CHAPTER 6

ACUTE AND CHRONIC TOXICITY STUDIES OF \( \gamma \)-SITOSTEROL IN NORMAL RATS

6.1. INTRODUCTION

Diabetes mellitus is one of the most predominant causes of disability and death in the world (Reiber et al., 1999; Pecoraro et al., 1990; Boulton, 2004). The management of diabetes is considered a global problem. The modern drugs including insulin and oral hypoglycemic agents, control the blood sugar level as long as they are regularly administered, and also produce a number of side effects (Upadhayay et al., 1996; Reynolds and Martindale, 1996). Treatment of diabetes has been attempted with different indigenous plants and poly herbal formulations (Upadhayay et al., 1996; Joy and Kuttan, 1998; Siddha MaruthuvaThirattu, 1989). Preliminary screening of poly herbal formulation showed significant hypoglycemic activity in streptozocin induced diabetic Swiss albino rats (Ramesh et al., 2003; Sharada et al., 1993). However preclinical toxicity studies are needed for determining a safe dose for human trials (Anoop et al., 2002). Therefore Acute and chronic toxicity studies were carried out to determine the toxic effects of \( \gamma \)-sitosterol using normal male albino wistar rats.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Adult male albino rats of Wistar strain weighing approximately 180-200g were obtained from Animal unit of Entomology Research Institute, Loyola College, Chennai, India. All the animals were kept and maintained under laboratory conditions of temperatures (22°C±2), humidity (45±5%), and 12 hours day: 12 hours night cycle: and fed with commercial pellet rat
chow (Hindusthan Lever Ltd., Bangalore, India) and had free access to water. The study was approved by the Animal Ethics Committee of the Institute (IAEC-ERI-LC-10).

6.2.2 Test for Drug

The test compound γ-sitosterol was prepared as given in chapter 2.

6.2.3 Acute toxicity test

An experiment was performed to know whether any toxic effect was produced by the isolated molecule γ-sitosterol on liver and kidney. Rats fasted for 12 hours were randomly divided into drug treated ‘test’ groups and vehicle treated ‘control’ group making up to 5 groups of 6 rats each. γ-sitosterol dissolved in DMSO (20, 50,100 and 200 mg/kg /b.wt) was separately administered orally to the rats in each of the test groups.

Each of the rats in the control groups was treated with vehicle alone (DMSO 10%). Then the rats in both the test and control groups were allowed access to food and water, and behavioral changes were observed over a period of 72 hours for sign of acute toxicity. The number of mortality caused by the compound within this period of time was observed in order to fix the lethal dose (LD50) of the compound (Lorke, 1983). A separate experiment was carried out to study the acute toxicity effect of γ-sitosterol.

6.2.4. Chronic toxicity study

To study the chronic toxicity (Witthawaskul et al., 2003) in Male albino wistar rats, 5 groups of animals were selected (6 rats each group). γ-sitosterol was dissolved in DMSO and administered to fasted rats in a dosage of effective dose (ED_{50}), 2.5 times of ED_{50}, 5 times of ED_{50}, 10 times of ED_{50}, (20, 50,100 and 200 mg/kg/ b.wt) separately orally to the rats in each group for 21 days. Each of the rats in control groups was treated with vehicle alone (DMSO 10%).
Then the rats in both the test and control groups were allowed access to food and water. At the end of the experiment all the animals were sacrificed after overnight fasting and the blood samples were collected. Hematological examinations like erythrocyte, leucocyte and hemoglobin content and biochemical parameters like blood glucose, urea, uric acid, protein, lactate dehydrogenase, alkaline phosphatase, acid phosphatase, aspartate amino transaminase and alanine amino transferase were carried out. Histopathological examinations of liver and kidney were carried out.

The experimental design is given below;

Group- I- normal control
Group- II- normal + γ-sitosterol 20 mg/ kg /b.wt
Group- III- normal + γ-sitosterol 50 mg/ kg /b.wt
Group- IV- normal + γ-sitosterol 100 mg/ kg /b.wt
Group- V- normal + γ-sitosterol 200 mg/ kg /b.wt

6.2.4.1. Collection of plasma and serum

As given in chapter 3.4.1.1.

