Chapter - III

Effect of *Hybanthus ennespermus* on mice liver GSTs treated with paracetamol
Introduction:

Oxidative stress and antioxidant deficiency have been implicated in the pathophysiology of a wide range of diseases and conditions. Consequently, over recent years many different supplementation trials have been implemented, aimed at improving clinical outcomes by boosting antioxidant levels. These trials have included supplementation with individual antioxidants, antioxidant combinations and antioxidant-rich foods, such as fruit and vegetable juices and other plant extracts. To ensure data from these trials is interpreted correctly, it is essential that suitable biomarkers are used to assess changes in in vivo antioxidant activity resulting from supplementation (Lisa et al., 2006). In this chapter some of the direct and indirect biomarkers of oxidative damage were measured to assess in vivo antioxidant activity of ethanolic extract of *Hybanthus enneaspermus* (EEHE). Direct biomarkers of oxidative stress such as antioxidant enzymes, detoxifying enzyme and non-enzymic antioxidants and indirect biomarkers of oxidative damage to lipids (lipid peroxidation) and DNA (comet assay) were measured. Some of the clinically important biomarkers such as ALP, amino transferases were also measured in serum and liver cytosol.

Animal models, which mimic specific characteristics of human oxidative stress, are useful in evaluating biomarkers as surrogate endpoints for oxidative stress incidence. Particularly, the correlation of surrogate endpoint modulation, to effect on oxidative stress incidence, in such models can provide strong evidence for validating the surrogate endpoint. This correlation can strengthen efficiency claims prior to definitive clinical validation (Kelloff et al., 1999). The animal models especially mice have been used extensively to study different deleterious effects of oxidative stress (Van Zwieten, 1984).

Paracetamol was selected as oxidative stress inducer to test antioxidant activity of active fraction of *Hybanthus enneaspermus*. As mentioned early, paracetamol is an effective hepatotoxin. In addition to its hepatotoxicity it also causes damage to kidneys (McGregor and Lang, 1996). Paracetamol is
metabolically activated by cytochrome P₄₅₀ into N-acetyl-p-benzoquinonimine (NAPQI). This conjugates with glutathione in the presence of GST and is excreted as a non-toxic conjugate in the urine. As glutathione is depleted, this reactive metabolite binds covalently to hepatic macromolecules like DNA, leads to cell death. These events ultimately establish a condition of oxidative stress in which the defense capabilities of cells against ROS, becomes insufficient (Nevin and Vijayammal, 2005). Therefore interventions (herbal medicines or plant extracts) favoring the scavenging of ROS and preventing oxidative stress, may mitigate the subsequent oxidative damage, is necessary for elucidation. Hence the following objectives were studied in our laboratory using the control and treated animals, mice.

Objectives:

1. To analyze the biochemical constituents of control and treated mice.
2. To study activities of glutathione s transferases of treated and control mice.

Materials and methods related to this chapter were described in “Materials and methods” chapter.

Experimental design:

A total of 42(6months old) male albino mice weighing 25±5.0g were obtained from Bangalore and treatments were given as follows. The treatments of samples were of for seven days.

Group I was control mice treated with saline 1ml /kg bw, ip. Group II were toxic control treated with paracetamol alone (300mg/kgbw in saline, ip). Group III and IV were treated with ethanolic Hybanthus enneaspermus extract alone, 100mg /kgbw and 200mg/ kg bw, in cmc, respectively, and Group V and VI were treated with ethanolic Hybanthus enneaspermus extract, 100mg /kgbw along with Paracetamol (300mg /kgbw), and 200mg /kgbw and Paracetamol 300mg /kgbw, respectively. Group VII mice were treated with Active fraction
(50mg /kgbw) and Paracetamol (300mg /kgbw). Paracetamol administration was observed for every 48 hrs intervals. Remaining treatments duration was about 7 days. On the eighth day mice were sacrificed by decapitation.

Results:

The mice of control, treated with Paracetamol, 300mg /kgbw, and also treated with EEHE 100mg /kgbw and 200 mg/kgbw as separate treatments and EEHE 100mg and 200mg /kgbw along with Paracetamol (300mg /kgbw) in combination treatment, respectively, were analyzed for the following results as various biochemical parameters such as GST, GPx, SOD, CAT, GR, GSH, transaminases, lipid peroxidation and DNA damage by comet assay in liver of mice.

