9. **IN VIVO PHARMACOLOGICAL STUDIES**

9.1 **Acute oral toxicity study**

The purpose of acute toxicity testing are to obtain information on the biologic activity of a chemical and gain insight into its mechanism of action. The information on acute systemic toxicity generated by the test is used in hazard identification and risk management in the context of production, handling, and use of chemicals. The LD50 value, defined as the statistically derived dose that, when administered in an acute toxicity test, is expected to cause death in 50% of the treated animals in a given period, is currently the basis for toxicological classification of chemicals.

9.1.1 **Materials and methods**

9.1.2 **Experimental animals**

Healthy adult female wistar Albino rat, weighing about 20-25 g were procured from Biogen, Bangalore. The study was approved by the Institutional Animal Ethical Committee, Sri Ramachandra University, Chennai (IAEC/XXXIV/SRU/294/2013Version-I) animals were housed individually in a well ventilated polypropylene cage. 12-hr light/12-hr dark artificial photo period was maintained. Room temperature of 25°C (±3°C) and relative humidity of 50–70 % were maintained in the room. Animals had free access to pelleted feed (Nutrilab rodent, Tetragon Chemie Pvt Ltd., India) and reverse osmosis (Rios, USA) purified water *ad libitum*. For experimental purpose the animals were kept fasting overnight but allowed for access to water.
9.1.3  **Acute toxic class method**

Acute oral toxicity study was performed according to the OECD (Organization for Economic Co-operation and Development) test guideline 423-Acute toxic class method. It is a stepwise procedure with three animals of single sex per step. Depending on the mortality and morbidity status of the animal, an average of 2-4 steps may be necessary to allow judgment on the test substance. The procedure is to fix a minimal number of animals, which allows acceptable database scientific conclusion. The method uses different defined doses (5, 50, 500, 2000 mg/kg body weight) and the results allow a substance to be ranked and classified according to the -Globally Harmonized System (GHS) for the classification of extracts which cause acute toxicity.

**Procedure**

Three healthy, wistar albino rats weighing 20-25 g were selected for the study. The rats were fasted over-night and provided with water *ad libitum*. Following the period of fasting, a dose limit at 2000 mg/kg body weight of ethanol extract dissolved in olive oil was administered orally to the animals of test group. The animals of control group received olive oil. In each case the volume administered was 10 ml/kg body weight. Following administration, the animals were closely observed during the first 3 hr, and 48 hr, thereafter, 14 days, for toxic sign and symptoms such as convulsions, salivation, diarrhoea, lethargy, sleep, coma and death. The weight of the animals was monitored throughout the experimental period.

9.1.4  **Results and discussion**

During the acute toxicity study, the ethanol extract was administered orally and animals were observed for mortality, changes in the autonomic nervous system, and other parameters as outlined in the procedure. The results were compared with the control group to determine the toxicity of the extract. The data were analyzed to determine the lethal dose (LD) values and other toxicological parameters according to the GHS classification system.
system, central nervous system and behavioral responses. The observations were tabulated in table 9.1.

Table 9.1 Observations of acute oral toxicity

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Animal No.</th>
<th>Organs</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>GP (2000 mg/kg body weight)</td>
<td>121</td>
<td>Skin, eyes, brain, lung, heart, liver, kidney, adrenals, spleen and sex glands</td>
<td>No abnormality detected</td>
</tr>
<tr>
<td></td>
<td></td>
<td>122</td>
<td></td>
<td>No abnormality detected</td>
</tr>
<tr>
<td></td>
<td></td>
<td>123</td>
<td></td>
<td>No abnormality detected</td>
</tr>
</tbody>
</table>

There was no mortality observed even at 2000 mg/kg body weight for the extract. No abnormality in the general behavior of the test animals either in the short term or long term, was observed. There was no body weight loss during the observation period. All the animals were found to be normal and there were no gross behavioral changes till the end of the observation period. This observation revealed that the ethanol extract of the plant was found to be very safe up to 2000 mg/kg of body weight known as maximum tolerated dose (MTD) by acute toxicity model study as per OECD guidelines 423. Hence from this, 1/10th and 1/5th of MTD was selected and the effective doses were fixed as 200 and 400 mg/kg for further pharmacological studies.
9.2 *IN VIVO* ANTI-ARTHRITIC ACTIVITY OF ETHANOL EXTRACT OF *Glycosmis pentaphylla*

9.2.1 Animals

Male Wister rats (150 - 250 g) were procured from *Biogen Laboratory Animal Facility* Bangalore. The animals were maintained in animal house at a room temperature of 19-23°C, with relative humidity of 50 to 70 % and alternating 12 Hr dark – light. The animals had free access to standard laboratory feed and water *ad libitum*. The rats were acclimatized to the environment for 1 week prior to experiment use. The study was approved by the institutional animal ethics committee (IAEC/XXXIV/SRU/294/2013 Version-I)