6.2.4.2. Hematological parameters

6.2.4.2.1. Estimation of hemoglobin

As given in chapter 3.5.3.

6.2.4.2.2. Red blood cell count

Red blood cell count was determined by the method of Chosbrough and Mc Arthur, (1972) in an improved Neubauer chamber. The count was expressed as millions/mm$^3$ of blood.
6.2.4.2.3 White blood cell count

White blood cell count was estimated as the number of cells per cubic millimeters of blood by the method of Chosbrough and Mc Arthur, (1972) in an improved Neubauer chamber. The count was expressed as thousand/mm$^3$ of blood.

6.2.5. Serum enzyme markers

Alkaline phosphatase (ALP), Acid phosphatase (ACP), Aspartate amino transaminase (AST) and Alanine aminotransferase (ALT) were analysed.

6.2.6. Estimation of Nephritic markers

As given in chapter 3.5.13.

6.2.7. Histopathological studies

As given in chapter 3.5.17.

6.3 RESULTS

6.3.1 Acute toxicity study

$\gamma$-sitosterol up to a high dose of 200mg/kg /b.wt did not cause any change in behavior of the animals. No mortality was observed in the drug treated rats. There was no lethality at any selected dose up to 200 mg/kg /b.wt until the end of the study.

6.3.2. Chronic toxicity study

Oral administration of doses of $\gamma$-sitosterol (200 mg/kg /b.wt) to the normal healthy rats did not show any significant alteration in food intake, body weight, hematological parameters like erythrocyte, leukocyte and hemoglobin content (Table 1).
Table 2 shows the changes of fasting blood glucose level in normal control and the rats treated with different doses of γ-sitosterol 20, 50,100 and 200 mg/kg /b.wt. γ-sitosterol treated rats showed no significant change in plasma glucose levels when compared to control rats.

The activities of plasma enzymes like AST, ALT, ACP and ALP appeared normal between the normal control and drug treated test groups (Table 3). Similarly, no changes were observed in the level of urinary urea, uric acid and creatinine levels in normal and drug treated groups (Table 4).

Histopathological observations in liver and kidney were found to be normal in control and γ-sitosterol treated groups (Figure 6.1 and 6.2). In chronic toxicity evaluation, the drug administration up to a high daily dose did not result in mortality or any change in behavior of the animals.

6.4. DISCUSSION

Numerous herbal preparations have been shown to affect blood glucose levels through various mechanisms, although they are usually limited by toxicity or relative lack of efficacy compared with standard medications. The lack of standardization of ingredients and preparation also cause problems (Batran et al., 2006). In our study oral administration of isolated molecules to normal rats revealed insignificant change in liver and kidney function during the experimental period.

In our acute toxicity study, there was no mortality until 200 mg/kg /b.wt of the dose of γ-sitosterol. In addition there was no marked change in food intake and behavioral changes like, irritation, restlessness, respiratory distress, abnormal locomotion and catalepsy over a period of
72 hours were not seen. Acute toxicity studies revealed the non-toxic nature of γ-sitosterol. Therefore LD\textsubscript{50} value of γ-sitosterol may be beyond 200 mg/kg /b.wt.

Most hepatotoxic chemicals damage liver cells mainly by inducing other oxidative damages and lipid peroxidation (Bhopata \textit{et al.}, 2011). Enzymes directly associated with the conversion of amino acids to ketoacids are ALT, AST, ALT and AST, these activities are used as the indicators of hepatocyte damage (Whitehead \textit{et al.}, 1999). The activities of ALT, AST, ALT and AST were observed in normal and treated groups and were found to be normal.

Urea is the major nitrogen containing metabolic product of protein metabolism; uric acid is the major product of purine nucleotides, adenosine and guanosine; creatinine is endogenously produced and released into body fluids and its clearance measured as an indicator of glomerular filtration rate (Pushpavalli \textit{et al.}, 2010). During the chronic toxicity study, the nephritic markers were observed to be normal in all the treated groups.