Analysis of Substrate specificities of GSTs:

The group I results of control were compared with the results of all six groups. The group II mice Paracetamol (300mg /kgbw) treated showed GST substrate specific activities with purification folds of DCNB, EPNP, and pNPA found to have an increase by 3.4, 6.4 and 2.5 folds. The EPNP was observed with highest purification fold, where as the specific activity of substrate of GST, CDNB and BSP showed highest activity 99.58 moles/min/mg protien 40.21 moles/min/mgprotien compared to other substrates of GSTs.

Extract of EEHE alone (100mg) on treated to mice showed GST substrate specific activities with purification folds of CDNB, DCNB, pNPA and A17 androsten 3,17 dione was increased by 1.47, 1.49, 5.2 and 2.6 folds. The pNPA was observed with highest purification fold, where as the specific activity of substrate of GST, CDNB and pNPA showed highest activity 118.96moles/min/mg protien and 70.42moles/min/mgprotien compared to other substrates of GSTs.
Extract of EEHE alone (200mg) on treated to mice showed GST substrate specific activities with purification folds of CDNB, pNPA, EPNP, $\Delta^5$, found to be increased by 1.3, 4.5, 4.2 and 6.1 folds. The $\Delta^5$ was observed with highest purification fold, where as the specific activity of substrate of GST, CDNB and BSP showed highest activity 120.54 moles/min/mg protein and 87.56 moles/min/mg protein compared to other substrates of GSTs.

The combinational studies of EEHE (100mg/ kgbw) and Paracetamol (300mg/ kgbw) showed GST substrate specific activities with purification folds of DCNB, pNPA, EPNP, $\Delta^5$, found to be increased by 1.28, 5.1, 1.6 and 5.1 folds. The pNPA and $\Delta^5$ were observed with highest purification folds, where as the specific activity of substrate of GST, CDNB and BSP showed highest activity 112.69 moles/min/mg protein and 59.96 moles/min/mg protein compared to other substrates of GSTs.

The combinational studies of EEHE (200mg/ kgbw) and Paracetamol (300mg/ kgbw) were showed GST substrate specific activities with purification folds of DCNB, EPNP, $\Delta^5$, found to be increased by 3.04, 1.0 and 3.9 folds. The $\Delta^5$ were observed with highest purification folds, where as the specific activity of substrate of GST, CDNB and BSP showed highest activity 117.11 moles/min/mg protein and 38.86 moles/min/mg protein compared to other substrates of GSTs.

The combinational studies of active fraction (50mg/ kgbw) and Paracetamol (300mg/ kgbw) showed GST substrate specific activities with purification folds of CDNB, DCNB, PNPA, $\Delta^5$, found to be increased by 1.47, 1.3, 5.2, and 6.1 folds, respectively. The pNPA and $\Delta^5$ were observed with highest purification folds, where as the specific activity of substrate of GST, CDNB showed highest activity 125.28 moles/min/mg protein and compared to other substrates of GSTs.
Table: 7 Effect of Paracetamal and EEHE on activity levels of GSTs of mice liver on different Substrates.

