3.1 TOXICITY STUDIES

3.1.1 Introduction:

All drugs are capable of producing harmful as well as beneficial effects. The important harmful effects due to drugs could be broadly classified into a hepatotoxicity, mutagenesis and carcinogenesis, teratogenesis and allergic reactions. It need not be emphasized that sustainable safe health care delivery system of standard quality must be developed to meet the changing needs of the people.

As natural products are inherently safer than the potent synthetic drugs, (which often produce undesirable side-effects), the scientists have turned their eyes on natural resources, which has resulted in radically new approaches and opportunities for drug discovery. After the isolation of flavonoidal glycoside from the investigated plants, keeping the usefulness of this class of compounds in mind it was proposed to evaluate the therapeutic efficacy of the plant drugs / their isolates. Hence a study was undertaken to screen the plant drugs / their isolates for pharmacological activities including their safety profile.

3.1.2 Toxicity studies:

Toxicity studies were carried out to determine the safe and the effective dose of administration of the test drugs viz., ethyl acetate fraction of flowers of *Rivea hypocrateriformis* (RHF), flowers of *Sarcostemma brevistigma* (SBF), root of *Ecbolium viride* (EVR) and flowers of *Clerodendrum philipinum* (CPF) to the
animals during their pharmacological screening. The determination of ED$_{50}$ (the dose effective in producing certain expected response in 50% of the animal group) values helps in ascertaining the potency of a drug in terms of a reference standard. The calculation of ED$_{50}$ value is done when a drug is showing graded response. But when the response is quantal or all-or-none, the ED$_{50}$ value becomes LD$_{50}$ (the dose lethal to 50% of the animal group). Both ED$_{50}$ and LD$_{50}$ values are important for knowing the safety of a drug.

The ratio between LD$_{50}$ and ED$_{50}$ ($LD_{50} / ED_{50}$) represents therapeutic index. Greater the therapeutic index, safer is the drug. The therapeutic index of most of the drugs, which have a low margin of safety, is generally close to unity. The most widely used methods to calculate these values are of Miller and Tainter$^1$ and Litchfield and Wilcoxon$^2$.

3.1.3. Materials and methods:

Healthy albino mice of either sex (20-25g) were used as experimental models. The animals were housed in standard microlon boxes under standard conditions of 12h light and 12h dark cycle at ambient temperature (35-36°C). The experiments were carried out during light cycle and were maintained in the Vinayaka Mission’s College of Pharmacy, Salem, Tamil Nadu. The animals were given commercial food supplied by Hindustan Lever Limited, Bangalore and water ad libitum. The cage numbers and individual markings on the tail identified the animals for the tests.

The overnight fasted animals were administered with different doses of test drugs viz., RHF, SBF, EVR and CPF suspended in 0.5%(w/v) solution of gum acacia through oral route by hit and trial method to find out the least tolerated (100% mortality) dose and the most tolerated (0% mortality) dose. For each dose a
set of 10 animals were used. After the administration of the test: drugs, the animals were observed continuously for the first 2h for death due to acute toxicity and then observed for further 4h and finally overnight mortality recorded.

The behavior of the drug-injected animal was observed carefully, by recording the following signs.

Increased motor activity, tremors, clonic convulsions, tonic extension, straub reaction, pilo-erection, muscle spasm, catatonia, spasticity, opisthotonus, hyperesthesia, loss of righting reflex, decreased motor activity, ataxia, sedation, muscle relaxation, hypnosis, analgesia, anaesthesia, arching and rolling, ptosis, lacrimation, exophthalmos, salivation: viscid, watery, diarrhoea, abdominal constriction, respiration, depression, stimulation, failure, skin colour: blanching, cyanosis and vasodilatation.

3.1.4. Results and discussion:

The acute toxicity studies of the plant drugs (RHF, SBF, EVR and CPF) were carried out to document the safety profile of them. The results of the studies carried out are given in Table 3.1, 3.2, 3.3 and 3.4.

Table 3.1

| LD50 and ED50 values of ethyl acetate extract of R.hypocrateriformis (RHF) |
|---|---|---|---|---|---|
| Group | Dose mg/kg | Log dose | Dead / Total | Dead % | Corrected % | Probit |
| 1 | 4000 | 3.6020 | 0/10 | 0 | 2.5 | 3.04 |
| 2 | 4500 | 3.6532 | 1/10 | 10 | 10 | 3.72 |
| 3 | 5000 | 3.6989 | 3/10 | 30 | 30 | 4.48 |
| 4 | 5500 | 3.7404 | 8/10 | 80 | 80 | 5.84 |
| 5 | 6000 | 3.7780 | 10/10 | 100 | 97.5 | 6.96 |

Log dose = 3.70 (trom graph)
Antilog = 5011.87
LD50 = 5011.87 mg/kg
ED50 = 501.18 mg/kg
Table 3.2
LD<sub>50</sub> and ED<sub>50</sub> values of ethyl acetate extract of <i>S.brevistigma</i> (SBF)

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose mg/kg</th>
<th>Log dose</th>
<th>Dead / Total</th>
<th>Dead %</th>
<th>Corrected %</th>
<th>Probit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4000</td>
<td>3.6020</td>
<td>0/10</td>
<td>0</td>
<td>2.5</td>
<td>3.04</td>
</tr>
<tr>
<td>2</td>
<td>5000</td>
<td>3.6989</td>
<td>1/10</td>
<td>10</td>
<td>10</td>
<td>3.72</td>
</tr>
<tr>
<td>3</td>
<td>6000</td>
<td>3.7780</td>
<td>3/10</td>
<td>30</td>
<td>30</td>
<td>4.48</td>
</tr>
<tr>
<td>4</td>
<td>7000</td>
<td>3.8450</td>
<td>6/10</td>
<td>60</td>
<td>60</td>
<td>5.25</td>
</tr>
<tr>
<td>5</td>
<td>8000</td>
<td>3.9030</td>
<td>10/10</td>
<td>100</td>
<td>97.5</td>
<td>6.96</td>
</tr>
</tbody>
</table>

Log close = 3.81 (from graph)
Antilog = 6456.54
LD<sub>50</sub> = 6456.54 mg/kg
ED<sub>50</sub> = 645.65 mg/kg

Table 3.3
LD<sub>50</sub> and ED<sub>50</sub> values of ethyl acetate extract of <i>E.viride</i> (EVR)

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose mg/kg</th>
<th>Log dose</th>
<th>Dead / Total</th>
<th>Dead %</th>
<th>Corrected %</th>
<th>Probit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4000</td>
<td>3.6020</td>
<td>0/10</td>
<td>0</td>
<td>2.5</td>
<td>3.04</td>
</tr>
<tr>
<td>2</td>
<td>4500</td>
<td>3.6532</td>
<td>2/10</td>
<td>20</td>
<td>20</td>
<td>4.16</td>
</tr>
<tr>
<td>3</td>
<td>5000</td>
<td>3.6989</td>
<td>5/10</td>
<td>50</td>
<td>50</td>
<td>5.0</td>
</tr>
<tr>
<td>4</td>
<td>5500</td>
<td>3.7404</td>
<td>7/10</td>
<td>70</td>
<td>70</td>
<td>5.52</td>
</tr>
<tr>
<td>5</td>
<td>6000</td>
<td>3.7780</td>
<td>10/10</td>
<td>100</td>
<td>97.5</td>
<td>6.96</td>
</tr>
</tbody>
</table>

Log dose = 3.69 (from graph)
Antilog = 5000
LD<sub>50</sub> = 5000 mg/kg
ED<sub>50</sub> = 500 mg/kg

Table 3.4
LD<sub>50</sub> and ED<sub>50</sub> values of ethyl acetate extract of <i>C.philipinum</i> (CPF)

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose mg/kg</th>
<th>Log dose</th>
<th>Dead / Total</th>
<th>Dead %</th>
<th>Corrected %</th>
<th>Probit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3000</td>
<td>3.4771</td>
<td>0/10</td>
<td>0</td>
<td>2.5</td>
<td>3.04</td>
</tr>
<tr>
<td>2</td>
<td>4000</td>
<td>3.6020</td>
<td>1/10</td>
<td>10</td>
<td>10</td>
<td>3.72</td>
</tr>
<tr>
<td>3</td>
<td>5000</td>
<td>3.6989</td>
<td>3/10</td>
<td>30</td>
<td>30</td>
<td>4.48</td>
</tr>
<tr>
<td>4</td>
<td>6000</td>
<td>3.7780</td>
<td>6/10</td>
<td>60</td>
<td>60</td>
<td>5.25</td>
</tr>
<tr>
<td>5</td>
<td>7000</td>
<td>3.8450</td>
<td>10/10</td>
<td>100</td>
<td>97.5</td>
<td>6.96</td>
</tr>
</tbody>
</table>

Log dose = 3.75 (from graph)
Antilog = 5623.41
LD<sub>50</sub> = 5623.41 mg/kg
ED<sub>50</sub> = 562.34 mg/kg
It can be inferred from the Tables 3.1, 3.2, 3.3 and 3.4 that these plants have LD₃₀ values exceeding the close of 5000 mg/kg. However the animals showed mild toxic symptoms such as abdominal constriction, muscle spasm, decreased motor activity and atoxia stretching of hind legs, which were observed half an hour after the drug administration. These toxic symptoms were more prominent in the case of *S. brevisigma*. The symptoms were moderate in the case of *C. philippinum*. But all the animals recovered completely after three hours. Thus all the four test drugs did not show much acute toxic symptoms. Arbitrarily the test dose for the extracts was identified as 250mg/kg by trial and error.

