\(^{th}\) day of percutaneous administration of SM, the body weight was 59.6% as compared to first day SM exposed group. Other then the decrease in body weight, the animals did not show erythema or edema. Due to severe body weight loss, the mice appeared emaciated. Weight loss was also observed in groups treated with chitosan microspheres and THC. Animal body weight decrease after 7 days post SM administration was significantly protected by THC-loaded chitosan (Table 5.1).

Vijayaraghavan et al. (2005) reported that SM is more toxic through the percutaneous route then oral and subcutaneous routes. For percutaneous application of SM, PEG-300 was used as a diluent, which is a high molecular weight liquid. It is possible that PEG may be
preventing SM evaporation and facilitating its absorption through percutaneous route which may be the reason for delayed deaths as well as high toxicity. Since the animals were kept in a well-ventilated cage, the possibility of inhalation exposure upon application was minimal. The body weight of animals decreased progressively after percutaneous administration of SM, and in some cases 50 to 60% reduction in initial body weight was reported (Gautam et al., 2007; Vijayaraghavan et al., 2008). The progressive decreases in animals body weight was partially due to the reduced food and water intake as a result of SM-induced injury to epithelial cells of the esophagus.

The protection against SM induced weight loss was observed when THC-loaded chitosan microspheres were administered but such protection was not observed when THC was administered. This may be attributed to the improved bioavailability, mucoadhesive, absorption enhancing and sustained-release property of THC-loaded chitosan microspheres which improves the interaction of THC with SM preventing the early responses in the body. Gautam et al. (2007) reported that mice died within a period of 14 days after SM (2LD$_{50}$) application and beyond this period they survived. At lower doses weight loss was seen up to 12 days after percutaneous SM application, and then body weight started improving. Gautam et al. (2007) reported that protection on SM-induced weight loss was observed only when flavonoids and natural antioxidants were administered as pre-treatment or simultaneous treatment but not as post-treatment. In
the case of post treatment, flavinoids and natural antioxidants has no role in reviving the affected cells. Similar observations were made in the protection of curcuminoids in ethanol induced oxidative stress injury in brain, liver, heart and kidney (Rajakrihna et al., 1999; Rukkumani et al., 2004). This shows that THC should be available to interact with the active metabolite of SM.

Reduction of body weight was used as a key indicator for the determination of animal general health status. SM exposure decreased the body weight, absolute and relative liver and spleen weights, leading to significant decrease in animal growth and alteration in organ to body weight ratio.

The effect of pretreatment of chitosan microspheres, THC-loaded chitosan microspheres and THC on organ to body weight change is presented in Table 5.2. Significant decrease was observed in liver and spleen weight after SM administration. This may be due to the effect of SM on fast growing cell i.e. liver and spleen. Vijayaraghavan et al. (2008) reported that dermally applied SM affected liver and spleen. There was no significant change in the weight of kidney, 7th day after SM exposure.

SM induced decrease in Organ to Body Weight Index (OBWI) of liver was significantly protected by THC-loaded chitosan microspheres but not by THC alone. Chitosan microspheres also showed significant protection in OBWI of liver. The liver is the main
organ for detoxification and beneficial effects of flavonoids and natural antioxidants may be related to their hepatoprotective potential. However, the spleen of SM administered mice was very small and pretreatment with THC-loaded chitosan microspheres and chitosan microspheres improved spleen weight but only marginally by THC alone. Vijayaraghavan et al. (2008) have reported that SM exposure has significantly induced disturbances of the intake of water and food, total body weight, OBWI of liver and spleen of animals. Accordingly, decrease in animal body weight and change in OBWI was significantly protected by pretreatment with THC-loaded chitosan microspheres.

**Table 5.1:** Effect of pretreatment of Chitosan MS, THC-loaded chitosan MS and THC on percent body weight change after administration of 2LD$_{20}$ (16.2 mg.kg$^{-1}$) SM

