CHAPTER V

Pharmacological investigations of 5,7-dihydroxy-2’,3’,5’,6’-tetramethoxy isoflavone isolated from *Dalbergia rubiginosa*

Flavonoids have a variety of biological activities and recently this group has gained much interest as active pharmacological agents. They are known for their anti-inflammatory and anti-allergic effects, for antithrombotic and vasoprotective properties, for inhibition of tumour promotion and as a protective for gastric mucosa. Many naturally occurring flavonoids are found to be diuretics or antispasmodics. Bae *et al.*\(^1\) have recently investigated the in-vitro anti helicobacter-pylori activity of a number of flavonoids.

Szent-Gyorgyi\(^2\) reported in 1938, a certain mixture of flavone and flavanone glycosides from *Capsicum annuum* and *Citrus Union* exhibiting prevention of capillary bleeding of scurvy. This mixture was later shown to consist of hesperidin, eriodictin and quercitrin. With their reported activities hesperidin and eriodictin have been introduced in clinical practice to increase the capillary resistance. Similarly other flavonoids like hesperitin, lutin, quercetin, naringenin, kaemferol etc. have been identified and found to possess capillary resistance increasing potential\(^3\). Such developments clearly indicate the necessity and advantages of characterizing pure isolates from plant sources.

5.1 Present work

As a part of our study we have investigated a few potential pharmacological activities of the crude extracts of *Dalbergia rubiginosci* Roxb and *Dalbergia malabarica* Prain and hence an isolate from the
benzene extract of leaves of *D. rubiginosa*, namely 5,7-dihydroxy-2′, 3′, 5′, 6′-tetramethoxy isoflavone (details of isolation and characterization are discussed already in Chapter III) has been investigated for various pharmacological activities like antioxidant, antilithiatic, antifibrotic, and hepatoprotective effects, for which the methodology followed has already been discussed in Chapter IV.

5.2 Antioxidant activity

Flavonoids are shown to possess pronounced antioxidant activity. Flavonoids occurring in fresh pepper, other vegetables and fruits, tea, soya bean, *Glycyrrhiza inflata*, stem bark of *Curdrania tricuspidata*, young green barley leaves and *Corylus columa* leaves have strong antioxidant activity and hence the test compound 5,7-dihydroxy-2′,3′,5′,6′-tetramethoxy isoflavone isolated from the benzene extract of the leaves of *D. rubiginosa* has been screened for its antioxidant activity.

5.2.1 Methodology

The free radical scavenging effect has been studied for the test compound. In this study, the animals were divided into 3 groups of six each, designated as Gl, G2 and G3. The treatment protocol followed is as follows:

Gl was kept as negative control, G2 as alcohol fed positive control with induced peroxidative liver damage and G3 was treated with the test compound (10 mg/kg) suspended in 1% CMC p.o. The method followed by Mahendran and Shyamaladevi, as described in Chapter IV has been followed to evaluate the free radical scavenging effect of the test compound. The results are shown in Table-5.1 and presented in Fig. 5.1 & 5.1.1.
Table 5.1 Effect of 5,7-dihydroxy-2,3,5,6-tetramethoxy isoflavone against ethanol induced Siver damage in rats

<table>
<thead>
<tr>
<th>Group/Treatment</th>
<th>Total Cholesterol (mg%)</th>
<th>Triglycerides (mg%)</th>
<th>AST (U/l)</th>
<th>ALT (U/l)</th>
<th>GSH (mmol/g tissue)</th>
<th>GP (mmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI Control</td>
<td>65.82 ± 0.79</td>
<td>126.82 + 1.57</td>
<td>140.15 ± 1.04</td>
<td>51.72 ± 1.18</td>
<td>4.50 + 0.01</td>
<td>170.0 + 2.1</td>
</tr>
<tr>
<td>G2 Positive control</td>
<td>89.6 ± 2.7*a</td>
<td>181.0 ± 3.4*a</td>
<td>281.0 + 4.7i*a</td>
<td>99.3 + 4.5*a</td>
<td>3.10 + 0.9*a</td>
<td>251.0 + 4.5**a</td>
</tr>
<tr>
<td>G3 Test compound</td>
<td>73.2 ± 1.8b</td>
<td>136.0 + 2.7 *b</td>
<td>156.3 + 6.9*b</td>
<td>71.0 ± 3.1 *b</td>
<td>4.30 + 0.1*b</td>
<td>154 + 2.7*b</td>
</tr>
</tbody>
</table>

*a different from G1  
*b different from G2

Newman keul’s multiple range test was used (*P<0.05)

5.2.2 Results

Chronic administration of alcohol to the animals in G2 resulted in abnormal raise in total cholesterol and triglycerides. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and glutathione peroxidase (GP) were also elevated and glutathione was depleted indicating liver cell damage. Treatment with the test compound reduced the levels of lipid, enzyme and glutathione peroxidase; but elevated the reduced glutathione (GSH).