<table>
<thead>
<tr>
<th>GROUPS(G1-G7)</th>
<th>CDNB</th>
<th>DCNB</th>
<th>pNBC</th>
<th>pNPA</th>
<th>EPNP</th>
<th>Androsten</th>
<th>BSP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>121.7±0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.6±0.42&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33.68±0.43&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38.55±0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.43±0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.8±0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.5±0.34&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Paracetamal(300mg)</td>
<td>99.58±0.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.32±0.28&lt;sup&gt;d&lt;/sup&gt;</td>
<td>29.1±0.34&lt;sup&gt;d&lt;/sup&gt;</td>
<td>32.6±0.37&lt;sup&gt;f&lt;/sup&gt;</td>
<td>20.19±0.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.54±0.41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>40.21±0.39&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>EEHE(100mg)</td>
<td>118.96±0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.1±0.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.93±0.37&lt;sup&gt;e&lt;/sup&gt;</td>
<td>70.42±0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.7±0.35&lt;sup&gt;c&lt;/sup&gt;</td>
<td>40.6±0.33&lt;sup&gt;e&lt;/sup&gt;</td>
<td>41.38±0.35&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>EEHE(200mg)</td>
<td>120.54±0.34&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5.43±0.42&lt;sup&gt;d&lt;/sup&gt;</td>
<td>37.36±0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>76.41±0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.59±0.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.34±0.37&lt;sup&gt;d&lt;/sup&gt;</td>
<td>87.56±0.41&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P+EEHE(100mg)</td>
<td>112.69±0.42&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.4±0.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59.51±0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55.88±0.37&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.25±0.37&lt;sup&gt;e&lt;/sup&gt;</td>
<td>16.5±0.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59.96±0.38&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P+EEHE(200mg)</td>
<td>117.11±0.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.4±0.37&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18.11±0.35&lt;sup&gt;d&lt;/sup&gt;</td>
<td>33.83±0.33&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4.8±0.36&lt;sup&gt;e&lt;/sup&gt;</td>
<td>15.6±0.34&lt;sup&gt;d&lt;/sup&gt;</td>
<td>38.66±0.36&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Active**

fraction+EEHE(200mg) | 125.28±0.30<sup>g</sup> | 17.32±0.22<sup>b</sup> | 25.0±0.32<sup>e</sup> | 40.04±0.30<sup>d</sup> | 8.99±0.33<sup>d</sup> | 14.22±0.39<sup>d</sup> | 42.5±0.28<sup>c</sup> |

**F value** | 487.329 | 996.305 | 2472.001 | 2938.034 | 517.319 | 902.517 | 2591.067 |

Values are expressed as Mean±SE by Duncan’s multiple range test (DMRT). Means having same subscripts in each column do not differ significantly at 0.01 level by Duncan’s Multiple range Test (DMRT). *p<0.01

Note: Specific activity observed moles/min/mg of protein.
Fig: 18 Effect of paracetamol and EEHE on levels of GSTs of mice liver with CDNB

Fig: 19 Effect of paracetamol and EEHE on levels of GSTs of mice liver with DCNB
Fig: 20 Effect of paracetamol and EEHE on levels of GSTs of mice liver with pNBC

Fig: 21 Effect of paracetamol and EEHE on levels of GSTs of mice liver with pNPA
Fig: 22 Effect of paracetamol and EEHE on levels of GSTs of mice liver with EPNP

![Graph showing the effect of paracetamol and EEHE on GST levels in mice liver treated with EPNP.]

Fig: 23 Effect of paracetamol and EEHE on levels of GSTs of mice liver with $\Delta^5$ Androstene

![Graph showing the effect of paracetamol and EEHE on GST levels in mice liver treated with $\Delta^5$ Androstene.]
Fig: 24 Effect of paracetamol and EEHE on levels of GSTs of mice liver with BSP
Antioxidant and enzymes:

Paracetamol treatment caused a significant (p<0.001) decrease in the level of SOD, catalase, GPx and GST in liver tissue when compared with normal group (Table 8). The treatment of Paracetamol and Hybanthus enneaspermus at the doses of 100 and 200 mg kg\(^{-1}\) resulted in a significant increase of SOD, Catalase, GPx, GR and GST when compared to Paracetamol treated mice. The liver of *active fraction* treated animals also showed a best significant increase in antioxidant enzymes levels compared to Paracetamol treated mice.

The localization of radical formation resulting in lipid peroxidation, measured as Malondialdehyde (MDA) contents in mice liver homogenate. MDA were increased in Paracetamol control group. The treatment of Paracetamol and *Hybanthus enneaspermus* 100 and 200 mg kg\(^{-1}\) were significantly inhibited MDA level when compared to Paracetamol induced hepatic damage. The liver of *active fraction* treated animals also showed a best-inhibited MDA levels compared to Paracetamol treated mice.