<table>
<thead>
<tr>
<th>Groups</th>
<th>1st Day (%)</th>
<th>4th Day (%)</th>
<th>7th Day (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 1.5</td>
<td>100 ± 2.4$^b$</td>
<td>100 ± 4.0$^b$</td>
</tr>
<tr>
<td>SM (2 LD$_{50}$) only</td>
<td>97.7 ± 1.0</td>
<td>71.4 ± 2.6$^a$</td>
<td>59.6 ± 4.2$^a$</td>
</tr>
<tr>
<td>Chitosan Microspheres (Placebo) + SM</td>
<td>97.7 ± 2.4</td>
<td>84.4 ± 5.7</td>
<td>62.8 ± 8.1$^a$</td>
</tr>
<tr>
<td>THC-loaded Chitosan Microspheres + SM</td>
<td>96.0 ± 0.7</td>
<td>95.0 ± 1.8</td>
<td>81.8 ± 0.9$^b$</td>
</tr>
<tr>
<td>THC + SM</td>
<td>92.5 ± 5.9</td>
<td>75.4 ± 4.3$^a$</td>
<td>60.5 ± 3.1$^a$</td>
</tr>
</tbody>
</table>

Mean ± SE (n = 4) have been shown, $^a$Significantly different from control group, $^b$Significantly different from SM group.
Table 5.2: Effect of pretreatment of Chitosan MS, THC-loaded chitosan MS and THC on organ to body weight change after administration of 2LD\textsubscript{20} (16.2 mg.kg\textsuperscript{-1}) SM

<table>
<thead>
<tr>
<th>Groups</th>
<th>Liver weight (%)</th>
<th>Spleen weight (%)</th>
<th>Kidney weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.31 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.79 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.12 ± 0.01</td>
</tr>
<tr>
<td>SM (2 LD&lt;sub&gt;50&lt;/sub&gt;) only</td>
<td>3.27 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.26 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.51 ± 0.11</td>
</tr>
<tr>
<td>Chitosan Microspheres (Placebo) + SM</td>
<td>5.25 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.70 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.35 ± 0.06</td>
</tr>
<tr>
<td>THC-loaded Chitosan Microspheres + SM</td>
<td>4.95 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.70 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.46 ± 0.22</td>
</tr>
<tr>
<td>THC + SM</td>
<td>4.74 ± 0.59</td>
<td>0.44 ± 0.08</td>
<td>1.53 ± 0.13</td>
</tr>
</tbody>
</table>

Mean ± SE (n = 4) have been shown, <sup>a</sup>Significantly different from control group, <sup>b</sup>Significantly different from SM group.

5.3.2 Protection studies on different biochemical variables and hematological variables

The effects of chitosan microspheres, THC-loaded chitosan microspheres and THC on oxidative stress markers are given in Table 5.3. Significant depletion in hepatic GSH and GSSG content was observed when compared to control group. In SM treated group, 36.5% and 48.6% reduction in GSH and GSSG was observed after 7 days post percutaneous administration of SM. This reduction in hepatic GSH and GSSG was significantly protected by THC-loaded chitosan microspheres and THC treated groups. However, GSH and
GSSG level in chitosan microsphere treated group were better than the SM group but still significantly lower than the control group. There was a significant increase in MDA level in SM group compared to control and this was protected by oral administration of THC-loaded chitosan microspheres. The chitosan microspheres and THC did not show any significant difference.

Percutaneous administration of SM decreased the WBC count (not statistically significant) and significantly increased the RBC count and the hemoglobin content (Table 5.4). This elevation in RBC count was significantly protected by THC-loaded chitosan microspheres and THC but not by chitosan microspheres. There was nearly 46% significant increase in hemoglobin level after 7 days post SM administration. This elevation in hemoglobin level was significantly protected by THC-loaded chitosan microspheres but not by chitosan microspheres and THC treated groups.

Sulphur Mustard is a potential alkylating agent with electrophilic property. This results in the production of reactive oxygen species (ROS) and oxidative stress with depletion of cellular detoxifying thiols including glutathione (Naghi, 2002). A number of mechanisms have been proposed for the toxic effect of SM and out of those mechanisms oxidative stress mediated mechanism is gaining importance (Vijayaraghavan et al., 1991; Elsayed et al., 1989; Elsayed et al., 1992; Kumar et al., 2001).
In the aqueous medium, SM is capable of undergoing nucleophilic substitution reactions to initiate free radicals either directly or indirectly and also generate reactive oxygen intermediates (Elsayed et al., 1989). Reduced glutathione, a cysteine containing tripeptide plays an important role in the detoxification of xenobiotics and in the scavenging of reactive species and free radicals. It accounts for almost 90% of cellular nonprotein thiols (Gentilhomme et al., 1992). GSH levels are reduced in human peripheral lymphocytes when incubated with SM (Ray et al., 1995). Davison et al. (1961) reported that the major urinary metabolites of SM are glutathione conjugates. Decrease in the level of GSH has been reported in vivo by several investigators as a toxic effect of SM and the monofunctional analogue chloroethylethyl sulphide (Vijayaraghavan et al., 1991; Elsayed et al., 1989; Das et al., 2003).