Histopathological examination of normal and γ-sitosterol treated rats also revealed apparently the same architecture in the liver and kidney. Phytosterols are non-toxic; they do not result in general immune suppression. Their high margin of safety makes them an attractive therapeutic tool for a variety of conditions (Bouic, 1999).

\textbf{6.5. CONCLUSION}

The different doses of γ-sitosterol did not exert any toxic effect during the present study. From the present study, it can be concluded that γ-sitosterol is not lethal in the usual range of oral antidiabetic drug ie 20mg to 200mg/kg /b.wt in experimental animal models. The 20 mg/kg /b. wt of γ-sitosterol is considered to be safe which is evidenced by our observation.
REFERENCES


Table 1: Effects of γ-sitosterol (200mg/kg /b.wt) on hematological levels in normal rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (gm/day)</th>
<th>WBC (x 1000/µl)</th>
<th>RBC (Million/uL)</th>
<th>Hb (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>42.63 ± 0.86</td>
<td>2.31 ± 0.06</td>
<td>9.09 ± 0.03</td>
<td>15.34 ± 0.58</td>
</tr>
<tr>
<td>Normal+ γ-sitosterol (200 mg/kg/b.wt)</td>
<td>45.51 ± 0.70'</td>
<td>2.55 ± 0.04'</td>
<td>9.72 ± 0.08'</td>
<td>15.55 ± 0.25'</td>
</tr>
</tbody>
</table>

Each value is mean ± S.E.M for 6 rats in each group
- No significance
Table 2: Effects of γ-sitosterol (200mg/kg/b.wt) on Fasting blood level levels in normal rat’s glucose

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fasting blood level (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>72.76 ± 1.35</td>
</tr>
<tr>
<td>Normal+ γ-sitosterol (20 mg/kg/b.wt)</td>
<td>75.28 ± 1.26*</td>
</tr>
<tr>
<td>Normal+ γ-sitosterol (50 mg/kg/b.wt)</td>
<td>74.16 ± 1.22*</td>
</tr>
<tr>
<td>Normal+ γ-sitosterol (100 mg/kg/b.wt)</td>
<td>75.10 ± 1.10*</td>
</tr>
<tr>
<td>Normal+ γ-sitosterol (200 mg/kg/b.wt)</td>
<td>74.82 ± 0.87*</td>
</tr>
</tbody>
</table>

Each value is mean ± S.E.M for 6 rats in each group
- No significance
Table 3: Effects of γ-sitosterol (200mg/kg /b.wt) on AST, ALT, ALP, ACP levels in normal rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (U/dl)</th>
<th>ALT(U/dl)</th>
<th>ALP (U/dl)</th>
<th>ACP (U/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>77.74 ± 0.95</td>
<td>23.36 ± 0.79</td>
<td>79.27 ± 0.59</td>
<td>24.94 ± 0.41</td>
</tr>
<tr>
<td>Normal+ γ-sitosterol</td>
<td>76.72 ± 0.45</td>
<td>20.84 ± 0.58</td>
<td>77.91 ± 0.95</td>
<td>26.00 ± 1.01</td>
</tr>
</tbody>
</table>

Each value is mean ± S.E.M for 6 rats in each group
- No significance
Table 4: Effects of \( \gamma \)-sitosterol (200mg/kg/b.wt) on urea, uric acid and creatinine levels in normal rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Urea (mg/dl)</th>
<th>Uric acid (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>124.52 ± 2.02</td>
<td>6.90 ± 0.16</td>
<td>2.35 ± 0.04</td>
</tr>
<tr>
<td>Normal + ( \gamma )-sitosterol (200mg/kg/b.wt)</td>
<td>130.47 ± 1.42</td>
<td>7.13 ± 0.10</td>
<td>2.18 ± 0.04</td>
</tr>
</tbody>
</table>

Each value is mean ± S.E.M for 6 rats in each group
- No significance
Figure 6.1 and 6.2: Histochemical changes on Liver and Kidney during chronic toxicity study in normal rats (H and E, 400x)

Fig 6.1(a): Normal liver

Fig 6.1(b): Normal + $\gamma$-sitosterol

Fig 6.2(a): Normal Kidney

Fig 6.2(a): Normal +$\gamma$-sitosterol