Serum and Liver marker enzymes:

The levels of serum AST, ALT, ALP were markedly elevated in Paracetamol treated animals, indicating liver damage (Table 9). Administrations of Paracetamol and *Hybanthus enneaspermus* extract at the doses of 100 and 200 mg kg\(^{-1}\) remarkably prevented Paracetamol-induced hepatotoxicity in a dose dependent manner. The liver of *active fraction* treated animals also showed best preventive levels compared to Paracetamol treated mice.
Table 8: Effect of extract of *Hybanthus enneaspermus* on enzymic antioxidants in liver of control and experimental mice against Paracetamol toxicity.

<table>
<thead>
<tr>
<th>Groups [G1-G7]</th>
<th>CAT</th>
<th>SOD</th>
<th>GPX</th>
<th>GR</th>
<th>GST</th>
<th>LPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48.22±0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.06±0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.156±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.72±0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.47±0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.39±0.41&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Paracetamol[300mg]</td>
<td>26.80±0.20&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.39±0.19&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.135±0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.38±0.44&lt;sup&gt;d&lt;/sup&gt;</td>
<td>19.55±0.30&lt;sup&gt;d&lt;/sup&gt;</td>
<td>61.43±0.41&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>EEHE[100mg]</td>
<td>41.88±0.34&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.34±0.44&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.146±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.49±0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.52±0.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>36.71±0.40&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>EEHE[200mg]</td>
<td>44.84±0.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.56±0.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.154±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.62±0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.64±0.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.48±0.37&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>P+EEHE[100mg]</td>
<td>35.99±0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.41±0.43&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.136±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.66±0.30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.71±0.45&lt;sup&gt;d&lt;/sup&gt;</td>
<td>55.59±0.36&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P+EEHE[200mg]</td>
<td>40.93±0.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.68±0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.144±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.53±0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.59±0.34&lt;sup&gt;c&lt;/sup&gt;</td>
<td>49.45±0.38&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>P+active fraction[50mg]</td>
<td>47.85±0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.49±0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.155±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.45±0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.69±0.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.55±0.38&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

F value: 661.128  104.936  4.85  9.535  367.185  554.221

Values are expressed as Mean±SE by Duncan’s multiple range test (DMRT). Means having same same subscripts in each column do not differ significantly at 0.01 level by Duncan’s Multiple range Test (DMRT). p<0.01

Note: μmoles of activity observed per min per mg of protein.
Fig: 24 Showing catalase levels in control and treated mice liver

Fig: 25 showing SOD levels in control and treated mice liver
Fig: 26 showing GPx levels in control and treated mice liver

Fig: 27 showing GST levels in control and treated mice liver
Fig: 28 showing GR levels in control and treated mice liver

![Bar graph showing GR levels in control and treated mice liver.](image)

Fig: 29 showing LPO levels in control and treated mice liver

![Bar graph showing LPO levels in control and treated mice liver.](image)
Table 9: Effect of extract of *Hybanthus enneaspermus* on serum and liver markers of control and experimental mice against Paracetamol toxicity.

<table>
<thead>
<tr>
<th>Groups (G1 - G7)</th>
<th>Serum</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AST</td>
<td>ALT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>52.63±0.42a</td>
<td>81.62±0.38a</td>
</tr>
<tr>
<td></td>
<td>24.20±0.32c</td>
<td>110.27±0.34c</td>
</tr>
<tr>
<td>EEHE[100mg]</td>
<td>45.37±0.28a</td>
<td>97.42±0.26a</td>
</tr>
<tr>
<td></td>
<td>31.80±0.40b</td>
<td>113.51±0.40b</td>
</tr>
<tr>
<td>EEHE[200mg]</td>
<td>42.24±0.38a</td>
<td>89.44±0.36a</td>
</tr>
<tr>
<td></td>
<td>20.37±0.43c</td>
<td>100.96±0.33b</td>
</tr>
<tr>
<td>Paracetamol[300mg]</td>
<td>91.52±0.33a</td>
<td>172.42±0.39a</td>
</tr>
<tr>
<td></td>
<td>79.80±0.34a</td>
<td>120.64±0.37a</td>
</tr>
<tr>
<td>P-EEHE[100mg]</td>
<td>82.43±0.41a</td>
<td>154.57±0.50a</td>
</tr>
<tr>
<td></td>
<td>74.41±0.40a</td>
<td>118.40±0.42c</td>
</tr>
<tr>
<td>P-EEHE[200mg]</td>
<td>79.46±0.25a</td>
<td>132.59±0.38a</td>
</tr>
<tr>
<td></td>
<td>70.33±0.40a</td>
<td>112.36±0.43a</td>
</tr>
<tr>
<td>P-active principle[50mg]</td>
<td>56.61±0.36a</td>
<td>90.50±0.33a</td>
</tr>
<tr>
<td></td>
<td>47.13±0.35a</td>
<td>109.76±0.43a</td>
</tr>
<tr>
<td>F value</td>
<td>2917.773</td>
<td>8834.65</td>
</tr>
<tr>
<td></td>
<td>4281.141</td>
<td>123.988</td>
</tr>
</tbody>
</table>

