Chapter 8

*Emilia sonchifolia (L.) DC*: Evaluation of the possible therapeutic application in conventional chemotherapy
8.1. Introduction

There is a need to develop protective agents to combat major side effects of chemotherapy, without a negative impact on the immune system while not negating the expected therapeutic outcome. This study is a pioneer attempt to find out the ameliorating effect of an active fraction from the medicinal plant *Emilia sonchifolia*, enriched with the major sesquiterpene γ-humulene (γ-hum). The major urological side effects of oxazaphosphorine cytostatic drug cyclophosphamide (CP) include urinary bladder oedema, urothelial damage, haemorrhage and disturbances in voiding (Corrow and Vizzard, 2009). Hepatic cytochrome p450 oxidase converts CP to 4-hydroxy cyclophosphamide (Huttunen et al., 2011), and these renally excreted 4-hydroxyl metabolites is responsible for its toxicity. In addition induction of oxidative stress by acrolein, one of the major metabolite of CP has deleterious effect on the bladder epithelium paving way to hemorrhagic cystitis (Emadi et al., 2009). An effective uroprotector either stabilize these toxic metabolites and thereby prevent its release or detoxify the acrolein being generated.

Lot of substances were screened for their ability to act as regional antidots and among them the compound sodium 2-mercaptoethanesulfonate (MESNA) found to be most promising (Brock, 1989). Administration of MESNA to alleviate the urotoxic effects of CP is not completely free of inauspicious events (Berlin and Mu Hugh, 1999). Although as a known uroprotector available today, MESNA was used as standard in the present study. The system is said to be under oxidative stress when the levels of antioxidants are low or the levels of oxidants are high in such a situation the oxygen free radicals will be higher than their normal levels. Antioxidant compounds can be used in chemotherapy to protect against these harmful free radicals. Considering this aspect, we have done a thorough investigation of the effect of our test material on the renal antioxidant system.
8.2. Materials and Methods

**Plant material:** - An active fraction from *Emilia sonchifolia*, enriched with γ-humulene (71%) (γ-hum).

**Dosage:** - γ-hum 5mg/kg body weight was intraperitoneally (i.p) administered for five consecutive days.

**Animals:** - Swiss Albino mice (6-8 weeks old)

**Experimental design**

Swiss Albino mice divided into 4 groups with a number of 24 animals/group. The mice in Group I was kept as normal without any treatment. Remaining 3 groups were treated with CP (1.5mmol/kg body weight, single acute dose). Group III was administered with γ-hum and Group IV with MESNA (150 mg/kg body weight, single dose) before CP administration. Group II kept as control with CP alone. From each group 8 mice were sacrificed at 4th hour, 24th hour and 48th hour after CP administration. Urine was collected prior to sacrifice (Wood et al., 2001). Blood was collected after sacrifice by heart puncture, and serum was separated and used for further analysis.

8.2.1. Biochemical investigations

The excised kidney and urinary bladder of the sacrificed animals were rinsed in ice cold normal saline. Tissue homogenate of these organs were prepared in chilled Phosphate buffered saline (PBS) in order to perform following biochemical parameters.

8.2.1.1. Reduced glutathione (GSH)

Briefly 125μl of 25% TCA was added to 0.5ml of the homogenate and kept cooled on ice for five minutes. Ellman’s reaction of GSH with Ellman’s reagent
dithionitrobenzene was used for estimation of reduced GSH according to the method of Moron et al. (1979).

8.2.1.2. Lipid peroxidation, total protein, serum creatinine and urea nitrogen

The levels of malondialdehyde (MDA) was estimated by extracting MDA-thiobarbituric acid (TBA) adduct using TBA and its subsequent measurement at 532nm (Ohkawa et al., 1979). Total protein, serum creatinine and urea nitrogen content was estimated using standard kits according to the manufacturer’s instructions.

8.2.1.3. Antioxidant enzyme measurements

The supernatant obtained after centrifugation (5,000g for 10 min at 4°C) of the kidney homogenate was assayed for superoxide dismutase (SOD) using nitroblue tetrazolium (NBT) reduction method of Mc Cord and Fridovich, 1969. Glutathione peroxidise (GPx) by the method of Hafeman et al., 1974 and catalase (CAT) by Aebi, 1984.