9.2.2 Induction of arthritis

Animals were injected intraperitoneal with 0.1 ml of Complete Freunds Adjuvant (CFA) into the right hind paw of each rat. Animals were assigned to groups of 6 animals. Arthritic control group received only intraperitoneal injection of CFA, but non-arthritic control (IFA group) received intraperitoneal injection of 0.1 ml Incomplete Freunds Adjuvant (IFA) (Sterile Paraffin Oil). ETGP (200 mg/ kg and 400 mg /kg), Methotrexate (0.1 ml/ kg) were administered to rats in the various groups respectively. Paw volume was measured by water displacement plethysmograph (Fereidoni, *et. al*, 2000) for both the ipsilateral (injected paw) and the contralateral paw (non- injected paw) before intraperitoneal injection of CFA (day 0) and every week. The edema component of inflammation was quantified by measuring the different in foot volume between day 0 and day 28. (Pearson *et.al*, 1956)
9.3 Two sets of experiments were carried out

9.3.1 The test drugs on established arthritis (Therapeutic protocol)

Intraperitoneal injection of 0.1 ml CFA was followed by drug administration on day 9 with the onset of arthritis.

Group 1 : Non Control/ IFA (Intraperitoneal injection of 0.1 ml of IFA)
Group 2 : Arthritic control/ CFA (Intraperitoneal injection of 0.1 ml CFA)
Group 3 and 4 : ETGP 200 mg/ kg and 400 mg/ kg respectively from day 9
Group 5 : Methotrexate (0.1 ml/ kg)

9.3.2 Test drug when given before inducing arthritis (Prophylactic protocol)

Drugs were administered on day 0 and CFA was injected intraperitoneal 24 hrs later.

Group 1 : Non Control/ IFA (Intraperitoneal injection of 0.1 ml of IFA)
Group 2 : Arthritic control/ CFA (Intraperitoneal injection of 0.1 ml CFA)
Group 3 and 4 : ETGP 200 mg/ kg and 400 mg/ kg respectively from day 0
Group 5 : Methotrexate (0.1 ml/ kg)

Scores for ipsilateral and contralateral paw volume were individually normalized as percentage of change from their value at day 0 and then averaged for each treatment group. The extract and standard was suspended in 0.5 % CMC. All the drugs were freshly prepared.
9.3.3 Arthritis index

Rats were evaluated every week for arthritis. The physical symptoms of arthritis were judged by the following grading system (Wooley PH, et al., 1981): 0 = normal paws; 1 = erythema of toes; 2 = erythema and swelling of paws; 3 = swelling of ankles; 4 = complete swelling of the whole leg and inability to bend it. The maximum achievable score is thus 16. Arthritis index for each rat was calculated by adding the four scores of individual paws. A sensitized animal was considered to have arthritis when at least one non-injected paw was inflammed (Philippe L, et al., 1997).

9.3.4 Measurement of body weight

Body weight for each rat was recorded before and every week after adjuvant inoculation to assess food intake and weight gain throughout the period of arthritis. The difference between body weight in each day and that of day 0 was calculated to determine the change in body weight in arthritic rats. Percentage of body weight changes was calculated.

9.3.5 Measurement of paw volume changes

Volumes of hind paws were measured before and daily after adjuvant inoculation by using water displacement plethysmometry (David LB, et al., 2001). The changes of volumes of hind paws, from those of day 0, were calculated.

9.3.6 Biochemical analysis

At the end of experiment, on the 28th day all animals were anaesthetized and blood was withdrawn by retro-orbital puncture and collected in plain and EDTA containing tubes, respectively for serum separation. The homogenized samples were
subjected to biochemical examination like BUN (Fischbach FT et al; 2004), CR, TP, ALT, AST, ALP, and CRP in blood and in liver SOD, GSH, LPO, GPX and PROTEIN.

9.3.7 Statistical analysis

The results are presented as the mean ± standard error. Clinical edema volumes measured in different treatment groups were compared with adjuvant non-treated control group (group II) and non-adjuvant control group (group I) by one way ANOVA and Dunnett's Multiple Comparison Test. Also, significance tests were calculated to determine the differences between the effects of different doses.

9.4 Immunohistochemistry

Immunohistochemistry (IHC) refers to the process of detecting antigens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. IHC takes its name from the roots "immuno," in reference to antibodies used in the procedure, and "histo," meaning tissue (compare to immunocytochemistry). The procedure was conceptualized and first implemented [Albert Coons, 1941]

Immunohistochemical staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death. IHC is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue. Visualising an antibody-antigen interaction can be accomplished in a number of ways. In the most common instance, an antibody is conjugated to an enzyme,
such as peroxidase, that can catalyse a colour-producing reaction. Alternatively, the antibody can also be tagged to a fluorophore, such as fluorescein or rhodamine.