The phytochemical investigation of these plant drugs has yielded flavonoidal glycosides (chapter II) and hence it can be said that the higher safety margin of these plant drugs may be due to the flavonoidal constituents.
3.2 ANTI-NOCICEPTIVE ACTIVITY

3.2.1 Introduction:

Pain is an unpleasant sensory and emotional experience associated with actual potential tissue damage or described in terms of such damage. The pain consists of two components namely the perception and the reaction. The perception is invariably uniform for everyone depending on the intensity of the stimulus. However, the reaction differs from person to person and it depends on factors like past experience, conditioning, memory and judgments. The exact mechanism of pain perception is yet to be understood clearly.

Clinically, pain can be classified into two types - acute and chronic. Acute pain is temporary instantaneous and eventually subsides by treatment or on its own accord (example: renal colic and headache).

In contrast, chronic pain is continuous, gradual in onset and rather refractory to treatment, (example: pain of rheumatoid arthritis and malignancy). Psychogenic pain exists, when no anatomical or physiological reason is available. The placebo therapy mostly is successful in the management of such pain. Pain is treated using analgesics. They are broadly classified into two groups, opioid and non-opioid analgesics.

Opioid analgesics:

Opioid analgesic agents are capable of relieving severe degree of pain, however, induces addiction liability. The opioid drugs are mainly phenanthrene derivatives, structurally related to morphine and synthetic compounds having similar pharmacological effects. Opioid analgesics exert their analgesic effect by acting on opiate receptors. These receptors are distributed both in the central and peripheral nervous systems. All opioids interact with the endogenous opioid
receptor system that presently includes four receptor subtype\(^6\) designated as mu, delta, kappa and sigma.

Non-opioid analgesics:

Non-opioid analgesic agents relieve mild to moderate degree of pain and are non-addictive. However, they produce serious side effects like gastritis. This group includes aspirin. They act independently by inhibiting prostaglandin synthesis. The site of action is on peripheral tissue\(^7\). Aspirin, phenacetin and aminopyrine and other related non-opioid analgesics are not only less effective in relieving pain but they are also much more selective in their action. These drugs relieve pain that usually accompanies inflammation\(^4\). The mechanism is by inhibiting cyclooxygenase enzyme and thereby inhibiting prostaglandin synthesis, chemical mediators that trigger the transmission of pain signals to the brain. The available analgesics at present are not free from side effects and hence not recommended for prolonged use.

3.2.2 Anti-nociceptive activity of plant extracts:

Many natural products exert anti-nociceptive response by acting peripherally. Anti-nociceptive activity for gossypin has been reported\(^9\). Vitexin, a flavone has been shown to be a potent anti-nociceptive agent\(^10\). A flavonoid glycoside, quercetin 3-O-(6\(^\prime\)-feruloyl) (3-D-galactopyranoside isolated from the aerial parts of \textit{Polygonum viscosum} inhibited acetic acid induced abdominal constriction in mice\(^9\). Flavonoids of \textit{Aerva torrentosa} exhibit anti-nociceptive activity by hot plate method\(^12\).

\textit{Achyrocline satureoides}\(^9\), \textit{Drimys winteri}\(^14\), \textit{Elaeagnus angustifolia}\(^15\), \textit{Erigeron floribundus}\(^5\), \textit{Plantago major}\(^11\), \textit{Proustia pyrifolia}\(^18\) \textit{Sambucus ebulus}\(^19\), \textit{Sideritis tciurica}\(^20\), \textit{Teucrium polium}\(^21\), \textit{Trigonella foenum-graecum}\(^22\), \textit{Wilbniindia}
are some of the other plants which are investigated for anti-nociceptive activity and found to possess such action.

In the last several decades, more anti-nociceptive substances have been purified from natural products resulting in novel structural classes and mechanism of actions. In this pursuit several other classes of compounds, especially from natural sources are being simultaneously screened to explore the possibility of potential analgesic property contained in them. The present study was aimed at determining the anti-nociceptive effect of the extracts obtained from the flowers of *R. hypocrateriformis* (RHF), *S. brevistigma* (SBF), *C. philippinum* (CPF) and roots of *E. viride* (EVR).

3.2.3 Materials and methods:

The anti-nociceptive activity of the test drugs was evaluated by using the following methods:

1. Eddy’s hot plate method

2. Acetic acid-induced abdominal constriction method.

3.2.4 Hot plate method:

Animals:

Male albino rats (Wistar strain) (150-200g) were used as experimental models. The animals were given food and water *ad libitum* (supplied by M/s. Hindustan Lever Limited, Bangalore). The animals were housed under standard conditions of 12h light and 12h dark cycle at ambient temperature (35-36°C). The experiments were carried out during light cycle.
Method:

Eddy’s hot plate method was employed. The control reaction time was noted by observing the time taken by the animal either to lick the hind paw or jump from the hot plate when placed on a hot plate maintained at constant temperature (55±0.5°C). Normally, animals responded within 6-8 seconds. A cut-off period of 15 seconds was maintained to avoid damage to the paws.

The reaction time of animals on the hot plate was recorded at 60, 120 and 180 minutes after the administration of the drug. The anti-nociceptive effect was expressed as the area under the time response curve obtained graphically by plotting the increase in reaction time vs the time after drug administration and counting the number of squares under the curve as cm². Each animal was used only once to avoid the development of behavioral tolerance, if any as reported for rats.

The rats were divided into fourteen groups. Each group consisted of 6 animals. Group 1, served as control, received gum acacia 0.5% (w/v) in saline (2ml/kg p.o.) while the second group was treated with pentazocine (5mg/kg i.p) (Ranbaxy, India). Group III, IV and V were treated with RHF at a dose of 125, 250 and 500 mg/kg p.o, respectively. Groups VI, VII and VII! were treated with SBF at a dose of 125, 250 and 500 mg/kg p.o. respectively. Groups IX, X and XI were treated with EVR at a dose of 125, 250 and 500 mg/kg p.o. respectively, while groups XII, XIII and XIV were treated with CPF at a dose of 125, 250 and 500 mg/kg p.o. respectively. Dosing in all groups was carried out at 10.00 A.M.

The percentage of anti-nociceptive activity was determined by noting the difference in reaction time of drug treated animals with that of the control from the reaction using the following relationship:
3.2.5 Acetic acid-induced abdominal constriction assay:

**Animals:**

Swiss male albino mice (20-25g) were used in this study. The animals were given food and water *ad libitum* (supplied by M/s, Hindustan Lever Limited, Bangalore) and were housed under standard conditions of 12h light and 12h dark cycle at ambient temperature (35-36°C). The experiments were carried out during light cycle.

The anti-nociceptive activity was evaluated using a modified Seigmund technique. The mice were divided into fourteen groups. Each group consisted of six animals. The animals received drug treatment as detailed under the hot-plate method except that group [I animals were administered with aspirin (Apex, India) of 100mg/kg p.o.

Thirty minutes later, the mice were given 0.6% (v/v) acetic acid (glacial, AR, Merck, India) at a dose of 10ml/kg i.p. to induce abdominal constriction. A typical abdominal constriction reaction sets in and is characterized by a wave of constriction of the abdominal musculature followed by extension of hind limbs. The abdominal constriction episodes in the animals were counted for 10 minutes and compared with those in the control group. The percentage protection of the compound was calculated, as follows,

\[
\text{% anti-nociceptive activity} = \left( \frac{\text{Reaction time (treated) \quad - \quad Reaction time (control)}}{\text{Reaction time (max 15 seconds)}} \right) \times 100
\]
\[
\% \text{ protection} = 100 - \left( \frac{\text{No. of abdominal constriction in drug treated animals}}{\text{No. of abdominal constriction in control animals}} \right) \times 100
\]

Statistical Analysis:

All values were expressed as mean ± SEM. The data were statistically analyzed by one-way ANOVA followed by Dunnett’s test.