Values are expressed as Mean=SE by Duncan’s multiple range test (DMRT). Means having same subscripts in each column don't differ significantly at 0.01 level by Duncan’s Multiple range Test (DMRT). P<0.01

Note: Activity observed in units/L.
Fig: 30 Showing the levels of serum AST in control and treated mice

Fig: 31 Showing the levels of serum ALT in control and treated mice
Fig: 32 Showing the levels of serum ALP in control and treated mice

Fig: 33 Showing the levels of AST in control and treated mice liver
Fig: 34 showing the levels of ALT in control and treated mice liver

![Bar diagram showing ALT levels in different treatments.]

Fig: 35 showing the levels of ALP in control and treated mice liver

![Bar diagram showing ALP levels in different treatments.]
DNA damage – Comet assay:

The alkaline version of the single cell gel electrophoresis assay (comet assay) is widely used to evaluate the genotoxic potential of chemicals and environmental contaminants that can induce oxidative stress leading to cellular injury. The comet assay was carried out to determine the protective role of the *Hybanthus enneaspermus* against paracetamol induced DNA damage in whole blood of animals. The percentage of damaged cells and the average tail lengths of comets in experimental groups were measured.

Blood drawn from the control mice (Group I) during acute treatment showed very few (4.32%) damaged cells. Paracetamol induced (Group II) showed 59.12% cells, having a distinct comet tail which came down significantly to 34.61% in *Hybanthus enneaspermus* (200mg/ kgbw) and paracetamol (Group VI) treated and 14.32% in active fraction (50mg/ kgbw) and paracetamol (Group VII) treated animals.

The tail length was also measured to assess the damage to erythrocyte. In the control group (Group I) the magnitude of the comet tail was very low and increased after paracetamol treatment. It might cause DNA damage of the cells that involved in greater elongation and diffused comet tail formation, resulting in an increase of tail momentum in single-gel-electrophoresis.

The microscopical image resulting from paracetamol (300mg/ kgbw) induced damaged blood cells (Group II) are comets with small or non-existent head and large, diffused tails in acute phase treatment. The average tail length was increased about 89.23 in (Group II) animals compared with (Group I) animals. Where as the tail lengths decreased by 41.6% in EEHE (100mg/ kgbw) and Paracetamol (Group III) treatments compared with (Group II) animals. The average tail length was decreased about 47.6% in (Group II) animals compared with (Group I) animals. Where as the tail lengths decreased
by 66.3% in EEHE (100mg/kgbw) and Paracetamol (Group III) compared with (Group II) animals.

(A)  
(B)  
(C)  
(D)  

Fig 36: Effect of EEHE on DNA damage in blood lymphocytes of control and treated mice during acute treatment.

A: normal mice  
B: Par 
C: HE alone  
D: B & C combination
A. Microphotograph of lymphocytes of control (Group I) in animals showed no significant DNA migration (damage), X 400.

B. Microphotograph of DNA damage in the animals treated with paracetamol (Group II) at a dose of 300mg/kg bw. Showing DNA migration with distinct comet tail formation, X400.

C. Microphotograph of DNA damage in the animals treated with EEHE (100mg/ kg bw) + paracetamol (300mg/kg bw) (Group III) showing less migration of DNA and tail formation with shorter tail length, X400.

D. Microphotograph of DNA damage in the animals treated with active fraction (50mg/ kg bw) + paracetamol (300mg/kg bw) (Group III) showing no or less migration of DNA and tail length is very short compared to control group, X400.