9.4.1 Materials and methods

CD4 (#SC7219, Santa Cruz, U.S.A.)

IL-2 (#SC7896 Santa Cruz, U.S.A.)

TGF-beta (#SC-146, Santa Cruz)

TNF-alpha 2 (#SC52746 Santa Cruz)

9.4.2 Protocol

1. Sections deparaffinized in xylene and hydrated through descending grades of alcohol.

2. Heat-induced antigen retrieval was carried out by microwaving at HI-90 for 20 minutes using citrate buffer (pH 6.8) for TNF-alpha or Tris-EDTA buffer (pH 8.0) for CD4, IL-2 and TGF-beta.

3. Each step herein is preceded by three washings in PBST (Phosphate buffered saline with 0.1% tween 20).

4. Endogenous peroxidase quenching by incubation with 3%H2O2 for 30 minutes.

5. Blocking with 5% goat serum in 1% BSA for 30 minutes.

6. Primary antibody incubation at 4°C overnight.

7. Biotinylated secondary antibody incubation for 30 minutes at room temperature.
8. Avidin-Biotin peroxidase incubation for 30 minutes at room temperature.

9. Staining with DAB (3,3'-diaminobenzidine)chromogen for 15-20 minutes.

10. Counterstaining with Harris hematoxylin for 30 seconds.

9.5 Histopathological examination

The rats from each group at the end of the experimental period were euthanized using anaesthetic ether. The left hind paw from all the rats were amputated proximal to the ankle joint and were fixed in 10% neutral buffered formalin for 24 hours followed by decalcification in 10% formic acid for 4 days approximately. After decalcification, the digits were trimmed off and the ankle joints and paw tissue were transected in a mid-sagittal plane. The ankle joint and paw tissues were passed through series of ascending grades of alcohol and embedded in paraffin. 3-4 micron thickness of tissue section were sectioned and then stained with Hematoxylin and Eosin (Bancroft and Gamble, 2008) for histopathological evaluation of joints and paw tissues for arthritis and other associated lesions.

9.6 Results and discussion

Presence of various phytochemical constituents may be responsible for many pharmacological actions like antiinflammatory, antioxidant and antiarthritic.

9.6.1 Effect of Drugs on Adjuvant Induced Arthritis on paw volume

The models of the adjuvant arthritis investigated in the present study were the acute and poly-arthritis/ chronic models corresponding to day 0-10 and days 10-18 post adjuvant inoculation respectively. The one way ANOVA (treatment
X time) revealed a significant (p< 0.05) effect of drug treatment. Total edema produced by each treatment is expressed in arbitrary units as AUC of the time course curves.

All arthritis control animals showed acute inflammatory edema at the ipsilateral (injected paw) around 4-6 days followed by subsequent chronic poly-arthritis model which begins around day 10-12. The progress of inflammatory edema in the contralateral (non-injected) paw was evident on day 12 with systemic inflammation. Throughout the 28 day experiment, there was no significant change in the paw volume of the non inflamed control group injected IFA. ETGP 200 mg/kg and 400 mg/kg modified the time course significantly (p< 0.05) and reduced the acute edema in the ipsilateral paw with the lowest dose table 9.5 used (200 mg/kg) significantly (p< 0.05) reducing the edema with percentage inhibition when compared with standard Methotrexate. (Table 9.5 and Figure 9.3 to 9.6)

This Methotrexate and ETGP completely presented the spread of the arthritic from the ipsilateral to the contralateral paws of the treatment animals. ETGP 400 mg/kg did not exhibit significant anti-arthritic activity. ETGP 200 mg/kg and 400 mg/kg did not show any significant effect compared to standard.

9.6.2 Arthritic score

Results of arthritic score for the therapeutic and prophylactic model showed significant (p< 0.05) score for the arthritic groups with wide spread erythemia and increased hind paw swelling particularly for the ETGP 200 mg/kg body weight as group and lower scores for the anti-arthritic groups 400 mg/kg and Methotrexate (0.1 ml/kg). (Table 9.2 and Figure 9.1)
Table 9.2 Effect on arthritic score in adjuvant induced arthritis

<table>
<thead>
<tr>
<th>Groups</th>
<th>Arthritic Index Score</th>
<th>Prophylactic Model</th>
<th>Therapeutic Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td>Arthritic control</td>
<td>4 ± 0.16</td>
<td>4 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>ETGP 200 mg/ kg</td>
<td>2.4 ± 0.14</td>
<td>2.4 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>ETGP 400 mg/ kg</td>
<td>3 ± 0.17*</td>
<td>2 ± 0.16*</td>
<td></td>
</tr>
<tr>
<td>Methotrexate 0.1 mg/ kg</td>
<td>1.6 ± 0.14**</td>
<td>1.4 ± 0.13**</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (n= 6) *p< 0.05, **p< 0.01 compared with arthritic control.