Significant reduction of GSH level due to SM is indicative of oxidative stress and cellular damage. In the present study, significant decrease not only in GSH but in GSSG was also observed. Since SM interacts with a wide variety of biomolecules causing multiorgan failure, decrease in GSH and GSSG is expected. Direct interaction of GSH with SM is also possible. Being electrophile in nature, SM has high affinity towards sulphhydryls groups and thus it depletes GSH in the body. The decrease in GSH and GSSG level was significantly protected by THC-loaded chitosan microspheres and THC but not by chitosan microspheres. In this study THC-loaded chitosan
microspheres and THC alone were administered 30 minutes prior to SM exposure. It has been already reported that natural antioxidants protects only when they are administered as a pre-treatment or simultaneously that means that antioxidants should be available before the SM metabolite reach the target molecule (Gautam et al., 2007). In such a case antioxidants donates its hydroxyl group to free radicals sparing GSH.

**Table 5.3:** Effect of pretreatment of Chitosan MS, THC-loaded chitosan MS and THC on biochemical parameters after percutaneous administration of SM 2LD$_{50}$ (16.2 mg.kg$^{-1}$)

<table>
<thead>
<tr>
<th>Groups</th>
<th>GSH (%)</th>
<th>GSSG (%)</th>
<th>MDA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 5.4$^b$</td>
<td>100 ± 2.2$^b$</td>
<td>100 ± 5.3$^b$</td>
</tr>
<tr>
<td>SM (2 LD$_{50}$) only</td>
<td>36.5 ± 5.3$^a$</td>
<td>48.6 ± 4.1$^a$</td>
<td>146.4 ± 9.9$^a$</td>
</tr>
<tr>
<td>Chitosan Microspheres (Placebo) + SM</td>
<td>63.1 ± 6.2$^a$</td>
<td>61.1 ± 7.9$^a$</td>
<td>135.7 ± 7.6</td>
</tr>
<tr>
<td>THC-loaded Chitosan Microspheres + SM</td>
<td>80.3 ± 6.3$^b$</td>
<td>84.7 ± 8.3$^b$</td>
<td>106.2 ± 5.4$^b$</td>
</tr>
<tr>
<td>THC+ SM</td>
<td>76.6 ± 10.1$^b$</td>
<td>81.4 ± 7.8$^b$</td>
<td>114.3 ± 8.8</td>
</tr>
</tbody>
</table>

Mean ± SE (n = 4) have been shown, $^a$Significantly different from control group, $^b$Significantly different from SM group. Chitosan microspheres, THC-loaded chitosan microspheres and THC (200 mg/kg, oral) were administered 30 min prior to SM exposure. The animals were sacrificed 7 days post SM administration. Control values: GSH=3.98±0.21 µ mole/gm of tissue, GSSG=1.76±0.04 µ mole/gm of tissue, MDA=4.17±0.22 n mole/ gm of tissue.
Normally lipid peroxidation takes place in normal cells to some extent and due to SM toxicity; there is increase in lipid peroxidation as shown by an increase in malondialdehyde (MDA) level. The MDA was increased in SM toxicity to 164% and this was recovered by pre-treatment with THC-loaded chitosan microspheres but not by THC alone may be due to its poor bioavailability result in limited adsorption through mucosal route. Usually lipid peroxidation takes place in the presence of iron molecules by the reactive oxygen species. Flavonoids are known to chelate iron, thereby removing the causal factor for the development of free radicals. Curcumonoids in particular is known for its iron chelating and iron stabilising property. Direct inhibition of lipid peroxidation is another protection measure of flavonoids. This may be the reason for the dose of flavonoids to be fairly high. Significant recovery in MDA levels is due to the antioxidant activity of THC.