5.2.3 Discussion

Continuous administration of ethanol induced hyperlipidemia that results in liver damage by elevating the levels of enzymes and lowering the glutathione (GSH). Glutathione is a tripeptide, which plays a major role in the protection of cells and tissues14. Chronic administration of alcohol resulted in depletion of GSH which was elevated in the test compound treated group, suggesting the restoration of balance between
Effect of 5,7-dihydroxy-2’,3’,5’,6’-tetramethoxyisoflavone against ethanol induced liver damage in rats

Fig. 5.1

Fig. 5.1.1
depletion and formation. Glutathione peroxidase catalyses the oxidation of GSH. A reduced glutathione peroxidase activity was observed in the test compound treated group. Probably through this mechanism, the test compound might be restoring the GSH levels indicating that it possesses free radical scavenging activity.

S3 Antilithiatic activity

Nephrolithiasis is a disorder indicating the presence of stones in kidney and these stones are found in all the parts of the urinary tract\textsuperscript{15}. Reduction in the concentration of inhibitors of crystallization in urine may be of great importance in renal stone pathogenesis. Magnesium is an effective competitor with calcium for oxalate ions, forming a soluble complex. Stone formers excreted more phosphorous and less magnesium than normal individuals. Study of biochemical parameters of urine provides a simple and non-invasive procedure to identify lithiasis.

5.3.1 Methodology

In the present study, antilithiatic activity was evaluated using male Wistar rats divided into three groups, each consisting of six animals, designated as Gl, G2 and G3. The treatment protocol followed is as follows:

Gl was kept as normal control and lithiasis was induced in G2 and G3 using ethylene glycol for 35 days. While G2 was reserved as lithiatic control, G3 received treatment with 10 mg/kg of the test compound (5,7-dihydroxy-2',3',5',6'-tetramethoxy isoflavone) suspended in 1% CMC p.o. Ethylene glycol induced hyperoxaluria and the protection by the test compound were studied as per the method followed by Christina et al.\textsuperscript{16}. The results are shown in Table-5.2 and presented in Fig. 5.2.
Table-5.2 Inhibitory effect of 5,7-dihydroxy-2,3,5',6'-tetramethoxy isoflavone on ethylene glycol induced hyperoxaluria

<table>
<thead>
<tr>
<th>Group/Treatment</th>
<th>Vol of urine ml/24 h</th>
<th>Calcium mg/24 h</th>
<th>Oxalate mg/24 h</th>
<th>Phosphate mg/24 h</th>
<th>Magnesium mg/24 h</th>
<th>Protein mg/24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL Control</td>
<td>5.30 ± 0.2</td>
<td>0.59 ± 0.29</td>
<td>0.38 ± 0.02</td>
<td>5.90 ± 0.52</td>
<td>0.89 ± 0.06</td>
<td>2.78 ± 0.39</td>
</tr>
<tr>
<td>G2 Lithiatic control</td>
<td>2.90 ± 0.1*</td>
<td>3.10 ± 0.7*</td>
<td>4.70 ± 0.3*</td>
<td>11.4 ± 0.1*</td>
<td>0.53 ± 0.2*</td>
<td>14.6 ± 1*</td>
</tr>
<tr>
<td>G3 Test compound</td>
<td>4.80 ± 0.4*</td>
<td>1.30 ± 1.2</td>
<td>0.91 ± 0.25*</td>
<td>8.40 ± 0.5</td>
<td>0.79 ± 0.5*</td>
<td>9.60 ± 1.2</td>
</tr>
</tbody>
</table>

Values are expressed as mg/24 h

Newman keul’s multiple range test was used (*P<0.05)

*a different from GL *b different from G2

5.3.2 Results

Ethylene glycol administration in G2 resulted in the elevation of levels of calcium, oxalate, phosphate and proteins confirming the induction of lithiassis. After treatment with the test compound, the risk parameter oxalate was considerably reduced while calcium, phosphate and protein levels were not reduced. However the magnesium level was found to have increased.