Figure 9.1 Effect on arthritic score in adjuvant induced arthritis
Table 9.3 Effect of root of ETGP on change in body weight

<table>
<thead>
<tr>
<th>Groups</th>
<th>% Body Weight Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prophylactic Model</td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
</tr>
<tr>
<td>Normal control</td>
<td>3.2</td>
</tr>
<tr>
<td>Arthritic control</td>
<td>3</td>
</tr>
<tr>
<td><em>ETGP</em> 200 mg/ kg</td>
<td>2.8</td>
</tr>
<tr>
<td><em>ETGP</em> 400 mg/ kg</td>
<td>2.61</td>
</tr>
<tr>
<td>Methotrexate 0.1mg/ kg</td>
<td>1.95</td>
</tr>
</tbody>
</table>

Figure 9.2 Effect of ETGP root on change in body weight

9.6.3 Effect of *Glycosmis pentaphylla* treatment on Paw volume

Effect of vehicle, *Glycosmis pentaphylla* 200 mg/ kg and 400 mg/ kg body weight, Methotrexate 0.1ml/kg body weight on paw volumes of CFA induced arthritic animals with untreated pathogenic arthritic animals are tabulated in table 9.5 and graphically represented in Figure 9.3 to 9.6.
Table 9.4 Effect of root of ETGP on paw volume on complete Freund’s adjuvant induced arthritic in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Prophylactic Model</th>
<th>Therapeutic Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
<td>0</td>
</tr>
<tr>
<td>Normal control</td>
<td>Days</td>
<td>0</td>
</tr>
<tr>
<td>Normal control</td>
<td>1.22 ± 0.04</td>
<td>1.32 ± 0.04</td>
</tr>
<tr>
<td>Arthritic control</td>
<td>1.37 ± 0.03</td>
<td>3.15 ± 0.06*</td>
</tr>
<tr>
<td>ETGP 200 mg/kg</td>
<td>1.24 ± 0.02</td>
<td>3.39 ± 0.09*</td>
</tr>
<tr>
<td>ETGP 400 mg/kg</td>
<td>1.31 ± 0.03</td>
<td>3.52 ± 0.06*</td>
</tr>
<tr>
<td>Methotrexate 0.1 mg/kg</td>
<td>1.31 ± 0.03</td>
<td>2.94 ± 0.05**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM; n=6 rats in each group, *p<0.05 compared with normal control, **p<0.01 compared with arthritic control.
Figure 9.3  Effect on paw volume changes in adjuvant induced arthritis (Prophylactic model)

Figure 9.4  Effect on paw volume changes in adjuvant induced arthritis (Therapeutic model)
Figure 9.5  Percentage reduction on anti-arthritic inflammation (prophylactic model)

Figure 9.6  Percentage reduction on anti-arthritic inflammation (Therapeutic model)
9.6.4 Biochemical parameters

Results for BUN showed significant (*p< 0.05) increase in 400 mg/ kg dose than 200 mg/ kg dose and standard in prophylactic model when compared to normal control.

The biochemical parameter of CR showed significant (*p< 0.05) increase in 400 mg/ kg dose and standard than 200 mg/ kg dose in prophylactic model. In the therapeutic model there is a significant (*p< 0.05) increase in both the dose and standard when compared to normal control.

The total protein showed significant (*p< 0.05) decrease in 200 mg/ kg and 400 mg/ kg dose than standard in prophylactic model and therapeutic model when compared to normal control.

Results for ALT, AST, ALP showed significant (*p< 0.05) increase in 400 mg/ kg dose than standard in prophylactic model and therapeutic model when compared to normal control.

The CRP showed significant (*p< 0.05) increase in 200 mg/ kg dose and 400 mg/kg than 400 mg/ kg dose and standard in prophylactic model and in therapeutic model significant (*p< 0.05) increase in 200 mg/kg dose only when compared to normal control.

Results for SOD showed significant (*p< 0.05) increase in 200 mg/ kg dose and 400 mg/ kg than standard in prophylactic model and in therapeutic model significant (*p< 0.05) increase in 400 mg/ kg dose and standard when compared to normal control. Results for GSH showed significant (*p< 0.05) increase in 200 mg/ kg, 400 mg/ kg and standard in prophylactic model and in therapeutic model significant (*p< 0.05) increase standard when compared to normal control.
Table 9.5 Effect on biochemical parameters in blood