3.2.6 Results and discussion:

The results obtained from the hot-plate test showed that animals treated with the extracts of RHF, SBF, EVR and CPF at a dose of 125, 250 and 500mg/kg exhibited the anti-nociceptive activity in a dose dependent manner. Among the four extracts, SBF showed the maximum anti-nociceptive effect at 500 mg/kg dose which was found to be comparable with that of the reference drug (Table 3.5 and Fig. 3.1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
<th>Dose (mg/kg)</th>
<th>Area under the time response curve (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control (saline)</td>
<td>2 ml/kg</td>
<td>14.15</td>
</tr>
<tr>
<td>II</td>
<td>Pentazocin</td>
<td>5</td>
<td>30.54</td>
</tr>
<tr>
<td>III</td>
<td>RHF</td>
<td>125</td>
<td>20.90</td>
</tr>
<tr>
<td>IV</td>
<td>RHF</td>
<td>250</td>
<td>21.98</td>
</tr>
<tr>
<td>V</td>
<td>RHF</td>
<td>500</td>
<td>22.39</td>
</tr>
<tr>
<td>VI</td>
<td>SBF</td>
<td>125</td>
<td>23.98</td>
</tr>
<tr>
<td>VII</td>
<td>SBF</td>
<td>250</td>
<td>25.15</td>
</tr>
<tr>
<td>VIII</td>
<td>SBF</td>
<td>500</td>
<td>26.35</td>
</tr>
<tr>
<td>IX</td>
<td>EVR</td>
<td>125</td>
<td>20.75</td>
</tr>
<tr>
<td>X</td>
<td>EVR</td>
<td>250</td>
<td>21.57</td>
</tr>
<tr>
<td>XI</td>
<td>EVR</td>
<td>500</td>
<td>22.29</td>
</tr>
<tr>
<td>XII</td>
<td>CPF</td>
<td>125</td>
<td>19.13</td>
</tr>
<tr>
<td>XIII</td>
<td>CPF</td>
<td>250</td>
<td>20.37</td>
</tr>
<tr>
<td>XIV</td>
<td>CPF</td>
<td>500</td>
<td>21.58</td>
</tr>
</tbody>
</table>

Table 3.5

Effect of test drugs on thermal stimulus - induced pain in rats
In acetic acid-induced abdominal constriction assay, extracts of RHF, SBF, EVR and CPF at a dose of 125, 250 and 500mg/kg reduced the number of abdominal constriction significantly in a dose dependent manner, which was comparable with the results obtained for the reference drug (Table 3.6 and Fig. 3.2). The standard drug at a dose 100mg/kg produced an anti-nociceptive activity effect of 62.38% whereas 500mg/kg of extracts of SBF, EVR, RHF and CPF produced an anti-nociceptive effect of 59.82%, 57.64%, 55.52% and 53.41% respectively. However, unlike aspirin, flavonoids do not have side effects such as ulcerogenic effect.
Table 3.6

Effect of test drugs on acetic acid induced abdominal constriction

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
<th>Dose (mg/kg)</th>
<th>No. of abdominal constriction (per 10 minutes)</th>
<th>% protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (saline)</td>
<td>2ml/kg</td>
<td>42.50±2.10</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>Aspirin 100</td>
<td>16.00±1.80</td>
<td>62.38</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>RHF 125</td>
<td>27.20±1.70</td>
<td>36.00</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>RHF 250</td>
<td>23.00±1.10</td>
<td>45.88</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>RHF 500</td>
<td>18.00±1.00</td>
<td>55.52</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>SBF 125</td>
<td>26.50±1.70</td>
<td>37.67</td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>SBF 250</td>
<td>22.80±1.20</td>
<td>46.35</td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td>SBF 500</td>
<td>19.20±1.00</td>
<td>59.82</td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td>EVR 125</td>
<td>27.80±1.80</td>
<td>34.58</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>EVR 250</td>
<td>23.50±1.20</td>
<td>44.70</td>
<td></td>
</tr>
<tr>
<td>XI</td>
<td>EVR 500</td>
<td>18.00±1.10</td>
<td>57.64</td>
<td></td>
</tr>
<tr>
<td>XII</td>
<td>CPF 125</td>
<td>28.30±1.20</td>
<td>33.41</td>
<td></td>
</tr>
<tr>
<td>XIII</td>
<td>CPF 250</td>
<td>23.60±1.10</td>
<td>44.47</td>
<td></td>
</tr>
<tr>
<td>XIV</td>
<td>CPF 500</td>
<td>19.80±1.00</td>
<td>53.41</td>
<td></td>
</tr>
</tbody>
</table>

n = 6 *P<0.01 Vs Control

Data were analyzed by one-way ANOVA followed by Dunnett’s test.

Values are expressed as mean ± SEM.
Thus the anti-nociceptive activity of test drugs against acute inflammatory pain was moderate as compared to potent inhibitory activity of aspirin.

Aspirin leads to a relief from inflammatory pain by suppressing the formation of pain inducing substances in the peripheral tissues. Prostaglandin and bradykinin were suggested to play an important role in the pain process. Non-opioid drugs like aspirin produce analgesic effect mainly by peripheral effects. The possibility of these anti-nociceptive agents exhibiting anti-inflammatory response cannot be excluded. Experiments carried out on these lines showed that all the four plant derived principles showed anti-inflammatory activity in experimental models. These data suggest that these plant-derived principles may act like aspirin. This suggestion warrants further experimentation.

Perusal of the table 3.5 shows that higher doses of drug were required to produce the activity in the hot plate method. This observation is in agreement with earlier reports. It has been previously found that ED$_{50}$ of morphine in the hot plate assay was 33 times more than that in the abdominal constriction assay. Similar phenomenon was observed in the assessment of gossipin for anti-nociceptive activity. Flavonoids have been shown to have anti-nociceptive activity through the inhibition of the prostaglandin synthetase.

The phytochemical investigation of the four chosen plants have already revealed the presence of luteolin 6'-prenyl 7-0 rhamnoside in RHF, quercetin 5'-prenyl 3-0-glucosyl 7-0 (4'' p-coumaroyl) neohesperidoside in SBF, vitexin, luteolin 7-0- (2'' sinapoyl) glucoside and luteolin 7-0- (4''senecioyl) neohesperidoside in EVR and apigenin 3-prenyl, 5-methyl ether, 7-0 rhamnoside in CPF (Chapter II). In view of this, it can be said that this bioflavonoid content in the investigated plant derived principles is likely to be responsible for the recorded
anti-nociceptive response. It is essential to mention here that bioflavonoids are unique as they exhibit anti-nociceptive, anti-inflammatory as well as anti-ulcerogenic properties. Usually, anti-inflammatory drugs are ulcerogenic in nature; only the degree of this side effect varies. This unique property of flavonoids viz., anti-ulcerogenic effect can be exploited for their effective therapeutic use for the management of pain.
3.3.1 Introduction:

Inflammation is a response of living tissues to injury. The inflammatory process involves series events that can be elicited by numerous stimuli such as infectious agents, ischemia, antigen antibody interactions and thermal or physical injury. The term inflammation originates from Latin word ‘inflammare’ meaning ‘to burn’.

Inflammatory responses occur in three distinct phases, each apparently mediated by different mechanisms viz., an acute transient phase, most prominently characterized by local vasodilatation and increased capillary permeability, a delayed sub-acute phase, most prominently characterized by infiltration of leucocytes and phagocytic cells and a chronic proliferative phase, in which tissue degeneration and fibrosis occur. Customarily, a distinction is made between acute and chronic inflammation. Acute inflammation is the response of tissues to severe but transient stimuli. Chronic or granulomatus inflammation occurs when the stimulus is persistent and usually weak. The appearance of a number of leucocytes at the site of injury is a characteristic feature of the inflammation. Two of the major manifestations of acute inflammation are vascular dilatation and increased vascular permeability, which involve changes in vasomotor function of small blood vessels. The chronic inflammatory response is characterized by cellular infiltration, fibrogenesis and neovascularisation.
The tissue damage that takes place during inflammation is accompanied by the release of several biochemical mediators such as histamine, bradykinin, platelet activity factor and a group of lipid materials known as leukotrienes and prostaglandins. These mediators are responsible for the symptoms that accompany inflammation process. While histamine, bradykinin and leukotriene cause the swelling and redness of the inflamed area (due to vasodilation and increased capillary permeability), prostaglandins, on the other hand, increase tissue sensitivity to pain and cause elevation of body temperature.

Sodium salicylate was first used for treatment of rheumatic fever. It was used as an antipyretic in 1875 by chemical manipulation of natural bitter glycoside, salicin, obtained from willow bark. Aspirin and sodium salicylate have been in wide use for almost a century. Modern chemical and pharmacological research to evaluate more effective and better tolerated anti-inflammatory drugs flourished only in the late 1940s after the dramatic effect of corticosteroids was discovered with a synthetic sample of hydrocortisone.

The attempts made to reduce hormonal and metabolic side effects of these steroidal drugs failed much to disappointment of the scientists. This led to the development of the non-steroidal anti-inflammatory drugs (NSAIDs). These were used for the treatment of broad range of pathophysiological conditions such as headache, discomfort associated with minor injuries, alleviation of severe pain caused by inflammatory and regenerative joint diseases such as osteo and rheumatoid arthritis.

The pharmacotherapy of inflammation is characterized by an apparent abundance of synthetic drugs, which do not actually cure large number of inflammatory diseases but often have dangerous side effects. The situation calls
for intensive efforts to unravel the mechanism of action of existing drugs, to improve their therapeutic safety and to develop concepts of more specific and less toxic anti-inflammatory compounds. This situation has necessitated in large scale screening of many plants.

Anti-inflammatory study has brought to light some potent and more specific anti-inflammatory agents with appreciable clinical utility.

3.3.2 The assay methods of anti-inflammatory agents:

The assay methods of anti-inflammatory agents are -

1. Erythema: In this method erythema caused by UV irritation was measured as an anti-inflammatory activity\(^\text{37}\).

2. Edema: Anti-inflammatory agents may be detected by their ability to diminish or prevent edema. Many different substances have been employed to induce edema. Formalin, dextran mustard, kaolin, egg albumin, 5-hydroxy tryptamine, histamine, silver nitrate, proteolytic enzymes, brewer's yeast and carrageenin are the different edematogens used.

3. Granuloma: This is based on preventing the formation of granulation tissue. The cotton pellet granuloma inhibition assay is the most commonly used method\(^\text{38}\).