The chemical moieties which are attributed to the antioxidant property of THC are hydroxyl and methoxyphenyl groups in 4, 4’ and 3, 3’ position in benzene ring and shows identical β-diketone of 3rd and 5th substitution in heptane moiety. THC showed the highest potency, implying that hydrogenation of curcuminoids increased their antioxidant activity. Moreover THC is a phenolic chain-breaking antioxidant, which donates H atoms from the phenolic OH groups and the H atom donation takes place at the active methylene groups in the diketone moiety. Phenolic groups in THC are essential
for activity, and are more effective at the \textit{para} position than at the \textit{ortho} position. In addition, an electron donating group at the \textit{ortho} position relative to the phenolic group is also required for activity. This theoretical approach favors the necessity of a phenolic OH group for the antioxidant activity of THC.

Following percutaneous administration of SM, the animals appeared emaciated and dehydrated, resulting in reduced blood volume. Due to generation of reactive oxygen species by SM, membrane loses its integrity and fluidity. The endothelial cells fail to retain plasma in blood, and the viscosity and density of blood increases, resulting in increase in RBC count and haemoglobin content. Many reports are available that RBC count and haemoglobin content are increased after SM exposure (Ghanei, 2004; Mahmaudi et al., 2005). To compensate, the blood from spleen also enters the circulation resulting in the shrinkage of the spleen. All these effects are significantly protected by THC-loaded chitosan microspheres. It is generally expected that following SM administration there should be a profound decrease in WBC count. But, due to hemoconcentration the decrease in WBC count following SM administration is not significant. Hemoconcentration occurring due to the increased permeability of the endothelial cells of the blood vessels may be the reason for the increased RBC count and Hb content. SM is bifunctional alkylating agent leading to DNA strand breaks and cell death. curcumonoids has also been reported to possess antimutagenic and anti-genotoxic effects
Therefore, it has the potential to revert DNA cross-linking, chromosome aberrations and abnormalities that are sequelae of SM toxicity. It concludes that SM induced toxicity can be significantly prevented by the antioxidant efficacy of THC-loaded chitosan microspheres.

Table 5.4: Effect of pretreatment of Chitosan MS, THC-loaded chitosan MS and THC on hematological parameters after percutaneous administration of SM 2LD$_{50}$ (16.2 mg.kg$^{-1}$)

<table>
<thead>
<tr>
<th>Groups</th>
<th>WBC (%)</th>
<th>RBC (%)</th>
<th>Hb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 9.1</td>
<td>100.0 ± 4.8$^b$</td>
<td>100.0 ± 5.9$^b$</td>
</tr>
<tr>
<td>SM (2 LD$_{50}$) only</td>
<td>73.9 ± 14.7</td>
<td>151.7 ± 6.7$^a$</td>
<td>147.1 ± 5.2$^a$</td>
</tr>
<tr>
<td>Chitosan Microspheres (Placebo) + SM</td>
<td>79.8 ± 11.1</td>
<td>133.1 ± 7.6</td>
<td>137.6 ± 8.5</td>
</tr>
<tr>
<td>THC-loaded Chitosan Microspheres + SM</td>
<td>88.4 ± 13.7</td>
<td>105.9 ± 8.8$^b$</td>
<td>99.6 ± 9.7$^b$</td>
</tr>
<tr>
<td>THC + SM</td>
<td>93.2 ± 17.8</td>
<td>115.9 ± 20.8</td>
<td>106.7 ± 4.3$^b$</td>
</tr>
</tbody>
</table>

Mean ± SE (n = 4) have been shown. *Significantly different from control group, $^b$Significantly different from SM group. Chitosan microspheres, THC-loaded chitosan microspheres and THC (200 mg/kg, oral) were administered 30 min prior to SM exposure. Control values: WBC=8.80±0.8 $\times$ 10$^3$ cells/mm$^3$, RBC=7.3±0.4 $\times$ 10$^6$ cells/mm$^3$, Hb=12.8±0.8 g/dl.
5.3.3 **Histological Studies in Various Groups**

Histological features of spleen from control mice showed normal structure having white pulp consisting of lymphatic follicles and red pulp consisting of sinusoids germinal centre and periartheriolar lymphatic sheath (PALS). Histological observation of spleen of SM applied mice showed reduced density of white pulp follicles and congestion in red pulp severe congestion and hypocellularity of white pulp follicles. The spleen capsule was occasionally detached from the underlying parenchyma. This was partly caused by parenchymal atrophy and degenerative changes in the extracellular space leading to an overall shrinkage of the parenchyma and hypocellularity (Figure 5.1B). Treatment with chitosan microspheres did not protect mice spleen and degenerative changes were observed. Treatment with THC showed regeneration of spleen lesions, and in the case of THC-loaded chitosan microspheres there were minimal changes and the spleen resembled like that of the control.