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>Arthritic control</th>
<th>Prophylactic Model</th>
<th>Therapeutic Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ETGP 200 mg/Kg</td>
<td>ETGP 400 mg/Kg</td>
</tr>
<tr>
<td>BUN</td>
<td>37.75±1.18</td>
<td>45.50±5.17**</td>
<td>40.50±0.50**</td>
<td>39.00±1.15*</td>
</tr>
<tr>
<td></td>
<td>40.00±0.50**</td>
<td>39.00±1.15*</td>
<td>41.75±1.31**</td>
<td>35.50±3.20**</td>
</tr>
<tr>
<td></td>
<td>35.50±3.20**</td>
<td>34.00±2.38**</td>
<td>39.25±2.06**</td>
<td></td>
</tr>
<tr>
<td>CR (mg/dl)</td>
<td>0.61±0.05</td>
<td>0.48±0.11**</td>
<td>0.50±0.014**</td>
<td>0.65±0.03*</td>
</tr>
<tr>
<td></td>
<td>0.50±0.014**</td>
<td>0.50±0.03*</td>
<td>0.57±0.06*</td>
<td>0.45±0.04*</td>
</tr>
<tr>
<td></td>
<td>0.45±0.04*</td>
<td>0.45±0.09*</td>
<td>0.51±0.05*</td>
<td>0.51±0.05*</td>
</tr>
<tr>
<td>TP (g/dl)</td>
<td>7.21±0.22</td>
<td>7.25±0.38*</td>
<td>6.48±0.08*</td>
<td>7.15±0.10*</td>
</tr>
<tr>
<td></td>
<td>7.38±0.08*</td>
<td>6.85±0.19</td>
<td>5.98±0.34*</td>
<td>6.38±0.44*</td>
</tr>
<tr>
<td></td>
<td>5.98±0.34*</td>
<td>5.98±0.34*</td>
<td>6.38±0.44*</td>
<td>6.53±0.19</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>96.50±5.72</td>
<td>99.75±6.16**</td>
<td>85.75±9.75*</td>
<td>92.00±3.32</td>
</tr>
<tr>
<td></td>
<td>99.75±6.16**</td>
<td>92.00±3.32</td>
<td>71.75±13.26</td>
<td>83.25±4.57*</td>
</tr>
<tr>
<td></td>
<td>83.25±4.57*</td>
<td>83.25±4.57*</td>
<td>79.50±7.46</td>
<td>71.25±11.33</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>142.50±3.57</td>
<td>186.25±30.92*</td>
<td>146.50±7.50**</td>
<td>127.00±6.04**</td>
</tr>
<tr>
<td></td>
<td>186.25±30.92*</td>
<td>127.00±6.04**</td>
<td>115.00±10.68*</td>
<td>139.0±4.08**</td>
</tr>
<tr>
<td></td>
<td>139.0±4.08**</td>
<td>115.00±5.46**</td>
<td>107.50±12.6*</td>
<td></td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>607.25±7.98</td>
<td>685.00±52.42**</td>
<td>823.50±27.50*</td>
<td>518.00±85.78</td>
</tr>
<tr>
<td></td>
<td>685.00±52.42**</td>
<td>518.00±85.78</td>
<td>567.75±13.14</td>
<td>813.50±27.50**</td>
</tr>
<tr>
<td></td>
<td>813.50±27.50**</td>
<td>813.50±27.50**</td>
<td>498.00±85.78</td>
<td>560.25±13.99</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>4.23±0.49</td>
<td>3.74±0.09**</td>
<td>3.64±0.49*</td>
<td>3.48±0.53*</td>
</tr>
<tr>
<td></td>
<td>3.74±0.09**</td>
<td>3.48±0.53*</td>
<td>3.74±0.37</td>
<td>3.11±0.42*</td>
</tr>
<tr>
<td></td>
<td>3.11±0.42*</td>
<td>3.11±0.42*</td>
<td>3.33±0.54</td>
<td>2.99±0.35</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>278.00±32.50</td>
<td>232.00±32.65*</td>
<td>250.00±24.00</td>
<td>265.00±35.69</td>
</tr>
<tr>
<td></td>
<td>232.00±32.65*</td>
<td>250.00±24.00</td>
<td>274.00±20.31</td>
<td>248.50±19.97</td>
</tr>
<tr>
<td></td>
<td>248.50±19.97</td>
<td>248.50±19.97</td>
<td>260.00±36.29</td>
<td>266.50±17.85</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n=6 rats per group. One way ANOVA followed by Tukey’s multiple comparison test. when compared with normal control group. *P<0.05, **P<0.01

ALP- Alkaline phosphatase, ALT- Alanine aminotransaminase, AST- Aspartate aminotransaminase, CR-Creatinine, LDH- Lactate dehydrogenase, TP- Total protein, CRP- hs- High sensitive C- reactive Protein
Table 9.6 Effect on antioxidant parameters in liver