Table: 10 Assessment of the protective activity of EEHE against paracetamol induced damage in blood lymphocytes of mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Damaged cells showing comet (%)</th>
<th>Average tail length (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>4.32 ± 1.02</td>
<td>6.91± 0.23</td>
</tr>
<tr>
<td>II</td>
<td>59.13 ± 1.62</td>
<td>64.15 ± 4.01</td>
</tr>
<tr>
<td>III</td>
<td>34.61± 1.21</td>
<td>37.5 ± 2.04</td>
</tr>
<tr>
<td>IV</td>
<td>14.32± 0.89</td>
<td>21.6 ± 3.08</td>
</tr>
</tbody>
</table>

Values are expressed as Mean±SE by Duncan’s multiple range test (DMRT). P<0.01.
Discussion:

GSTs are a major group of phase-II drug detoxification enzymes which play a pivotal role in the detoxification of an array of xenobiotics. GSTs are multifunctional, multigene and dimeric proteins. These enzymes detoxify the compounds that are hydrophobic or electrophilic in nature either endogenous or exogenous in origin. The GSTs detoxify these compounds by conjugating with the reduced glutathione and there by making them hydrophilic and more easily excretable compounds (Mantle et al, 1990).

Induction of the hepato carcinogenetic process by the administration of chemicals provides a system for characterizing alterations in the liver at early stages. Human hepatocarcinogenesis cells (HCC) and both spontaneous and chemically induced HCC in rodents exhibit considerable similarities with regards to morphology, genomic alterations and gene expression, despite their different etiologies. Therefore investigation of the development of HCC in mice provides valuable insight in to the human condition (Thorgeirson et al., 2002).

The GST isozymes differed in their specificity toward xenobiotic or endogenous substrates such as paracetamol shows variation in the expression of GSTs. The CDNB is the main substrate, which undergo nucleophilic displacement of the chloride moiety by GSH. All classes (α, μ, π) of GSTs except ß catalyze this reaction.

Control showed highest activity with CDNB and pNPA, this shows the induction of π, α & μ GSTs subunits'.

Paracetamol alone showed highest activity with CDNB. This shows the induction of π GST subunit, which indicates the toxic effect.

Extract alone (100mg /kgbw) showed highest activity with CDNB and pNPA. The result indicates the presence of π, α & μ GSTs subunits'.
Extract alone (200mg /kgbw) showed highest activity with CDNB and BSP. The result indicates the induction of 'π & μ GST subunits'.

EEHE (100mg /kgbw) and Paracetamol (300mg /kgbw) showed highest activity with CDNB and BSP. The result indicates the induction of 'π & μ GST subunits'.

EEHE (200mg /kgbw) and Paracetamol (300mg /kgbw) showed highest activity with CDNB and BSP. The result indicates the induction of 'π & μ GST subunits'.

Active fraction EEHE (50mg /kgbw) and Paracetamol (300mg /kgbw) showed highest activity with CDNB. The result indicates the induction of 'π & μ GST subunits'.

These results indicate with the above expressed subunits that plant extract is having protective activity as it regained 'μ -GST subunit', which deals with detoxification by conjugation reactions, it combats oxidative stress by thiolysis. There fore the effect of toxicity has been removed.

Liver marker enzymes in serum such as transaminases (aspartate amino transferase (AST), alanine amino transferase (ALT) and alkaline phosphatase (ALP) are specific indicators of damage (Manju et al., 2002). The increase in the activities of these enzymes in serum may be due to the increased cell damage of the internal organs (Martin et al., 1981; Patil et al., 2009). The abnormal variation in these marker enzymes reflects the overall change in metabolism that occurs during pathological conditions (Rajkapoor et al., 2005). To examine general systemic toxicity of paracetamol, serum biochemical indicators of liver function were assessed in the present study.

Serum transaminases have been reported to be sensitive indicators of liver injury (Molander et al., 1995). Paracetamol a potent hepatotoxin causes rise in these enzymes in serum. The disturbances in the transport function of the hepatocytes as a result of hepatic injury causes the leakage of these enzymes from cells due to altered permeability of membrane (Zimmerman and Seeff, 1970). In the present study, elevated levels of AST, ALT in the serum
was observed. The altered activities of ALT, AST in serum were normalized in a dose and time dependent manner after treatment with active fraction. Raja et al also observe similar results in 2007.