5.3.3 Discussion

Ethylene glycol gets oxidised to oxalic acid and results in hyperoxaluria, which is a significant risk factor in the pathogenesis of renal stones. This, along with increased excretion of calcium, forms calcium oxalate renal stones. The test compound perhaps reduces supersaturation of oxalate in urine, thereby reducing stone formation. As
Inhibitory effect of 5,7-dihydroxy-2',3',5',6'-tetramethoxyisoflavone on ethylene glycol induced hyperoxaluria

Fig. 5.2
observed in this study, high urinary oxalate and phosphate were conducive for stone formation. Urinary magnesium is increased after treatment with the test compound and is responsible in the formation of a soluble complex with oxalate ions competing with calcium and there by reduces the incidence of lithiasis.

5.4 Antifibrotic activity

Liver fibrosis resulting from repeated chronic liver damage is one of the most common causes of death in humans and hence the development of methods to prevent fibrotic changes in liver or to repair fibrosis is therefore important. In the present study the liver fibrosis is assessed by measuring levels of liver hydroxyproline, lipid peroxidation and serum enzyme activities.

5.4.1 Methodology

Liver fibrosis was induced following method of Nan et al. The animals were randomized into three groups designated G1, G2 and G3 of six animals each. G1 was reserved as normal negative control; G2 as positive fibrotic control with CCl4 treatment only and G3 as fibrotic batch along with the test compound 5,7-dihydroxy-2',3',5',6'- tetramethoxy isoflavone. CCl4 was given to rats orally (1 ml/100 g) mixed with an equal volume of liquid paraffin twice a week for 28 days. The test compound suspended in 1% CMC was given for 28 days by gavage at a daily dose of 100 mg/kg. Three days after the administration of the last dose, the rats were sacrificed and the liver and blood samples were collected and the serum biochemical parameters were estimated following the method of Jamal et al and the results are tabulated in Table-5.3 and presented in Fig. 5.3 & 5.3.1.
Table 5.3  Effect of 5,7-dihydroxy-2',3',5',6'-tetramethoxy isoﬂavone on biochemical parameters altered by CCl₄ administration

<table>
<thead>
<tr>
<th>Group/ Treatment</th>
<th>AST U/l</th>
<th>ALT U/l</th>
<th>ALP U/l</th>
<th>TBL mg%</th>
<th>HP Mg(g liver)⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 Control</td>
<td>169.6 ± 1.80</td>
<td>56.0 ± 0.63</td>
<td>216.4 ± 2.9</td>
<td>0.50 ± 0.02</td>
<td>50.0 ± 0.3</td>
</tr>
<tr>
<td>G2 Fibrotic control</td>
<td>261.0 ± 4.9* ²=³</td>
<td>91.0 ± 4.5* ³</td>
<td>439.0 ± 5.6* ³</td>
<td>1.30 ± 0.1* ³</td>
<td>141.0 ± 4.4* ³</td>
</tr>
<tr>
<td>G3 Test compound</td>
<td>170.0 ± 5.4* ³b</td>
<td>74.0 ± 3.6* ³b</td>
<td>274.0 ± 6.5* ³b</td>
<td>0.60 ± 0.2* ³b</td>
<td>79.0 ± 5.8* ³b</td>
</tr>
</tbody>
</table>

Newman keul’s multiple range test was used (*P<0.05)
*a different from G1  *b different from G2

5.4.2 Results

The biochemical parameters viz. AST, ALT, ALP, TBL and HP are found to increase in G2 following administration of CC₁₄, indicating the liver damage. The results of G3 reveal that the values of all the above mentioned parameters are brought to normal or near normal levels showing the antifibrotic action of the test compound.

5.43 Discussion

The trichloromethyl radical formed from CC₁₄ by the liver cytochrome P450s, initiates lipid peroxidation resulting in liver damage. In liver fibrosis, the transaminases AST and ALT, the alkaline phosphatase ALP and the total bilirubin TBL are elevated. Estimation of hydroxyproline (HP) serves as a biochemical marker to assess the extent of chronic liver damage. It is obvious that the test compound is able to show antifibrotic protection of the liver by keeping CC₁₄, elevated AST, ALT, ALP and TBL at near normalcy. Infact it has kept the biochemical
Effect of 5,7-dihydroxy-2',3',5',6'-tetramethoxyisoflavone on biochemical parameters altered by CCl₄

Fig. 5.3

Fig. 5.3.1
marker HP at a well reduced level nearer to the normal value of a healthy functional liver indicating a reduction in lipid peroxidation associated with liver damage.