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal control</th>
<th>Arthritic control</th>
<th>Prophylactic Model</th>
<th>Therapeutic Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ETGP 200 mg/kg</td>
<td>ETGP 400 mg/kg</td>
</tr>
<tr>
<td>SOD</td>
<td>0.35±0.02</td>
<td>0.11±0.006**</td>
<td>0.26±0.005*</td>
<td>0.25±0.005*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.26±0.005*</td>
<td>0.25±0.005*</td>
</tr>
<tr>
<td>GSH</td>
<td>3.13±0.008</td>
<td>1.03±0.15**</td>
<td>2.41±0.29*</td>
<td>2.4±0.07*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.4±0.07*</td>
<td>2.4±0.09*</td>
</tr>
<tr>
<td>LPO</td>
<td>0.11±0.008</td>
<td>0.24±0.04**</td>
<td>0.14±0.02**</td>
<td>0.12±0.02*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.12±0.02*</td>
<td>0.12±0.03*</td>
</tr>
<tr>
<td>GPX</td>
<td>8.55±0.32</td>
<td>17.93±0.65**</td>
<td>11.28±0.66*</td>
<td>10.12±0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10.12±0.06</td>
<td>10.02±0.39</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n=6 rats per group. One way ANOVA followed by Tukey’s multiple comparison test. when compared with vehicle control group, *P<0.05, **P<0.01

**SOD**- Superoxide dismutase, **GSH**- Glutathione, **LPO**- Lipid peroxidase, **GPX**- Glutamate peroxidase.
Results for protein showed significant (*p< 0.05) increase in 200 and 400 mg/ kg than standard in prophylactic model and 200 mg/ kg therapeutic model when compared to normal control.

The biochemical parameter of LPO showed significant (*p< 0.05) increase in 400 mg/ kg and standard than 200 mg/ kg dose in prophylactic model and in therapeutic model significant (*p< 0.05) increase in 200 mg/ kg, 400 mg/ kg and standard when compared to normal control.

The GPX showed significant (*p< 0.05) increase in 200 mg/ kg than standard and 200 mg/ kg dose in therapeutic model when compared to normal control. All the bio chemical parameters and antioxidant parameter in liver value showed in table 9.6 and 9.7.

9.6.5 Immunohistochemistry - IHC Inflammatory markers - CD4

The number of cells of CD4 in case of the group induced with CFA was found to be high compared with the groups treated with the standard drug and the extract treated groups. When compared with in groups treated with the extract of 400 mg and 200 mg, the group which received 400mg of the extract showed less number of cells thereby confirming the efficacy of the extract at the higher dose as well as dose dependent effect showed in Figure 9.7 to 9.11.
IHC Inflammatory markers – IL 2

The number of cells of IL 2 in case of the group induced with CFA was found to be more when compared with the groups treated with the standard drug and the extract treated groups. The comparison with in groups treated with the extract of 400 mg and 200 mg, the group which received 400mg of the extract showed less number of cells thereby confirming the efficacy of the extract at the higher dose as well as dose dependent effect showed in Figure 9.12 to 9.16.
Figure 9.12 Normal control

Figure 9.13 CFA Induced

Figure 9.14 CFA+ Methotrexate

Figure 9.15 CFA+ETGP 200 mg

Figure 9.16 CFA+ ETGP400 mg
IHC (Tissue) Inflammatory markers – TGFβ1

The number of cells of TGFβ1 in case of the group induced with CFA was found to be high compared with the groups treated with the standard drug and the extract treated groups. When compared with in groups treated with the extract of 400 mg and 200 mg, the group which received 400mg of the extract showed less number of cells thereby confirming the efficacy of the extract at the higher dose as well as dose dependent effect showed in Figure 9.17 to 9.21.

![Figure 9.17 Normal control](image1)

![Figure 9.18 CFA Induced](image2)

![Figure 9.19 CFA+ Methotrexate](image3)
**IHC Inflammatory markers – TNF-α**

The number of cells of TNF-α in case of the group induced with CFA was found to be more when compared with the groups treated with the standard drug and the extract treated groups. The comparison with in groups treated with the extract of 400 mg and 200 mg, the group which received 400mg of the extract showed less number of cells thereby confirming the efficacy of the extract at the higher dose as well as dose dependent effect showed in Figure 9.22 to 9.26.
Histopathology

- The H&E section of the left ankle joints and paw tissue from normal control group revealed normal histology of joint capsule, synovium, periarticular tissues and paw tissue structure.

- The H&E section of left ankle joints and paw tissue of the CFA induced group revealed severe degree of dermal infiltration by polymorphonuclear cells and panniculitis with mononuclear cells infiltration. Skeletal muscular necrosis infiltrated by mononuclear cells. Chronic proliferative synovitis characterized by hyperplasia of synovium, proliferation of connective tissues,
lymphoplasmocytic infiltration around the synctical blood vessels and fibrosis. Moderate degrees of multinucleated giant cells infiltration were also observed in panniculitis and synovitis. Moderate degree of articular cartilage erosion and synovial invasion with mononuclear cells infiltration in the eroded lesions along with moderate degree of oestoclasts cells infiltration in the bone components. Paw tissues revealed edema and severe infiltration of mixed population of inflammatory cells (neutrophils and mononuclear cells) in the subcutaneous tissues.