Alkaline phosphatase is a membrane bound enzyme and its alterations likely to affect the membrane permeability and produced derangement in the transport of metabolites. In the present investigation, an elevated activity of ALP was observed in the serum of paracetamol treated mice. The raise in the activity of ALP in paracetamol mice may be due to disturbance in the secretory activity or in transport of metabolites or may be due to altered synthesis of certain enzymes in these conditions. Active fraction treatment throughout the study, in group VII treated animals significantly lowered the enzyme activity when compared to paracetamol treated group.

My reports are in accordance with the results of Rajesh Bhattacharjee and Parames Sil, (2007) in CCl₄ induced toxicity in mice when treated with a protein isolate of Phyllanthus niruri.

**Biomarker of oxidative stress**

**Lipid peroxidation**

Lipids are easily attacked by the activated metabolites of paracetamol resulting in damage to intracellular membranes and plasma membrane (Cheeseman et al., 1985). Lipid peroxidation, a process induced by free radicals, leads to oxidative deterioration of polyunsaturated lipids. Under normal physiological conditions, only low levels of lipid peroxides occur in body tissues. The excessive generation of free radicals leads to peroxidative changes that ultimately result in enhanced lipid peroxidation (Rikans and Hombrook, 1997). Malondialdehyde (MDA) is a major reactive aldehyde resulting from the peroxidation of biological membrane polyunsaturated fatty acid (PUFA) (Vaca et al., 1988). MDA, a secondary product of lipid peroxidation, is used as an indicator, of tissue damage involving a series of chain reactions (Okhawa et al., 1979). It reacts with thiobarbituric acid,
producing red-coloured products. Lipid peroxidation has been implicated in the pathogenesis of increased membrane rigidity, osmotic fragility, reduced erythrocyte survival and perturbations in lipid fluidity. It has been hypothesized that one of the principle causes of paracetamol-induced toxicity is lipid peroxidation of cell membranes by free radical derivatives of paracetamol (Recknagel et al., 1989, 1991). The observation of elevated levels of MDA in serum and liver treatments in paracetamol treated groups; the present study is consistent with this hypothesis. Thus, the maintenance of near normal levels of MDA in active fraction treated groups, is of great interest since, it provides additional evidence to suggest a protective role of the active fraction. The increased accumulation of lipid peroxides in liver and serum of with oxidative stress has been demonstrated. Many reports have been shown that the administration of natural antioxidants reduces the accumulation of lipid peroxides in the tissues (Aruna Kanase et al., 1997). The result obtained in the present study has a correlation with these reports.

Antioxidant enzymes:

Living tissues are endowed with innate antioxidant defense mechanisms, such as the presence of these enzymes SOD, catalase, GPx, GR and GST.

SOD catalyzes the removal of superoxide radical $O_2^-$ to $H_2O_2$, which would otherwise damage the membrane and biological structures. Catalase has been shown to be responsible for the detoxification of significant amounts of $H_2O_2$ (Cheng et al., 1981). GPx catalyses the reduction of $H_2O_2$ to $H_2O$ and $O_2$ and also reduce lipid hydroperoxides. A reduction in the activities of these enzymes is associated with the accumulation of highly reactive free radicals, leading to deleterious effects such as a loss of integrity and function of cell membranes (Reedy and Lokesh, 1992). A reduction in the activity of these enzymes in paracetamol toxicity has been well documented. Administration of paracetamol leads to generation of peroxy radical, which is associated with inactivation of catalase and SOD enzymes in liver tissue were studied. This probably explains the significantly reduced activities of catalase, SOD and GPx.
observed in mice challenged with paracetamol treated. In mice receiving paracetamol and active fraction for a period of 7 days, the activities of catalase, SOD and GPx were significantly higher than in paracetamol treated mice. And were very similar to the values of control. This suggests a protective effect of the *Hybanthus enneaspermus* active fraction.