3.3.3 Anti-inflammatory activity of plant extracts:

In view of the universal requirements for NSAIDs many plants have been utilized for this purpose in traditional medicine. A systematic study of anti-inflammatory effects of Indian medicinal plants was started by Gujral and co-workers\(^\text{39-40}\), who screened a number of plants for their anti-arthritic effect.
The anti-inflammatory activity of flavonoids has been extensively studied and many flavonoids do exhibit significant activity. The active constituents of *Proustia pyrifolia* could diminish cyclo-oxygenase and lipo-oxygenases enzyme activities. The crude hydroalcoholic extract of *Pfaffia glomerata* showed a potent activity on chronic inflammation. Kaempferol 3,7-O-a-dirhamnoside and quercetin 3,7-O-a-dirhamnoside isolated from *Tilia argentea* showed potent antinociceptive and anti-inflammatory effects. Kaempferol and its glycosides of *Lilium longiflorum* inhibited COX-1 enzyme. 60% methanol fraction of *Clerodendron drichotomum* leaves showed potent anti-inflammatory activity. Quercetin, dicatalenol and caryolan-1,9 (3-diol isolated from *Heterotheca inuloides* showed dose dependent anti-inflammatory activity. The methanolic extract of *Crescentia cdata* exerted a significant in-vivo anti-inflammatory activity on carrageenin induced paw edema. Quercetin 3-O-methyl ether isolated from *Rhamnus nakaharai* possesses potent anti-inflammatory effects. Polymethoxy flavonoid, nobiletin isolated from citrus fruits possess the anti-inflammatory and antitumour activity. Kaempferol and quercetin possess a strong and prolonged anti-inflammatory activity. Rutin isolated from *Phyllanthus emblica* has inhibitory activity on human polymorpho nuclear leukocyte and platelets. Flavonoids and coumarin obtained from *SantoHna oblong ifolia* have been reported to exhibit anti-inflammatory activity. Flavonoid glycosides, myricetin and quercetin 3-O-[1-D-xylopyranosyl(1-2) L-a-rhamnopyranosides], isolated from *Eugenia jambos* were more effective than indomethacin and phenylbutazone. Quercetin 3-O-xilosyl (1-2) rhamnoside and quercetin 3-O-rhamnoside, isolated from *Erythrospernum monticolum* are effective on TPA (12-O-tetradecanoylphorbol 13-acetate) induced mouse ear...
edema. Quercetagetin, kaempferol 3-O-galactoside, scutellarein, scuteliarein 7-O-glucuronide, hispidulin and hibifolin have been shown to inhibit TPA induced ear edema in mice. Propolis extracts (natural product produced by honey bee) containing flavonoids as phytoconstituents exhibited anti-inflammatory activity. 5,7,3’ Trihydroxy 3,6,4’ trimethoxyflavone (centaureidin) and 5,3’ dihydroxy, 4’-methoxy 7-carbomethoxy flavonol isolated from Tcmacetum microphyllum possess the anti inflammatory activity. A biflavonoidal fraction, kolaviron isolated from Garcinia kola possessed anti-inflammatory activity comparable to that of phenyl butazone and acetyl salicylic acid. The ethanol extract of Cassia occidentalis and Cardiospermum halicacabam showed a dose dependent response on anti-inflammatory activity. Delonix elata and Peltophorum pterocarpum were shown to exhibit anti-inflammatory activity. Apigenin dimethyl ether isolated from Rhus undulata, baicalin, baicalein and wogonin, isolated from Scutellaria baicalensis showed anti-inflammatory effects. The flavonoid hedychenone isolated from hexane - soluble extract of Hedychium spicatum showed a significant activity with less ulcerogenic index than phenylbutazone. Gossypin isolated from Hibiscus vitifolius seems to reduce vascular permeability. The flavonoid glycoside, chrysoeriol, 7-O-(3-D-glucopyranosyl (2-1) D-apiofuranoside isolated from Deilbergia volubilis showed anti-inflammatory activity.

The present study was undertaken to evaluate anti-inflammatory effects of the ethyl acetate fraction obtained from the flowers of R.hypocrateriformis (RHF), S.brevistigma (SBF), C.philippinum (CPF) and roots of E.viride (EVR).
3.3.4 Materials and methods:

The anti-inflammatory activity was evaluated using the following methods.

1. Carrageenin-induced rat hind paw edema.

2. Cotton pellet granuloma.

Animals:

Male albino rats (Wistar strain) (150-200g) were used as experimental models. The animals were given food and water ad libitum (supplied by M/s. Hindustan Lever Limited, Bangalore). The animals were housed under standard conditions of 12h light and 12h dark cycle at ambient temperature (35-36°C). The experiments were carried out during light cycle.

3.3.5 Carrageenin-induced rat hind paw edema:

The anti-inflammatory activities were evaluated using Carrageenin-induced rat hind paw edema method. The rats were divided into fourteen groups. Each group consisted of six animals. Group I served as control and received 2ml/kg p.o. of gum acacia in saline (0.5% w/v) while the second group was treated with indomethacin (Tablets India, Chennai), (1Omg/kg p.o). Group III, IV and V were treated with RHF at a dose of 125, 250 and 500 mg/kg p.o. respectively. Group VI, VII and VIII were treated with SBF at a dose of 125, 250 and 500 mg/kg p.o. respectively. Group IX, X and XI were treated with EVR at a dose of 125, 250 and 500 mg/kg p.o. respectively, while group XII, XIII and XIV were treated with CPF at a dose of 125, 250 and 500 mg/kg p.o. respectively, one hour before the carrageenin injection.
Inflammation was caused by injecting 0.1 ml of 1% carrageenin (Sigma Chemical Co.) suspension (in 0.5% w/v gum acacia) into the right hind paw of each rat in the sub plantar region. The paw volume was measured using a plethysmometer at 0 h and 3 h after injection of carrageenin. A single dose of drug pretreatment was given 1 h before the injection of carrageenin in the morning at 9.00 am. The percentage inhibition of edema was calculated.

\[
\text{% inhibition} = 100 - \left( \frac{\text{Increase in paw volume of the drug treated animals}}{\text{Increase in paw volume of the control animals}} \right) \times 100
\]

3.3.6 Cotton pellet granuloma:

The anti-inflammatory activities were evaluated using Cotton pellet granuloma method. The rats were divided into fourteen groups. Each group consisted of six animals. The experimental groups were designated as discussed in section 3.3.5.

In cotton pellet granuloma model the rats were anaesthetized with pentobarbitone (30 mg/kg). After removing the fur on the back, through a single midline incision on the dorsal surface, sterilized pre-weighed cotton pellets were implanted in both axillae and groin regions. The standard and test drugs were administered daily in the morning at 9.00 am for 10 days (0 to 9 days). On the 10th day the rats were sacrificed and the pellets were dissected out, weighed, dried at 60°C and the dry weights were determined.

The percentage of anti-inflammatory activity was determined by noting the difference in drug treated granuloma weight with that of the control from the relation \( \text{viz.} \).
Statistical Analysis:

All values were expressed as mean ± SEM. The data were statistically analyzed by one-way ANOVA followed by Dunnett’s test.

3.3.7 Results and discussion:

Table 3.7 shows the effect of the ethyl acetate fraction of RHF, SBF, EVR and CPF, containing the flavonoidal glycosides on carrageenin induced rat paw edema. A dose dependent inhibition of carrageenin induced rat hind paw edema with maximum activity was observed during the second phase of inflammation. The edema suppressant effect was significant (P<0.01) with doses of 250 and 500 mg/kg of RHF, SBF, EVR and CPF when compared with control by one-way ANOVA followed by Dunnett’s test. In the case of rats treated with 125 mg/kg of RHF, SBF and EVR the edema suppressant effects were less, but statistically significant. (Table 3.7 and Fig.3.3), But in the case of rats treated with 125mg/kg of CPF the edema suppressant effect was nonsignificant as compared with control.

Group II treated with indonithacin (10 mg/kg) exerted 65% anti-inflammatory activity. The maximum anti-inflammatory effect was obtained with 500 mg/kg doses of all the extract.

Carrageenin-induced paw edema was taken as a prototype of exudative phase of inflammation. The development of edema had been described as biphasic. The initial phase is due to release of histamine, serotonin and kinin in the first hour after injection of carrageenin. More pronounced second phase is
related to the release of prostaglandin tike substance in 2-3 h\textsuperscript{76}. It has been reported that the second phase of edema is sensitive to both clinically useful steroidal and non-steroidal anti-inflammatory agents\textsuperscript{77}. Diinsininol, diinsinin 5-glucoside isolated from \textit{Sarcophyte piriei} inhibit prostaglandin synthesis\textsuperscript{78}. Flavonoids have been shown to inhibit prostaglandin synthatase\textsuperscript{31}.