- The H&E section of the left ankle joints and paw tissues of the reference control (methotrexate) has reduced the severity of synovitis and inflammatory cells infiltration, muscular necrosis, panniculitis and articular cartilage erosions and paw tissue inflammatory cells infiltration to mild to moderate degree when compared to that of the CFA induced arthritic group.

- The H&E section of left ankle joint and paw tissue of arthritic group treated with Glycosmis pentaphylla at the dose level of 400 mg/kg b.wt (Both prophylactic and therapeutic dose) has revealed mild to moderate degree of the panniculitis, moderate dermis and subcutaneous infiltration of polymorphonuclear cell in the skin, minimal to mild degree of synovitis, no articular erosions and decreased proliferation of connective tissues, lymphocytic infiltration around the blood vessels, decreased inflammatory cells infiltration in the subcutaneous paw tissues when compared to the arthritic rats treated with Glycosmis pentaphylla at the dose of 200mg/kg b.wt (both prophylactic and therapeutic dose)
Figure 9.27 Water Displacement plethysmograph

Figure 9.28 Measurement of Paw volume

Figure 9.29 Induction of arthritis

Figure 9.30 Paw after ETGP treatment
Histopathology of synovial joint in \textit{in-vivo} anti-arthritic activity of GP

Figure 9.31 Normal control

Figure 9.32 CFA Induced

Figure 9.33 CFA+ Methotrexate
Histopathology of paw tissues *in vivo* antiarthritic activity of GP

Figure 9.34 CFA+ETGP 200 mg

Figure 9.35 CFA+ ETGP400 mg

Figure 9.36 Normal control

Figure 9.37 CFA Induced
9.6.6.2 Result and Discussion

The *in vivo* anti arthritic activity of the ETGP was evaluated in the Complete Freund’s Adjuvant (CFA) induced arthritis model in rats. The Complete
Freund’s Adjuvant (CFA) induced arthritis model is considered as a chronic, immunological, cellular and proliferative arthritis similar to clinical RA. In adjuvant induced arthritis model, rats develop a chronic swelling in multiple joints with influence of inflammatory cells, erosion of joint cartilage and bone destruction and remodeling. These inflammatory changes ultimately result in the complete destruction of joint integrity and function in affected animals. Complete Freund’s Adjuvant (CFA) induced is considered as cell mediated autoimmunity due to the structural mimicry between mycobacteria and cartilage proteoglycans in rats. This is a most frequently used model for screening the anti–arthritic agents, especially NSAIDs, as the inflammation associated with CFA is highly dependent on Prostaglandin E2 (PGE2) generated by Cycloxygenase (COX-2) [Anderson GD, et. al, 1996].

The arthritis observed in rats is associated with a hyperalgesia phenomenon and spontaneous behaviors, such as protection of the affected paw, evidenced by curving and/ or elevation of the paw, as well as a voidance of supporting the body on the paw (Claworthy et al, 1995). The hyperalgesia is more evident during the acute inflammatory model, when spontaneous behaviors, indicative of painful response are more pronounced. Increased paws diameter (ipsilateral and contralateral paw), due to inflammation and edema is also observed (Cain et al, 1997). The initial inflammatory response is developed within hours, but more critical signals emerged from the 10th post inoculation day and thereafter and the alteration remain detectable for several weeks (Cloparet et al, 1992). The results of the present study indicate that ETGP exhibits anti-arhritic effects in rat with Freund’s Adjuvant- Induced arthritis, either on its acute as well as its chronic model.

Pathogenesis of rheumatoid arthritis is multifactorial and recent research has implicated oxygen free radicals as mediators of cartilage damage. Oxygen free
radicals such as superoxide and hydrogen peroxide are produced by polymorphonuclear leukocytes when they ingest bacteria or immune complexes (Gutteridge, 1986). In rheumatoid arthritis, it has been suggested that OH radical or a similar oxidizing species, contribute to membrane damage, alteration in the protein structure, conformation and antigenicity, production of auto-antibodies, hyaluronic degradation and destruction of antioxidants within the synovial joints (Gutteridge, 1986). Many cellular defense mechanisms are afforded against the toxic effect of these radicals in inflammation including serum sulphydryl groups, cerulopasmin, and albumin and blood glutathione (Fahim et al, 1995).