8.2.2. Serum cytokine levels

Serum collected from the sacrificed animals were quantified for various murine cytokines such as IL-2, IFN-γ and TNF-α on the same day using highly specific quantitative sandwich enzyme-linked immunosorbent assay kits according to the instructions of the manufacturer.

8.2.3. Bladder morphology and histopathology

Urinary bladder removed immediately after sacrificing the animals was examined on three different time intervals 4, 24 and 48 hours respectively. The morphological changes like colour, inflammation etc. Were observed and were confirmed by three different persons (Davis and Kuttan, 2000). Histopathology was done by fixing the bladder in 10% formaldehyde. Different concentrations
of alcohol were used for further treatments, and then the tissue was fixed in paraffin wax. Staining of sections with 4μm thickness was done using eosin and hematoxylin and thereafter subjected to microscopic analysis.

8.3. Results

8.3.1. Effect of γ-hum on BUN and UUN

There was an elevation in the blood and urine urea nitrogen level in animals by CP treatment when compared to normal animals (BUN: 24.48±2.58mg/dl; UUN: 14.48±1.3mg/dl). In the CP treated animals the BUN levels were 105.08±8.91mg/dl (4h), 76.26±4.94mg/dl (24h), 52.51±5.31mg/dl (48h) and UUN levels were 28.13±1.93mg/dl (4h), 25.37±3.95mg/dl (24h), 24.27±3.3mg/dl (48h) indicating renal damage. γ-hum treatment significantly reduced (P<0.001) these elevated levels to (BUN 35.12±4.67mg/dl) (4h), 28.7±3.64mg/dl (24h), 26.81±3.18mg/dl (48h)) and (UUN 18.35±2.55mg/dl (4h), 16.22±1.84mg/dl (24h), 15.25±3.47mg/dl (48h)) as shown in figure 8.1. These results were almost similar to the established uroprotector MESNA indicating their protective effect from the CP induced toxicity.

8.3.2. Effect of γ-hum on the total protein and serum creatinine levels

The CP administration drastically enhanced the total protein levels in serum to 10.81±0.67g/dl at 4h, 9.95±0.8g/dl at 24h and 8.19±0.96g/dl at 48h compared to the normal serum protein level 6.92± 0.57g/dl. Similarly the urine protein levels were elevated to 7.98±0.7g/dl at 4h, 8.95±0.85g/dl at 24h and 6.21±0.94g/dl at 48h compared to the normal urine protein level 4.38±0.53g/dl. The γ-hum treatment significantly diminished (P<0.001) these serum protein levels to 7.5±0.45g/dl at 4h, 6.94±0.71g/dl at 24h and 6.39±0.31g/dl at 48h and the protein in the urine to 5.44±0.35g/dl at 4h, 5.53±0.51g/dl at 24h and 4.85±0.32 g/dl at 48h and these results were almost comparable to the uroprotector MESNA. Similarly there was a hike in creatinine level in the serum by CP treatment (0.93±0.14mg/dl), (0.79±0.08mg/dl) (0.65±0.07mg/dl) at 4, 24 and 48h respectively. By γ-hum treatment this increased creatinine content was
Figure 8.1. Blood and urine urea nitrogen level

\[ a \text{p < 0.001, } b \text{p < 0.01} \]
Figure 8.2. A. Total protein

![Bar chart for total protein levels over time periods with annotations for statistical significance.]

\( ^a p < 0.001, \ ^b p < 0.01 \)

B. Serum creatinine

![Bar chart for serum creatinine levels over time periods with annotations for statistical significance.]

\( ^a p < 0.001, \ ^b p < 0.01 \)
reduced in a time dependent manner (0.64±0.13mg/dl), (0.53±0.11mg/dl) in 4h 24h and by 48th h attained a near normal value (0.39±0.05mg/dl), demonstrating its promising regulatory effect (figure 8.2).