Table 3.7
Effect of test drugs on carrageenin-induced rat paw edema

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
<th>Dose (mg/kg)</th>
<th>Increase in paw volume after 3h (ml)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control (saline)</td>
<td>2ml/kg</td>
<td>0.52±0.02</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Indomethacin</td>
<td>10</td>
<td>0.18±0.02\textsuperscript{a}</td>
<td>65.38</td>
</tr>
<tr>
<td>III</td>
<td>RHF</td>
<td>125</td>
<td>0.39±0.04\textsuperscript{a}</td>
<td>25.00</td>
</tr>
<tr>
<td>IV</td>
<td>RHF</td>
<td>250</td>
<td>0.32±0.04\textsuperscript{a}</td>
<td>38.47</td>
</tr>
<tr>
<td>V</td>
<td>RHF</td>
<td>500</td>
<td>0.28±0.0 r\textsuperscript{i}</td>
<td>46.16</td>
</tr>
<tr>
<td>VI</td>
<td>SBF</td>
<td>125</td>
<td>0.39±0.04\textsuperscript{a}</td>
<td>25.00</td>
</tr>
<tr>
<td>VII</td>
<td>SBF</td>
<td>250</td>
<td>0.30±0.03\textsuperscript{a}</td>
<td>42.31</td>
</tr>
<tr>
<td>VIII</td>
<td>SBF</td>
<td>500</td>
<td>0.24±0.02\textsuperscript{a}</td>
<td>53.85</td>
</tr>
<tr>
<td>IX</td>
<td>EVR</td>
<td>125</td>
<td>0.40±0.03\textsuperscript{a}</td>
<td>23.08</td>
</tr>
<tr>
<td>X</td>
<td>EVR</td>
<td>250</td>
<td>0.31±0.04\textsuperscript{a}</td>
<td>40.04</td>
</tr>
<tr>
<td>XI</td>
<td>EVR</td>
<td>500</td>
<td>0.27±0.01\textsuperscript{a}</td>
<td>48.08</td>
</tr>
<tr>
<td>XII</td>
<td>CPF</td>
<td>125</td>
<td>0.42±0.03</td>
<td>19.23</td>
</tr>
<tr>
<td>XIII</td>
<td>CPF</td>
<td>250</td>
<td>0.32±0.04\textsuperscript{a}</td>
<td>38.47</td>
</tr>
<tr>
<td>XIV</td>
<td>CPF</td>
<td>500</td>
<td>0.28±0.01\textsuperscript{a}</td>
<td>46.16</td>
</tr>
</tbody>
</table>

n = 6 \textsuperscript{a}P<0.01 Vs Control.
Data were analysed by one-way ANOVA followed by Dunnett’s test.
Values are expressed as mean ± SEM
The cotton pellet granuloma method has been widely employed to assess the transudative, exudative and proliferative components of chronic inflammation. The ethylacetate extract of RHF, SBF, EVR and CPF exerted dose related effects and inhibition of granuloma was significant (P<0.01) in the doses of 125, 250 and 500 mg/kg in case of all test drugs when compared to control by one-way ANOVA followed Dunnet (Table 3.8),

In the cotton pellet granuloma model inflammation, the granuloma develops during a period of several days. So the anti-inflammatory activity of extracts was assessed after the ninth day by measuring the weight of granuloma. The fluid absorbed by the pellet greatly influences the wet weight of granuloma and the dry weight correlates well with the amount of granulomatous tissue formed\textsuperscript{79}. Three phases of inflammatory responses to subcutaneous implantation of cotton pellets have been described\textsuperscript{80}. Pellets with fluid of low protein content
characterize the first, phase of short duration and the second phase is due to the exudation of fluid containing protein. The last phase is characterized by proliferative fibroplasts capable of synthesizing collagen.

Table 3.8

Effect of test drugs on cotton pellet granuloma in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
<th>Dose (mg/kg)</th>
<th>Granuloma weight (wet weight in mg)</th>
<th>% inhibition wet weight</th>
<th>Granuloma weight (dry weight in mg)</th>
<th>% inhibition dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control (saline)</td>
<td>2 ml/kg</td>
<td>226.0±1.20</td>
<td>56.70±1.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Indo methacin</td>
<td>10</td>
<td>80.20±1.14*</td>
<td>64.51</td>
<td>18.60±1.45*</td>
<td>67.20</td>
</tr>
<tr>
<td>III</td>
<td>RHF</td>
<td>125</td>
<td>130.12±1.84*</td>
<td>41.15</td>
<td>34.80±1.64*</td>
<td>38.62</td>
</tr>
<tr>
<td>IV</td>
<td>RHF</td>
<td>250</td>
<td>126.40±2.12**</td>
<td>57.03</td>
<td>32.50±1.20*</td>
<td>42.68</td>
</tr>
<tr>
<td>V</td>
<td>RHF</td>
<td>500</td>
<td>88.08±1.08a</td>
<td>60.02</td>
<td>22.50±0.83a</td>
<td>60.30</td>
</tr>
<tr>
<td>VI</td>
<td>SBF</td>
<td>125</td>
<td>146.30±1.15*</td>
<td>33.98</td>
<td>38.00±1.15</td>
<td>33.00</td>
</tr>
<tr>
<td>VII</td>
<td>SBF</td>
<td>250</td>
<td>122.06±1.26*</td>
<td>44.90</td>
<td>29.40±1.10*</td>
<td>48.14</td>
</tr>
<tr>
<td>VIII</td>
<td>SBF</td>
<td>500</td>
<td>84.29±1.10a</td>
<td>61.96</td>
<td>19.83±0.70a</td>
<td>65.03</td>
</tr>
<tr>
<td>IX</td>
<td>EVR</td>
<td>125</td>
<td>140.21±1.44**</td>
<td>36.72</td>
<td>38.00±1.15*</td>
<td>33.00</td>
</tr>
<tr>
<td>X</td>
<td>EVR</td>
<td>250</td>
<td>118.73±1.16*</td>
<td>46.42</td>
<td>32.00±1.15*</td>
<td>43.57</td>
</tr>
<tr>
<td>XI</td>
<td>EVR</td>
<td>500</td>
<td>82.04±1.10a</td>
<td>62.97</td>
<td>21.33±1.76a</td>
<td>62.39</td>
</tr>
<tr>
<td>XU</td>
<td>CPF</td>
<td>125</td>
<td>129.04±3.12a</td>
<td>41.60</td>
<td>33.60±1.89a</td>
<td>46.04</td>
</tr>
<tr>
<td>XIII</td>
<td>CPF</td>
<td>250</td>
<td>125.34±1.44a</td>
<td>43.43</td>
<td>30.40±1.32a</td>
<td>46.35</td>
</tr>
<tr>
<td>XIV</td>
<td>CPF</td>
<td>500</td>
<td>97.23±1.84a</td>
<td>56.12</td>
<td>23.16±1.07a</td>
<td>59.16</td>
</tr>
</tbody>
</table>

n=6 *P<0.01 Vs Control. Data were analysed by one-way ANOVA followed by Dunnett’s test. Values are expressed as mean ± SEM.
This model is the induction for proliferate phase of inflammation. Inflammation involves proliferation of macrophages, neutrophages, neutrophils and fibroblasts, which are basic sources for granuloma formation. It can be noted from Table 3.8 and Fig. 3.4 that the extracts obtained from EVR showed better activity (62.97%) than SBF (61.92%), RHF (60.02%) and CPF (56.12%) at a dose of 500 mg/kg in inhibiting wet weight of cotton pellet granuloma. But in the case of the standard drug indomethacin the inhibition produced was 64.51%. On a dry weight basis, SBF exerted better effect (65.03%) than EVR (62.39%), RHF (60.30%) and CPF (59.16%). The activity shown by the extracts was comparable with standard drug (67.02%). Hence, it can be said that all extracts effectively inhibited the proliferation of granulomatous tissue. There are numerous reports already cited for the anti-inflammatory activity of flavonoids.

The phytochemical investigation of the four chosen plants have already revealed the presence of luteolin 6- prenyl 7-0- rhamnoside in RHF, quercetin 5'-prenyl 3-0- glucosyl 7-0- (4p-coumaroyl) neohesperidoside in SBF, vitexin,
luteolin 7-0- (2"-sinapoyl) glucoside and luteolin 7-0- (4" seneciroyl) neohesperidoside in EVR and apigenin 5-methyl ether, 3-prenyl 7-0- rhamnoside in CPF (Chapter II).

Hence it can be concluded that the anti-inflammatory activity of RHF, SBF, EVR and CPF could be due to the presence of bio flavonoidal constituents. It was found that SBF extract displayed promising anti-inflammatory activity than other extracts. Normally, the anti-inflammatory drugs, though potent, produce gastritis as one of the major side effects. This limits their use either as analgesic or anti-inflammatory agents. Flavonoids have been shown to be unique in possessing anti-nociceptive and anti-inflammatory activity along with anti-ulcer property. Such a unique combination of these three activities in a single class of compounds is not documented for any other type of compounds. Flavonoids have been reported to have high margin of safety, lacking side effects like ulcerogenicity. Flavonoids, which are usual dietary supplements, can be safely recommended for use either as analgesic or anti-inflammatory or anti-ulcer whenever needed. The identification of anti-nociceptive and anti-inflammatory activities of these plant drugs containing flavonoidal compounds is considered significant. The use of carrageenin edema and cotton pellet granuloma assay models of experimental inflammation has led to detection of a number of clinically useful anti-inflammatory compounds. It is possible to exploit the therapeutic potential of these drugs especially the anti-inflammatory activity of these flavonoidal constituents after detailed clinical trial.
3.4 HEPATOPROTECTIVE ACTIVITY

3.4.1 Introduction:

The liver is the principal organ for the metabolism of carbohydrates, proteins, lipids, porphyrins and bile acids. The liver is also the major site for storage of iron, glycogen, lipids and vitamins. The liver plays an important role in the detoxification of xenobiotics and excretion of metabolic end products such as bilirubin, ammonia and urea.