The increased lipid peroxide level noticed in arthritic rats in our study (group 2 in both therapeutic and prophylactic model) may be due to its release from neutrophils and monocytes during inflammation (Greenwald, et al; 1980). At the onset of inflammation, there is a rapid fall in the total iron content of blood plasma followed by an increased deposition of iron protein in the synovial fluid. The drop in plasma iron correlates closely with the activity of the inflammatory process. In the synovial fluid of inflamed joints, the iron released during necrosis, might catalyze the formation of OH (hydroxyl) radicals from H₂O₂, thus contributing increased lipid peroxidation in arthritis. From the literature review, it is apparent that RA is exposed to oxidative stress and is prone to lipid peroxidation (Heliovaara et al, 1994). In the present study, the concentration of lipid peroxidation was significantly altered in arthritic rat after the administration of ETGP, when compared with arthritic controlled rats.

Many cellular defense mechanisms are directed against the toxic effects of these radicals in inflammatory process. SOD which converts superoxide radicals to H₂O₂ is widely distributed in cells having oxidative metabolism and it is believed to protect such cells against the toxic effects of superoxide anion. Increase delivery
of NADPH from stimulated HMB shunt during inflammation is proposed to lead to the activation of SOD in arthritic rat. Increase production of NADPH from HMB shunt during arthritis may cause and increase in SOD activity (Marklund, et al, 1987). This increase in enzyme activity appears to be protective against the intracellular oxygen free radical (Kasama et al, 1988). Administration of ETGP to arthritic rat caused a significant decrease in elevated SOD activity.

Superoxide anion is thought to be involved in inflammatory reactions as they are produced by phagocytic cells (Babior et al, 1973). These cells are reported to produce hydroxyl radical (Salin, et al; 1975) and singlet oxygen (Allen et al, 1972). Glutathione peroxidase is localized in cytoplasm and mitochondria, which catalyses the degradation of various peroxides by oxidizing glutathione with the formation of its conjugates. The increased activity of glutathione peroxides in liver of arthritic rat, are shown by the present results, indicate the free radical defense system against oxidative stress and health to explain the pathogenesis associated with arthritis. After ETGP treatment, GPX level were significantly decreased.

After ETGP treatment, the alterations produced in arthritic rats with respect to lipid peroxidation and antioxidant concentrations were modulated nearly to normal levels. This antiperoxidative action observed in ETGP treated arthritic rats might be due the presence of compounds like flavonoids and phenol in ETGP. These compound shave been shown to scavenge free radicals, including hydroxyl and superoxide anions and reduce the level of lipid peroxidation in stress induced animals (Jovanovic, et al; 2000).

In the present investigation, reduction of paw swelling in the drug treated rats from the third week onwards may be due to immunological protection rendered by the plant extract. Adjuvant inoculation triggers the production of activated
macrophages and lymphocytes or their products like monokines, cytokines and chemokines. These in turn produce lipid peroxides due to abnormal lipid peroxidation leading to increased inflammation. In the current study, a significant reduction in the inflammation was shown in Group IV and Group V of both therapeutic and prophylactic treated animals when compared to that of Group II both therapeutic and prophylactic treated animal’s toxin intoxicated animals indicating the antiperoxidative nature of the plant extract. Complex network of cytokines and growth factors with overlapping biological effects are observed in the perpetuation of RA.

As a consequence of the inflammatory processes, several immunological mediators such as CD4, TGFβ1, TNF–α and IL–2 have been implicated in the pathological mechanism of synovial tissue proliferation, joint destruction and programmed cell death in rheumatoid joint. It was reported that the expression of inflammatory cytokines such as TNF–α and IL–2 and the tissue enzymes such as metalloproteinase were observed to be increased in the sub–chondral bone region of the knee joint samples from human osteoarthritis or rheumatoid arthritis patients. Biological agents that specifically inhibit the effects of TNF–α or IL–2 or leukocyte migration and accumulation in arthritis may have beneficial effects for joint preservation. Thus, in this study, observing the in vitro experiments such as inhibition of protein denaturation, by the plant extracts and in vivo anti arthritic effect analysis indicates proof towards the above theory of joint preservation. Plant phenolic compounds have been found to possess potent anti inflammatory activity (G Rao, et al, 2009). It has also been reported that the flavonoids significantly inhibit the leukocyte migration in a dose dependant manner

[Tian et al.1995 ] The presence of high content of flavonoids and phenol in ethyl acetate extract could be responsible for the antiarthritic activity.
The pathological findings showed Figure 9.31 to 9.40 and suggest the treatment of CFA induced arthritic group with *Glycosmsis pentaphylla* at the dose level of 400 mg/kg b.wt (both at therapeutic and prophylactic level) have reduced the severity of the arthritis and other associated tissue lesions in ankle joints and paw tissues than the arthritic group treated with *Glycosmsis pentaphylla* at the dose level of 200 mg/kg body weight.
9.7 References


Gutteridge, J. M. (1986). Antioxidant properties of the proteins caeruloplasmin, albumin and transferrin. A study of their activity in
serum and synovial fluid from patients with rheumatoid arthritis. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, 869(2), 119-127.


and liver of young and old mice with collagen-induced arthritis. Life sciences, 43(23), 1887-1896.