Histological features of control mice showed normal hepatic chord, hepatic lobules and hepatocytes (Figure 5.2A). Liver histology of mice after SM administration revealed hepatocellular cytoplasmic vacuolar changes characterised by granulovacuolar degeneration and perinuclear clumping of cytoplasm. Vacuolar degeneration of hepatocytes was predominantly observed in the intermediate zone with vacuolization of centrilobular zone. The prominent lesions were
cell swelling, cytoplasmic vacuole formation, and accumulation of inflammatory cells composed of lymphocytes and mononuclear cells. Sporadic neutrophils were also observed in the degenerated hepatic parenchyma (Figure 5.2B). Treatment with chitosan microspheres did not offer any protection and the same magnitude of hepatic degradation was observed, while treatment with THC showed fewer disturbance in the lobular pattern and less infiltration of mononuclear cells. The hepatic lesions were minimal in mice liver treated with THC-loaded chitosan microspheres at 30 min pre-treatment. However, few inflammatory cells were observed in the vicinity of the central canal. Histological examination of spleen and liver showed that THC-loaded chitosan microspheres can protect SM-induced histological lesions which may be due to its free radical scavenging properties.
**Figure 5.1:** Effect of placebo, THC and THC-loaded chitosan microspheres (200 mg/kg, p.o.) against percutaneously administered SM (2 LD$_{50}$) on mice spleen. Chitosan, THC and THC-loaded chitosan microspheres were administered 30 minutes prior to SM administration. H&H, 40X. (A) Control spleen showing normal histology with germinal center, red and white pulp follicles. (B) Spleen of mouse exposed to SM showing congestion, hypocellularity and degradation of white pulp follicles. (C) Chitosan treated mouse spleen showing less protection and degenerative changes were observed. (D) THC treated mouse spleen showing lesser magnitude of splenic degeneration. (E) THC-loaded chitosan microspheres showing minimal changes in the spleen and resembling more like that of control.
Figure 5.2: Effect of placebo, THC and THC-loaded chitosan microspheres (200 mg/kg, p.o.) against percutaneously administered SM (2 LD$_{50}$) on mice liver. Chitosan, THC and THC-loaded chitosan microspheres were administered 30 minutes prior to SM administration. H&H, 40X. (A) Control liver showing normal hepatic chord, hepatocytes, central canal and kupffer cells. (B) SM administered mice liver (2 LD$_{50}$) showing hepatocellular vacuolation clumping of cytoplasm, necrosis and infiltration of inflammatory cells. (C) Chitosan treated mouse liver showing marginal protection. (D) THC treated mouse liver showing lesser magnitude of hepatic lesions. (E) THC-loaded chitosan microspheres treated mouse liver showing protection of hepatic degeneration.

The histopathological changes observed in the present study after percutaneous administration may be due to the systemic toxicity of some unidentified toxic molecule that is generated more in
percutaneous route of administration. All these changes were significantly reduced in mice treated with THC-loaded chitosan microspheres. As the alterations produced in the GSH indicates involvement of deleterious oxidative changes, increase levels of GSH would therefore be important in protecting cells against SM toxicity. Reports are available that pretreatment or simultaneous treatment with flavonoids can protect SM-induced histological lesions and this may be due to its free radical scavenging and anti-inflammatory or antioxidant property (Vijayaraghavan et al., 2008; Gautam et al., 2007; Vijayaraghavan et al., 2006). Similarly, Muthumani (2013) has reported that various pathological changes were observed in the liver of arsenic intoxicated rats such as portal triad with mild inflammation, cell infiltration, focal necrosis, and giant cell formation. Pre-administration of THC recovered the histological alterations induced by arsenic in the liver. Based on the above results, it could be concluded that THC-loaded chitosan microspheres is a hepatostimulant and exerts a significant hepatoprotective against SM-induced toxicity. THC-loaded chitosan microsphere show batter protection against SM induced toxicity than THC alone, due to its improved bioavailability owing to its high surface to volume ratio of microspheres, mucoadhesive and absorption enhancement property of chitosan microspheres.