The functional unit of the liver is a lobule. The liver is a highly vascularized organ and receives one quarter of the cardiac output. Hepatocytes represent about 65% of the total cell population and 90% of the volume of the liver. Hepatocytes are richly endowed with drug-metabolizing enzymes, which convert chemicals to more water-soluble forms, which can be secreted readily by the body. Mitochondria are abundantly numerous in the liver cells for energy production. Lysosomes are common to the hepatocytes. Both the rough-surfaced and the smooth-surfaced varieties of endoplasmic reticulum are well developed in the hepatocytes. Most of the hepatic drug metabolizing enzyme activity is confined to the smooth endoplasmic reticulum.

Liver diseases constitute a major medical problem of worldwide proportions. They can be caused by a variety of agents, mainly viruses, parasites and toxins. The central role-played by the liver in the removal of the chemicals from the portal circulation and subsequent metabolism and disposition makes it susceptible to first and often persistent attack by the offending chemicals, culminating in toxic injury.
Hepatotoxicity may be defined as the effect of any agent on the liver, which results in a deviation from the normal function and morphology of this organ. More than 600 compounds are suspected of being possibly hepatotoxic.

Hepatotoxicity may be inflicted by synthetic chemicals, drugs and naturally occurring chemicals such as bacterial, fungal, plant and animal toxins. The list of most hepatotoxic compounds and a brief description of the lesions induced by them are well reviewed. Some examples of hepatotoxins include carbon tetrachloride (CC\textsubscript{4}), DDT, mirex, phenobarbital, diphenylhydantoin, phosphorus, thioacetamide, dimethylnitrosoamine (DMN), ethionine, dimethylaminobenzene (DMAB), pyrrolizidine alkaloids and galactosamine.

The diverse variety of toxic chemicals that are capable of inflicting hepatotoxicity may be categorized based on the circumstances of exposure, property of the toxin or the host characteristics. The hepatic toxicity can be

a) Acute toxicity,

b) Subchronic toxicity, and

c) Chronic toxicity.

The term ‘acute’ may be used to indicate exposure to a single dose and the severity of toxicity. The term chronic is used to indicate either repeated exposure over an extended period of time or the presence of a hepatotoxic response over an extended period of time. While acute toxicity may result in necrosis of the liver, continued exposure may lead to additional cell death and withdrawal may lead to a partial or complete recovery. Repeated and prolonged exposure results in sub chronic expression of toxicity such as necrosis. Continued and repeated exposure may lead to cirrhosis.
Zimmerman\textsuperscript{86} classified hepatotoxic agents also on the basis of mechanisms. He divided intrinsic hepatotoxins into either direct hepatotoxins (injure liver cells by affecting a number of organelles such as the endoplasmic reticulum, mitochondria and lysosomes, example: CCl\textsubscript{4}) or indirect hepatotoxins (affect particular metabolic pathways, which uncouples the metabolic organization of hepatocytes, example: galactosamine).

All the hepatotoxins impair liver functions. Many toxic chemicals may alter hepatocellular permeability. Necrosis leads to high levels of serum transaminases (serum glutamate-oxalate transaminase (SGOT) and serum glutamate-pyruvate transaminase (SGPT), now renamed as aspartate amino transferase (AST) and alanine transaminase (ALT) respectively), alkaline and acid phosphatases, lactic dehydrogenase isoenzymes, isocitric dehydrogenase and sorbitol dehydrogenase\textsuperscript{9}\textsuperscript{9} Other responses include overproduction of acute phase proteins, fatty acid accumulation, increased production and deposition of collagen, hepatitis, allergic and hypersensitivity reactions, storage diseases (vitamin A, iron), vascular and sinusoidal alterations and tumors. In CCL\textsubscript{4}-induced hepatocellular necrosis, accumulation of Ca\textsuperscript{2+} has also been demonstrated.

Cirrhosis can be defined as a chronic disease condition representing morphological alterations of the lobular structure characterized by destruction and regeneration of parenchymal cells and increased connective tissue\textsuperscript{86}. Major morphological changes include granular or nodular appearance and are characterized by the presence of septae of collagen throughout the liver. The aggregated liver cells circumscribed by sheath-like fibrous growth of collagenized connective tissue give the appearance of nodules of hepatic cirrhosis. Necrosis or cell death may be zonal, massive or diffuse.
The toxin itself, a stable metabolite, or a reactive metabolite can induce toxicity. The formation of chemically reactive metabolites by oxidation mediated by cytochrome P-450 or in few cases by reduction may lead to three main initial molecular lesions: covalent binding to proteins and nucleic acids, covalent binding to lipids and lipid peroxidation produced by free radicals or activated oxygen and glutathione depletion\(^8\).

Accumulation of lipid in the liver has been variously referred to as fatty infiltration, fatty degeneration and liposis\(^9\). Liposis refers to excessive accumulation of fat or lipid material prominently consisting of triglycerides and fatty acids. Normal liver may contain 5% of its weight as fat. Lipidotic liver may contain up to 50% of its weight as fat, most of it being triglycerides. Under conditions of hepatic triglyceride accumulation, lipid droplets appear surrounded by membranes to form liposomes.

3.4.2 Hepatoprotective activity of plant extracts:

In traditional medicines, various herbal preparations are being used for treating liver disorders. In the absence of an effective treatment in modern medicine, efforts are being made to find out suitable herbal drugs. Polyherbal formulations reputed to have hepatoprotective activity that are available on the Indian market comprise of about 100 Indian Medicinal Plants\(^9\). Reports on the hepatoprotective activity of the many species have been published: some of the plants investigated are Anastatica hierochuntica\(^9\), Andrographis paniculata\(^9\), Apium graveolens\(^9\), Butea frondosa\(^9\), Carica papaya\(^9\), Caseria esculenta\(^9\), Cichorium intybus\(^9\), Cachlospermum tinctorium\(^9\), Croton oblongifolius\(^9\), Daccus carta\(^9\), Emblica officinalis\(^9\), Fumaria indica\(^9\), Hygrophyla auriculata\(^9\), Kalachoe pinnata\(^9\), Lawsonia alba\(^9\), Moringa oleifera\(^9\), Moringa.
pterygosperma, Orthosiphon thymiflorus, Petroselinum crispum,
Ptreocarpus marsupium, Trichopus zeylanicus, Turkis folk remedies,
Ventilctgo leiocarpa and Ziziphus mauritiana.

The hepatoprotective activities of flavonoids have been extensively studied and many flavonoids do exhibit significant activity. In a study carried out to investigate silymarin, apigenin, quercetin and naringenin as putative therapeutic agents against microcrystin LR - induced hepatotoxicity, silymarin was found to be the most effective one. Rutin and venorutin showed regenerative and hepatoprotetive effects in experimental cirrhosis.

The hepatoprotective activity of Ginkgo biloba on paracetamol induced hepatic damage in rats has been attributed to the presence of flavone glycoside.

Flavonoids and phenolic acids have been shown to be responsible for hepatoprotective activity of Rosmarinus tomentosus and R. officinalis.

Hepatoprotective activity of kaempferol 3-O-glucoside from methanol extract of Equisetum arvense on tacrine - induced cytotoxicity in human liver-derived Hep G2 cells has been reported. The hepatoprotective activity of silymarin was studied in androgenic anabolic steroid induced liver damage, which showed a significant increase in the glycogen density in hepatocytes. Quercetin has been found to be a potent inhibitor of human liver phenol sulfotransferase and estrogen sulfotransferase activities and sulfation of resveratrol. Smitilbin, engeletin, astilbin and dihydroquercetin isolated from rhizomes of Smilax glabra are capable of preventing immunological hepatocyte damage.

Kaempferol, naringenin and isohelichrysin were most active components in increasing bile secretion. Eupatolin and arcapallin isolated from Artemisia capillaris were attributed to the liver protective activity on CCl4 induced lesion in
3.4.3 Present study:

Liver is the most important organ concerned with metabolic activities in the human body. It has tremendous capacity to detoxify and synthesize useful principles. Therefore, damage to the liver inflicted by hepatotoxic agents is of serious concern. There is an ever-increasing need for an agent, which could protect liver from such damages. A great deal of research has been carried out to evaluate the scientific basis for hepatoprotective activity of herbal agents as a single agent or in combination. Many of the analgesic drugs when taken for a prolonged period induce hepatotoxicity. Therefore, in this study, ethyl acetate extract (mainly of flavonoid constituents) from the flowers of *R. hypocrateriformis* (RHF), *S. brevistigma* (SBF), *C. philippinum* (CPF) and root of *E. viride* (EVR) have been evaluated for their effect on liver functions.

The present study was thus aimed at determining the effect of the extracts of the plants on drug metabolizing enzymes in CCl₄ induced liver toxicity in experimental animal models. Silymarin (a flavonoidal mixture), a known hepatoprotective drug obtained from the fruits of *Silybum marianum* (milk thistle) (Compositae) was used as a standard drug in the present study.

3.4.4 Materials and methods:

**Animals:**

Male albino rats (Wistar strain) (150-200g) were used as experimental models. The animals were given food and water *ad libitum* (supplied by M/s. Hindustan Lever Limited, Bangalore). The animals were housed under...
standard conditions of 12h light and 12h dark cycle at ambient temperature (35-36°C). The experiments were carried out during light cycle.