8.3.3. Effect of γ-hum on GSH levels

CP treatment reduced the bladder GSH levels (1.54±0.4nmol/mg protein (4h), 1.87±0.09nmol/mg protein (24h), 2.43±0.33nmol/mg protein (48h)) and renal GSH levels 2±0.35nmol/mg protein, 2.42±0.27nmol/mg protein, 3.01±0.17nmol/mg protein at 4, 24 and 48 hour respectively compared with normal levels of GSH in bladder 4.44±0.29nmol/mg protein and kidney 5.87±0.07nmol/mg protein. The CP along with γ-hum significantly increased the bladder glutathione levels to 2.41±0.24nmol/mg protein (4h), 3.57±0.35 nmol/mg protein (24h), 4.07±0.27nmol/mg protein (48h) similar to the results obtained by MESNA treatment. The renal glutathione levels were also increased by γ-hum treatment to 3.48±0.51nmol/mg protein (4h), 4.66±0.17nmol/mg protein (24h), 5.41±0.43nmol/mg protein (48h) as shown in figure 8.3.

8.3.4. Effect of γ-hum on lipid peroxidation and renal antioxidant system

The renal antioxidant levels of SOD, CAT and GPx in 3 different time intervals were depicted in figure 8.4. The renal antioxidant status was very much lowered in the CP treated animals. The γ-hum treatment enhanced this lowered levels showing its potent antioxidant and free radical scavenging effect. CP elevated the lipid peroxidation 1.16±0.32nmoles/mg protein, 2.5±0.47nmoles/mg protein, 2.25±0.34nmoles/mg protein, 2.09±0.27nmoles/mg protein at time intervals of 4, 24 and 48h. γ-hum significantly (P<0.001) reduced this levels to 1.75±0.12nmoles/mg protein, 1.26±0.21nmoles/mg protein, 1.13±0.33 nmoles/mg protein in the respective time intervals pointing out towards a cell level protection (Figure 8.4).
Figure 8.3. Bladder and kidney GSH level

\[ a^{p<0.001}, \ b^{p<0.01} \]
Figure 8.4. Renal antioxidant levels

A. SOD

B. Catalase

\[ a \ p<0.001, \ b \ p<0.01, \ c \ p<0.05 \]
C. GPx

D. Lipid peroxidation

\( ^a p<0.001, \quad ^b p<0.01, \quad ^c p<0.05 \)
8.3.5. Effect of γ-hum on cytokine markers and histopathology

The three serum cytokine markers were positively regulated by γ-hum treatment. IFN-γ in CP+γ-hum and in CP+MESNA showed a time dependent increase that finally normalized in about 48 hours. IL-2 was also performed a similar strategy both compared to the decreased level in CP alone treatment. The effect was reversed in the case of TNF-α with a diminished level in the γ-hum and MESNA treated groups (figure 8.5). Apparent differences in the morphology of urinary bladder between control and treated groups were evident at different time intervals of sacrifice. There was an inflamed outlook with dark colouration at 4 h of CP treatment; in CP+γ-hum slight inflammation with usual colour was observed. Severe haemorrhage was shown at 24h of treatment in control compared to γ-hum+CP treated bladder which showed a tendency toward normalisation with slight haemorrhage. At 48h the condition became worse in CP group in comparison to γ-hum+CP that looked like normal. Use of the uroprotective agent MESNA produced appreciable results as well in the bladders. Bladder pathology was almost normalised by γ-hum treatment along with CP administration as shown in the histopathological sections at different time intervals (figure 8.6). In combination with γ-hum the necrosis of cells were reduced and the appearance was like normal epithelium with numerous folds and rugae.