5.5 Hepatoprotective activity

Liver is an important organ for the detoxification and deposition of endogenous and exogenous substances. Its function may readily be impaired by viruses, hepatotoxins and xenobiotics. The functional integrity of liver can be assessed by measuring the enzyme levels, bile flow and bile pigments. CC14, a well-known model to induce hepatic injury, requires biotransformation by hepatic microsomal P450 to produce the hepatotoxic metabolite, trichloromethyl radical. Management of hepatic damage is still a major problem in developing countries as the drugs used for it are either not readily available or expensive and ineffective.

5.5.1 Methodology

Hepatoprotective activity was evaluated by the method of Anand et al. using male Wistar albino rats. The animals were divided into three groups, of six animals each and designated as Gl, G2 and G3. The treatment protocol given is as follows:

Gl was kept as normal control that received the vehicle orally. G2 and G3 received CC14 challenge (0.75 ml/kg p.o.), of which G2 was kept as hepatotoxic control and G3 was treated with the test compound 5,7-dihydroxy-2',3',5',6'-tetramethoxy isoflavone at a dose level of 10 mg/kg orally 2 hours before CC14 intoxication. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline
phosphatase (ALP), total bilirubin (TBL) and total proteins were estimated 18 hours after CC1\(_4\) administration. The results are tabulated in Table-5.4 and presented in Fig. 5.4 & 5.4.1.

**Table-5.4 Effect of S,7-dihydroxy-2',3',5',6'- tetramethoxy isoflavone on CCl\(_4\) induced hepato-toxicity in rats**

<table>
<thead>
<tr>
<th>Group/Treatment</th>
<th>AST U/l</th>
<th>ALT U/l</th>
<th>ALP U/l</th>
<th>Total Bilirubin mg%</th>
<th>Total Protein g%</th>
<th>Liver weight Kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gl Control</td>
<td>138.2 ± 2.1</td>
<td>47.2 ± 1.8</td>
<td>180.7 ± 5.2</td>
<td>0.69 ± 0.02</td>
<td>9.43 ± 0.31</td>
<td>3.80 ± 0.08</td>
</tr>
<tr>
<td>G2 Positive control</td>
<td>281.0 ± 2.4(^a)</td>
<td>134.0 ± 7.6(^a)</td>
<td>399.0 ± 7.4(^a)</td>
<td>1.40 ± 0.1 (^a)</td>
<td>9.81 ± 2.1</td>
<td>6.90 ± 0.4(^a)</td>
</tr>
<tr>
<td>G3 Test compound</td>
<td>149.0 ± 7.6(^b)</td>
<td>61.0 ± 5.6(^b)</td>
<td>219.0 ± 6.5(^b)</td>
<td>0.70 ± 0.14(^b)</td>
<td>8.90 ± 1.7</td>
<td>4.80 ± 0.2(^b)</td>
</tr>
</tbody>
</table>

Newman keul's multiple range test was used (*P<0.05)

*\(^a\) different from Gl *\(^b\) different from G2

5.5.2 Results

Following treatment with the test compound, all the levels of elevated enzymes were reduced. Bile is synthesised in liver and concentrated in gall bladder. Estimation of bile pigments adds support to the extent of liver injury. The test compound could bring down the bilirubin value towards normal. However the total protein was not altered by CCl\(_4\) administration.

5.5.3 Discussion

The trichloromethyl radical formed from CC1, initiates lipid peroxidation which damages the hepatic cells. As a result the levels of all enzymes were elevated, particularly AST is released in large amounts into circulation\(^{28}\). The test compound seems to maintain the integrity of
Protective effect of 5,7-dihydroxy-2’,3’,5’,6’-tetramethoxyisoflavone on \( \text{CCl}_4 \) induced hepatotoxicity in rats

**Fig. 5.4**

**Fig. 5.4.1**
liver cell membrane as evidenced by a significant reduction in the AST and ALT levels, elevated by CC1, administration. Furthermore, reduction in serum bilirubin indicated the restoration of the functional status of liver. The increase in liver weight after CC1, treatment is due to its enlargement. The test compound was able to reduce the size of the enlarged liver with restoration of all parameters and hence is found to possess hepatoprotective effect.
References