Experiments:

Hepatoprotective activity was evaluated using CCl₄-induced model¹⁴. The rats were divided into eleven groups. Each group consisted of six animals. Dosing in all groups was carried out once a day for a period of seven days. All dosing was done by oral (p.o.) route. Group I served as control, which received vehicle (gum acacia 0.5%w/v in saline at a dose of 2 ml/leg). Group II was treated similar to group one except for being administered with CCl₄ in liquid paraffin (1:4) at a dose of 1.25ml/kg(E. merck, India) on day seven, Group III was treated with silymarin (100 mg/kg) (Microlabs, Osshur) daily for seven days. Extracts obtained from RHF, SBF, EVR and CPF were administered to groups IV, VI, VIII and X at a dose of 125 mg/kg for seven days. Groups V, VII, IX, and XI received a dose of 250 mg/kg from the same extract in that order.

On the seventh day, the animals of the groups III to XI were given CCU orally 30 minutes after the administration of the standard and test drugs. After 36 hours of CCl₄ administration, the rats of all the eleven groups were anaesthetized and the blood was collected by puncturing the retro orbital plexus. The blood samples were allowed to clot for 30 minutes and serum was separated by centrifugation at 2500 rpm for 15 minutes. The separated serum samples were analyzed for various biochemical parameters.

3.4.5 Assessment of liver function:

Bio-chemical parameters:

The liver function tests were carried out using following biochemical parameters.
a) **Serum glutamate-oxaloacetate transaminase (SGOT):**

Serum glutamate oxaloacetate transaminase also called as Serum aspartate transaminase present in serum was estimated by using kinetic method\(^{115}\). SGOT catalyses the transformation of amino group from L-aspartate to 2-oxoglutarate by forming oxaloacetate and L-glutamate. This reaction is coupled to the reduction of the oxaloacetate to malate by malate dehydrogenase in the presence of reduced nicotinamide adenine dinucleotide (NADH\(_2\)). The oxidation of NADH\(_2\) and hence transaminase reaction was followed by measuring the decrease in the light absorption at 340nm. The enzyme activity was expressed as U/l.

Enzyme reagent 1 for the analysis of SGOT contained TRIS buffer (pH 7.8) (80 mmol/l), L-aspartate (240 mmol/l), lactate dehydrogenase (≥ 600 U/l) and malate dehydrogenase (≥ 600 U/l) while reagent 2 had 2-oxoglutarate (12 mmol/l) and NADH\(_2\) (0.18 mmol/l).

To 200 \(\mu\)l of serum samples, 1000 \(\mu\)l of enzyme reagent 1 was added and incubated for 5 minutes at 37\(^\circ\)C. To this 250 \(\mu\)l of reagent 2 was added and mixed well so as to form L-malate and NAD\(^+\). The absorbance was measured after a minute at 340 nm using an auto analyzer.

b) **Serum glutamate-pyruvate transaminase (SGPT):**

Serum glutamate pyruvate transaminase also called as Serum alanine transaminase was estimated by using a kinetic method\(^{115}\). SGPT catalyses the transfer of amino group from L-alanine to 2-oxoglutarate with the formation of pyruvate and L-glutamate. The pyruvate so formed is allowed to react with NADH\(_2\) to produce L-lactate. The rate of this reaction was monitored by an indicator reaction that was coupled with lactate dehydrogenase in the presence of
NADH<sub>2</sub>. The oxidation of NADH<sub>2</sub> in this reaction was measured as the decrease in the absorbance of NADH<sub>2</sub> at 340nm, which was proportional to SGPT activity. The enzyme activity was expressed as U/l.

The reagent-1 for the analysis of SGPT contained TRIS buffer (pH 7.5) (100 mmol/l), L-alanine (500 mmol/l) and lactate dehydrogenase (≥ 1200 U/l) while reagent-2 had 2-oxoglutarates (15 mmol/l) and NADH<sub>2</sub> (0.18 mmol/l).

To 200 µl of serum sample 1000 µl of reagent-1 was added and the mixture incubated for 5 minutes at 37°C. To this, 250 µl of reagent-2 was added, mixed well so as to form L-lactate and NAD<sup>+</sup>. The absorbance was measured after a minute at 340 nm using an autoanalyser. The SGPT content was calculated in U/l.

**c) Alkaline phosphatase (ALP):**

Alkaline phosphatase also called as orthophosphoric monoester phosphohydrolase was estimated by colorimetric method using “optimized standard method”<sup>116</sup>. The estimation was on the principle that serum alkaline phosphatase hydrolyses p-nitro phenyl phosphate into p-nitro phenol and phosphate under defined conditions of time, temperature and pH.

Reagent-1 for the analysis of ALP had diethanol amine buffer solution (pH 9.8) (1.0 mol/l) and magnesium chloride (0.5 mmol/l) while reagent-2 had p-nitro phenyl phosphate (10 mmol/l). To 20 µl of serum sample 1000 µl of reagent-1 was added and mixed well. The mixture was incubated for a minute at 37°C. To this mixture 250 µl of reagent-2 was added and the absorbance was measured at 405 nm using an autoanalyser.
d) **Total bilirubin and direct bilirubin:**

Serum bilirubin was estimated by a colorimetric method. The estimation of bilirubin in serum was based on the formation of a purple compound of azo-bilirubin where bilirubin in serum was allowed to react with a freshly prepared solution of diazo reagent.

Reagent-1 had sulfanilic acid (29 mmol/l) and HCl (0.17N) while reagent-2 contained sodium nitrite (25 mmol/l). Reagent-3 contained caffeine (0.26 mol/l) and sodium benzoate (0.52 mol/l) whereas reagent-4 had tartrate (0.93 mol/l) and NaOH (1.9 N) and finally reagent-5 contains of sodium chloride solution (0.9%w/v).

Total bilirubin was estimated by mixing 100 μl of serum sample with 100 μl of reagent-1, 25 μl of reagent-2 and 500 μl of reagent-3, mixed well and incubated at 37°C for 10 minutes. To this 500 μl of reagent-4 was added and mixed well. The mixture was then incubated at 37°C for 5 minutes to complete the reaction. The absorbance was measured at 578 nm using an auto analyzer.

Direct bilirubin was determined by mixing 100 μl of serum sample with 100 μl of reagent-1, 25 μl of reagent-2 and 1ml of reagent-5. The thoroughly mixed mixture was incubated at 37°C for 5 minutes and absorbance was measured at 578 nm.

e) **γ-glutamyl transferase (GGT) or γ-glutamyl transpeptidase (GGTP):**

Quantitative in vitro measurement of γ-glutamyl transferase (γ-GT) activity in serum was done using a method reported by Szasz. γ-glutamyl transferase catalyses the transfer of the glutamyl group from L-γ-glutamyl 3-carboxy 4-nitroanilide to glycylglycine and 5-amino 2-nitro benzoate.
The substrate has no colour while 5-amino 2-nitro benzoate absorbs strongly at 405 nm. The amount of 5-amino 2-nitro benzoate liberated is proportional to γ-GT activity and may be measured kinetically at 405 nm by increasing intensity of the yellow colour formed.

The reagent contained L-γ-glutamyl-3-carboxy-4-nitroanilide (3.3 mmol/l) and glycylglycine (110 mmol/l). The substrate was prepared by mixing serum sample (100 μl) and reagent (2 ml) glutamyl glycylglycine and 5-amino 2-nitro benzoate was formed and absorbance measured at 405 nm using an auto analyzer.

3.4.6 Histopathological studies:

After collection of blood samples, the rats were sacrificed and their liver was isolated, perfused with chilled normal saline and taken for biopsy studies. The ratio of wet liver weight per 100 g of animal body weight was calculated. The liver was examined grossly and weighed, preserved in 10% (v/v) formalin for further studies. Liver slices were fixed in Bouin’s solution for 12 hours and then processed for paraffin embedding using a standard technique. A 5 μm section of the liver was sliced and stained with alum haemotoxylin and eosin. The slides were placed under an optical microscope and observed (45x) for any histopathological changes, i.e., normal, damaged and recovered liver between the treated and control group of animals.

Statistical Analysis:

All values were expressed as mean ± SEM. The data were statistically analyzed by one-way ANOVA followed by Dunnett’s test.
3.4.7 Results and discussion:

Liver cells participate in a variety of metabolic activities and thus contain a host of enzymes. CCl₄ is one of the most commonly used hepatotoxin in the experimental study of liver diseases. The hepatotoxicity induced by CCl₄ is due to its metabolites \textit{viz.}, trichloromethyl (CCl₃⁺) and trichloromethyl peroxy (CCl₃O₂⁺), radicals that bind to lipoprotein and lead to peroxidation of lipids of endoplasmic reticulum\textsuperscript{120}. These activated radicals bind covalently to the macro molecules and induce peroxidative degradation of membrane lipids of endo-plasmic reticulum rich in poly unsaturated fatty acids. This leads to the formation of lipid peroxides. This lipid peroxidative degradation of biomembranes is one of the principal causes of hepatotoxicity of CCl₄\textsuperscript{121}. In severe acute liver damage, serum transaminase levels are high indicating that both cellular and mitochondrial membranes have been damaged. It is reported that large doses of CCl₄ results in cell lysis and cytoplasmic hepatic enzymes are released into blood circulation\textsuperscript{122}.