8.4. Discussion

The toxic side effects of CP on the hepatic, urinary (Morandi et al., 2005), renal (Amudha et al., 2007) and haematopoietic (Papaldo et al., 2005; Schwartz et al., 2005) systems of the body often masks its beneficial therapeutic effects. Results obtained in the current study were found to be very promising for the usage of γ-hum, as an ameliorating agent against CP induced damages. γ-hum is found to be equal to or may be greater promising in comparison to MESNA because of its null side effects. The protective effects of γ-hum can be attributed to the already known free radical scavenging and anti-inflammatory effects of E. sonchifolia. The severely impaired bladder morphology along with bladder inflammation and oedema due to CP administration was markedly revamped by
Figure 8.5. Serum cytokine levels

A. TNF-α

B. IL-2

C. IFN-γ

\(^a p<0.001, \ ^b p<0.01\)
Figure 8.6. Histopathological analysis of urinary bladder in cross section after different time points

A. Normal
B. 4h after CP administration
C. 4h after CP+γ-hum administration
D. 24h after CP administration
E. 24h after CP+γ-hum administration
F. 48h after CP administration
G. 48h after CP+γ-hum administration
γ-hum treatment as evident from the histopathology. Histology revealed about normal structural organization along with functional recovery of the bladder tissues.

The inflammation and shifts in overall redox cycling is due to the pro oxidative metabolites of CP and also by peroxidation and additional engendering of free radicals (Kehrer and Biswal, 2000). GSH maintains cell integrity by protecting cellular constituents from oxidising and alkylating agents or by its involvement in detoxification mechanisms (Masella et al., 2004) and its reduction make mitochondria susceptible to free radicals prodded by itself leading to severe after effects (Hatono et al., 1996). Interactions of acrolein with GSH and one of the constituent aminoacid of GSH will lead to the reduction in GSH level (Manesh and Kuttan, 2005) and the resultant death or apoptosis of the cells lining the bladder. This hike in lipid peroxidation and reduction in GSH in CP administered animals can be reversed remarkably by γ-hum administration as evident from the graphical depiction of the results.

Apart from this the antioxidant levels were significantly increased suggesting the role of the antioxidant system in alleviating the CP induced toxic effects. When the levels of antioxidant enzymes are elevated the vulnerability to cell injury will subsequently diminish (Werts and Gould, 1986). Cellular enzymatic antioxidants SOD, CAT and GPx offer significant protective effects (Cerutti et al., 1994). These antioxidants also regulate intra cellular LPO hike and reduced antioxidant enzyme content well exhibited while in CP administration. Impediment in peroxidase and catalase enzyme levels along with a hike in malondialdehyde (MDA) denotes the harm caused by the ROS produced and the resulting severe oxidative stress on the urinary bladder (Sulkowska et al., 1998). γ-hum significantly negate toxic effects as confirmed from the results of the renal antioxidant status after treatment with this test material in CP induced animals. This regulatory effect γ-hum on the antioxidant system may be attributed to its free radical scavenging activity or by inhibiting seepage of lipid peroxides that well complements the previous studies reporting the antioxidant effects of the plant E. sonchifolia.
In chronic bladder inflammatory conditions cytokines play a major role in enhancing or diminishing the severity by acting as targets of therapy or as markers to denote up to date status (Erickson et al., 2005). TNF-α is such a candidate that contribute to the pathogenic complications in various renal dysfunctions (Vielhauer and Mayadas, 2007). This situation of TNF-α elevation indicating toxicity of CP is found in the control animals. Our studies have demonstrated that γ-hum could reduce this elevated level denoting its protective action. The excessive release of IFN-γ is also a marker in severe inflammation and autoimmune conditions and its elevated level can check the length of inflammatory process (Muhl and Pfeilschifter, 2003). IL-2 inhibit IL-17 dependent inflammatory process by suppressing the formation of inflammatory T helper 17 (Th17) with modification of sites in the genes involved in Th 17 differentiation with STAT 5 substitution, in place of STAT 3 transcription factor and also by down regulating the expression of IL-6 receptor, thereby checking its further signalling processes. IL-2 also involved in the Tregs (Regulatoy T cells) development and maintenance (Laurence et al., 2007; Yang et al., 2011). Thus IL-2 limits overall inflammation by opposing actions on Tregs and the Th 17 cells. Studies revealed small amount of IL-2 may reduce inflammation and alleviate the ailment (Koreth et al., 2011; Saadoun et al., 2011). The levels of these two cytokines were significantly increased by γ-hum as well as MESNA treatment revealing the significant ameliorating effects of γ-hum my modulating the immune response complimenting the results obtained in our recently published immunomodulatory study on the plant (George and Kuttan, 2015).