GPx coupled with GR, catalyse the conversion of oxidized glutathione (GSSG), to reduced glutathione (GSH) and simultaneously NADPH is oxidized to NADP⁺. GPx activity was significantly reduced after paracetamol treatment when compared with control, which indicates the damaging of the cells (Singh et al., 1999). The reversal of GPx activity to normal level after treatment with active fraction is due to antioxidant property by scavenging / detoxifying the endogenous metabolic peroxides generated during paracetamol injury in the internal organs.

GST plays a physiological role in initiating the detoxification of potential alkylating agents. Chemicals like chloroform and paracetamol alter the hepatic GST activity (Aniya and Anders, 1985). GST level was significantly reduced in paracetamol treated mice and upward reversal was observed after treatment with the active fraction.

GSSG is reduced to GSH by glutathione reductase, which is NADPH-dependent. GR plays a role in maintaining adequate amounts of GSH. Accordingly, the reduction of GR results in decreasing GSH (Recknagel et al., 1991). In paracetamol treated mice, the activity of GR is markedly decreased. An increase in GR activity indicates that the active fraction protects the tissues from oxidative damage. Increased levels of SOD, CAT, GPx, GST ad GR enzymes in liver were reported by Rajesh and Latha (2004).

In the present study, we have found that there is a significant reduction in the activities of enzymic antioxidants in liver tissues treated with paracetamol when compared with normal animals. This is a quite common phenomenon as
the exposure to xenobiotic leads to the condition of oxidative stress and the antioxidant defence of the body is utilized to combat the oxidative stress and to quench the enormous amounts of free radicals produced during such conditions.

Antioxidants are known to up regulate the ability of liver to intercept and metabolize xenobiotics (Jeon et al., 2003). Induction of hepatic antioxidant enzymes by antioxidative agents has been reported to alter oxidative stress development at extra hepatic sites. Numerous studies have shown that antioxidants derived from plant sources were able to increase the activities of enzymic antioxidants also to improve the antioxidant status in various experimental models (Hsieh and Yen, 2000). In the present study treatment with active fraction was able to significantly improve the overall antioxidant status of the animals and combat the oxidative damage induced by paracetamol.

DNA damage:

Oxidative damage to DNA seems to be an important factor in developing many human diseases. DNA strand breaks can originate from the direct modification of DNA by chemical agents to their metabolites, from the processes of DNA excision repair, replication and recombination or from the process of apoptosis (Eastman and Barry, 1992). Direct breakage of the DNA strands occurs when ROS interact with DNA (Collins et al., 1993). When the alkaline unwinding step in the comet assay is performed above Ph13.1, some adducts are converted to strand breaks (i.e. alkali labile sites). An alkali labile site can be generated by depurination of an adducted base of the nucleotide and a subsequent conversion to strand break by alkali treatment (Ilanas et al., 1995). The majority of publications on the comet assay state that it detects strand breaks and alkali labile sites. However, in most situations, intermediate repair sites could be the most important contributors to the DNA damage detected by the comet assay. Paracetamol is metabolized to reactive intermediates that then covalently bind to DNA and initiate oxidative stress and the formation of ROS,
resulting in the oxidation of DNA (Boll et al., 2001; Christian Huber et al., 2009).

Possible DNA damage induced by paracetamol in control and experimental mice was detected using alkaline single cell gel electrophoresis (SCG) comet assay following a simplified protocol with slight modifications (Endoh et al., 2002). The use of this alkaline SCG assay as a method to detect genotoxicity and cytotoxicity in vivo is well documented and DNA damage thus detected has been used to predict the presence of genotoxic metabolites in specific organs (Henderson et al., 1998). This method has several advantages: it is highly sensitive for detecting the DNA damage at the level of individual cells, expressed in the form of single strand breaks, alkali liable site formation and disordered DNA fragmentation (Hartmann et al., 2003). And few cells are required for the experiment.

In the present investigation, the observations suggest that there was a high statistical difference in the level of DNA damage between oxidative stress bearing mice and active fraction treated mice. It was found that treatment with active fraction reduced the extent of paracetamol DNA damage (genotoxic stress) of blood cells in comparison with paracetamol treated groups in a duration and dose dependent manner. These results show that active fraction provides a beneficial effect against paracetamol induced oxidative damage. The protective effect may be due to the active compounds 1,3 butadienes and unsaturated carboxylic compounds.
Chapter - IV

Histopathology