The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic mechanisms, which have been disturbed by a hepatotoxin, is the index of its protective effects. Protection of hepatic damage caused by CCl₄ administration was observed by changes in the marker enzymes such as SGPT, SGOT, ALP, total bilirubin and GGTP in normal, toxin treated, standard drug (silymarin) treated and extract treated (RHF, SBF, EVR and CPF in both 125 mg/kg and 250 mg/kg) groups. The disturbance in the transport function of the hepatocytes as a result of hepatic damage causes the leakage of enzymes from cells due to altered permeability of membrane\textsuperscript{123}.
Table 3.9

Effect of test drugs on the levels of marker enzymes in CCl₄ treated rats.

<table>
<thead>
<tr>
<th>Pre treatment</th>
<th>Treatment</th>
<th>Dose mg/kg</th>
<th>SGPT U/L</th>
<th>SGOT U/L</th>
<th>ALP U/L</th>
<th>Total Bil mg%</th>
<th>Direct Bil mg%</th>
<th>GGTP U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Saline)</td>
<td>-</td>
<td>2 ml/kg</td>
<td>131.50±0.80</td>
<td>45.30±0.80</td>
<td>195.60±10.60</td>
<td>0.70±0.03</td>
<td>0.20±0.07</td>
<td>123.00±4.10</td>
</tr>
<tr>
<td>Saline</td>
<td>CCl₄</td>
<td>1.25 ml/kg</td>
<td>217.30±4.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>341.00±3.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>388.60±19.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.20±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.50±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>257.30±5.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Silymarin</td>
<td>CCl₄</td>
<td>100</td>
<td>138.00±2.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81.30±9.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>218.60±5.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.80±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.28±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>124.60±5.20&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RHF</td>
<td>CCl₄</td>
<td>125</td>
<td>178.60±2.32</td>
<td>120.50±3.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>288.60±3.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.90±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>135.50±2.61&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RHF</td>
<td>CCl₄</td>
<td>250</td>
<td>167.60±2.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>104.40±3.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>253.30±4.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.81±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.30±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>126.00±2.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SBF</td>
<td>CCl₄</td>
<td>125</td>
<td>142.10±1.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90.10±3.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>324.00±4.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.94±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.30±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>130.30±2.40&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SBF</td>
<td>CCl₄</td>
<td>250</td>
<td>132.30±1.90</td>
<td>76.00±1.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>222.60±5.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.90±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.22±0.01</td>
<td>126.00±2.40&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>EVR</td>
<td>CCl₄</td>
<td>125</td>
<td>158.10±3.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>125.50±2.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>219.60±4.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.82±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.31±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>128.40±1.90&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>EVR</td>
<td>CCl₄</td>
<td>250</td>
<td>135.00±3.70</td>
<td>110.20±2.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>270.30±4.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.75±0.01</td>
<td>0.24±0.02</td>
<td>126.50±1.90&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CPF</td>
<td>CCl₄</td>
<td>125</td>
<td>172.50±3.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>190.20±4.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>341.80±9.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.96±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.36±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>129.20±1.90&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CPF</td>
<td>CCl₄</td>
<td>250</td>
<td>156.50±0.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>179.00±6.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>301.80±10.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.84±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>126.40±1.80&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

n = 6; <sup>a</sup>P < 0.01
Data were analyzed by one way ANOVA to Dunnett’s test
Values are expressed as mean ± SEM
**Fig 3.5**
Effect of test drugs on SGPT level in CCl4 treated rats.

**Fig 3.6**
Effect of test drugs on SGOT level in CCl4 treated rats.

**Fig 3.7**
Effect of test drugs on ALP level in CCl4 treated rats.
Fig 3.8
Effect of test drugs on total bilirubin level in CCl₄ treated rats.

% increase in total bilirubin

Dose treatment (mg/kg)

Fig 3.9
Effect of test drugs on direct bilirubin level in CCl₄ treated rats

% increase in direct bilirubin

Dose treatment (mg/kg)

Fig 3.10
Effect of test drugs on GGTP level in CCl₄ treated rats

% increase in GGTP

Dose treatment (mg/kg)
This results in decreased levels of SGOT and SGPT in the hepatic cells and a raise in their serum level. From Table 3.9, it is clear that extracts obtained from RHF, SBF, EVR and CPF show greater hepatoprotective effect at a dose of 250 mg/kg and the values are comparable to those of silymarin, the standard drug used in the present study.

The serum alkaline phosphatase and glutamyl transpeptidase levels act as indicators to any obstruction in the biliary system, either within the liver or in the larger bile channels outside the liver. These enzymes are elevated in large number of disorders that affect the drainage of bile, such as gallstone or tumour blocking the common bile duct, or alcoholic liver disease or drug induced hepatitis, blocking the flow of bile in smaller bile ducts within the liver124.

Table 3.10
Effect of test drugs on variation of liver weight in CCl4 treated rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose mg/kg</th>
<th>Liver wt/1OOg body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control (Saline)</td>
<td>2 ml/kg</td>
<td>4.10±0.01</td>
</tr>
<tr>
<td>ri</td>
<td>CCl4, 1.25 ml/kg</td>
<td></td>
<td>6.50±0.28*</td>
</tr>
<tr>
<td>II</td>
<td>Silymarin 100</td>
<td></td>
<td>4.10±0.26</td>
</tr>
<tr>
<td>IV</td>
<td>RHF 125</td>
<td></td>
<td>4.80±0.22*</td>
</tr>
<tr>
<td>V</td>
<td>RHF 250</td>
<td></td>
<td>4.10±0.08</td>
</tr>
<tr>
<td>VI</td>
<td>SBF 125</td>
<td></td>
<td>4.60±0.18*</td>
</tr>
<tr>
<td>VII</td>
<td>SBF 250</td>
<td></td>
<td>4.10±0.10</td>
</tr>
<tr>
<td>VIII</td>
<td>EVR 125</td>
<td></td>
<td>4.80±0.21*</td>
</tr>
<tr>
<td>IX</td>
<td>EVR 250</td>
<td></td>
<td>4.10±0.16</td>
</tr>
<tr>
<td>X</td>
<td>CPF 125</td>
<td></td>
<td>5.00±0.14*</td>
</tr>
<tr>
<td>XI</td>
<td>CPF 250</td>
<td></td>
<td>4.60±0.26*</td>
</tr>
</tbody>
</table>

n = 6; *P < 0.01 Vs normal control
Data were analyzed by one - way ANOVA followed Dunnett’s test
Values were expressed as mean ± SEM
Levels of marker enzymes like ALP and GGTP show marked increase in the CCl$_4$ treated while the levels are brought to near normal in the drug treated group indicating that the flow of bile in the smaller bile duct within the liver is restored.

Bilirubin, the main bile pigment in human beings when elevated, causes jaundice. Bilirubin results from the enzymatic breakdown of haeme. Bilirubin is conjugated with glucuronic acid in hepatocytes to increase its water solubility and is then rapidly transported into bile. Liver disease mainly impairs the secretion of conjugated bilirubin into bile. Some conditions including liver disease or the destruction of red blood cells, causes increased levels of bilirubin in the bloodstream.$^{124}$

It was observed that the colour of the liver was pale reddish brown and size enlarged in CCl$_4$ intoxicated rats but it was normal in drug treated groups. A significant reduction (P < 0.01) in liver weight when compared with normal control supports this finding (Table 3.10 and Fig 3.11). Histopathological studies offer direct evidences for the efficacy of a drug as a hepatoprotective agent. The extract treated groups showed good recovery of the hepatocytes.
Fig. 3.12 Biopsy study of the liver of normal group

Fig. 3.13 Biopsy study of the CCl₄ intoxicated liver

Fig. 3.14 Biopsy study of the CCl₄ intoxicated liver treated with the standard drug silymarin

Fig. 3.15 Biopsy study of the CCl₄ intoxicated liver treated with the ethyl acetate fraction of *R. hypocrateriformis*

Fig. 3.16 Biopsy study of the CCl₄ intoxicated liver treated with ethyl acetate fraction of *S. brevicollis*

Fig. 3.17 Biopsy study of the CCl₄ intoxicated liver treated with ethyl acetate fraction of *E. viride*

Fig. 3.18 Biopsy study of the CCl₄ intoxicated liver treated with ethyl acetate fraction of *C. philippinum*
The liver sections showed almost disappearance of fatty deposit and necrosis, comparable to the standard and drug treated group (Fig 3.14 to Fig 3.18). The lesions developed on treatment with CCl₄ (Fig 3.13) were found to be normalized with near normal histo-architecture of liver cells, in standard drug (Fig 3.14) and extract (RHF, SBF, EVR and CPF) treated animals (Fig 3.15 to Fig 3.18).

Results obtained from enzyme analysis show that among the four extracts taken up for the present study, extract of SBF has remarkable hepatoprotective activity (Table 3.9) The phytochemical investigations of the plants have already been investigated and the study has revealed the presence of flavonoids (Chapter II). It is quite likely that the hepatoprotective activity may be due to the presence of flavonoidal constituents in the extract.

Rajnarayana et al., have observed that quercetin has better radical scavenging activity than other flavonoids. It can be noted from Table 3.9 that SBF showed better hepatoprotective activity than EVR, RHF and CPF at a dose of 250 mg/kg. The greater hepatoprotective activity of SBF may be attributed to the higher scavenging activity of quercetin glycosides compared to the flavone glycosides present in the other plants. Flavonoids with tri or ortho-dihydroxyl groups in the B ring and/or in A ring can scavenge radicals more effectively.

Thus the radical quenching ability of the flavonoidal constituents of these plant drugs may be responsible for the hepatoprotective effect shown by them.
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