3. MATERIALS AND METHODS

MAINTENANCE OF PARASITE

A rodent malaria parasite, *Plasmodium berghei* (NK-65 strain) was maintained in white Swiss mice, *Mus musculus* (BALB/c strain). The mice were obtained from Central Animal House, Panjab University, Chandigarh and were fed the normal pellet diet. Six to eight weeks old mice of either sex, weighing about 20-25 g each, were used for the study. Approval for the present study was obtained from the Animal Ethical Committee (CPCSEA/45/1999), Panjab University, Chandigarh. Normal mice were injected intraperitoneally (i.p.) with \( 1 \times 10^6 \) *Plasmodium berghei* infected erythrocytes in citrate saline [0.85% (w/v) sodium chloride; 3.8% (w/v) sodium citrate].

The course of infection in mice was monitored daily by preparing thin blood smears from the tail of infected mice. The blood smears were air dried and fixed in methanol for 1 min, stained with Giemsa's stain [25% Giemsa stock in 0.67M potassium dihydrogen orthophosphate (\( \text{KH}_2\text{PO}_4 \)) and 0.67M disodium hydrogen orthophosphate (\( \text{Na}_2\text{HPO}_4 \)) buffer] for 35min. Blood smears were washed with water after staining and air dried. The smears were observed under microscope using immersion oil. About five hundred erythrocytes were counted to calculate the percent infection (parasitaemia). The parasitaemia was calculated as:

\[
\frac{\text{Number of infected erythrocytes}}{\text{Total number of erythrocytes}} \times 100
\]
PLANT MATERIALS

The leaves of *Ajuga bracteosa*, *Xanthium strumarium* and root bark of *Berberis aristata* were collected from the Mandi district of Himachal Pradesh, India. Voucher specimen of *Ajuga bracteosa* (Voucher No. 8895) has been deposited in the herbarium of Dr Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (Fig. 3.1A, B), where identification of plant was confirmed by Taxonomist in the Department of Horticulture and Forestry. *Xanthium strumarium* (Fig. 3.1C, D), and *Berberis aristata* (Fig. 3.1E, F), has been identified in the Department of Botany, Panjab University, Chandigarh. Voucher No. 17920 and 17919 has been deposited in the herbarium of same department. The root bark of *Berberis aristata*, leaves of *Ajuga bracteosa* and *Xanthium strumarium* were air dried at room temperature and then powdered.

Preparation of extract

Dried and powdered leaves of *Ajuga bracteosa* and *Xanthium strumarium* were found to be soluble in ethanol. Thus ethanolic leaves extract of *Ajuga bracteosa* (ELEAB) and *Xanthium strumarium* (ELEXS) was prepared by Soxhlet extraction method. Approximately 1500 ml of 70% ethanol was added to 500g dried and powdered leaves in a glass flask. The solvent extract was evaporated to dryness in Rota evaporator. The dried residues thus obtained were stored in screw capped vials at −4°C.

The dried root bark of *Berberis aristata* (350g) was macerated in 1000 ml distilled water in round neck flask and agitated for 24 hr at 70°C. The aqueous root extract of *Berberis aristata* (AREBA) was evaporated to dryness in Rota evaporator. The dried residue thus obtained was stored in screw capped vials at −4°C.

PHYTOCHEMICAL SCREENING OF PLANT EXTRACTS

Phytochemical screening of the plant extracts was carried out
Fig. 3.1: Photographs of medicinal plants *Ajuga bracteosa* (A, B), *Xanthium strumarium* (C, D) and *Berberis aristata* (E, F) along with deposited specimens and dried root bark.
by employing standard procedures (Harbone, 1983; Trease and Evans, 1989). Qualitative tests were performed to detect alkaloids, saponins, phenolic compounds, anthraquinones, steroids and cardiac glycosides. The dried residue (ELEAB and ELEXS) was dissolved in 70% acetone, while AREBA was dissolved in distilled water to a final concentration of 50 mg/ml.

**Determination of Alkaloids**

Presence of alkaloids was determined in test solution of extracts with a few drops of Dragendorff’s reagent. The formation of reddish brown precipitates indicated the presence of alkaloids in test solution of extracts.

**Determination of Saponins**

1 ml test solution of plant extracts was combined with 5 ml of water (60°C) and shaken for 2 min. The volume of froth produced in this experiment was observed and recorded every 10 min for a total time period of 30 min to detect the presence of saponins.

**Determination of Phenolic Compounds**

1 ml test solution of each extract was treated with 10% ethanolic ferric chloride. Presence of phenolic compounds is indicated by change in colour of test solutions from bluish-green to dark blue.

**Determination of Anthraquinones**

The Borntrager test was performed for the detection of anthraquinones. 2 ml of test sample was shaken with 4 ml of hexane. The upper lipophilic layer was separated and treated with 4 ml of dilute ammonia. Change in colour of lower layer from violet to pink indicated the presence of anthraquinones.

**Determination of Steroids**

1 ml test solution of respective plant extracts was treated with
three drops of acetic anhydride and one drop of concentrated sulphuric acid. Change in colour from dark green to dark brown indicated the presence of steroids.

**Cardiac glycosides**

Keller-Killiani test was performed for the detection of cardiac glycosides. 1 ml of respective test solution of employed plant extract was mixed with 1 ml glacial acetic acid and treated with one drop of 5% ethanolic ferric chloride solution. 1 ml of concentrated sulphuric acid was carefully poured down from the sides of test tubes. Brown ring formation at interface of two layers along with change in colour of lower acidic layer from brown to bluish-green with the passage of the time indicated the presence of cardiac glycosides.

**SCHIZONT MATURATION INHIBITION ASSAY**

Short term *in vitro* culture of *P. berghei* blood stages was maintained in candle jar by the modified method of Trager and Jensen (1976). *In vitro* antimalarial efficacy of plant extracts was determined by schizont maturation inhibition assay (WHO, 2001).

**Short term *in vitro* culture**

**Culture medium:** RPMI-1640 (Gibco) was used as culture medium. It was supplemented with 0.06% (w/v) HEPES, 5% (w/v) sodium bicarbonate; antibiotics – gentamycin (50 μg/ml), penicillin (100 μl/ml) streptomycin (100 μg/ml). The pH of incomplete medium was adjusted to 7.4. It was filtered through 0.22μ Millipore syringe filters under sterile conditions. 10% (v/v) inactivated fetal calf serum (FCS) was added to incomplete medium for the formation of complete medium.

**Preparation of normal and *P. berghei* infected erythrocytes:** Blood from normal mice was collected aseptically in citrate saline and centrifuged at 1,000 g for 10 min. The plasma and
buffy coat were removed and erythrocyte pellet was washed with incomplete medium. Suspension of red blood cells (50%) was made in incomplete medium. *P. berghei* infected blood was collected in citrate saline. It was then centrifuged at 1,000g for 10 min and parasite pellet containing rings at lower layer was aspirated. It was centrifuged for 5 min at 1,000g. The supernatant was discarded and pellet was washed twice with incomplete medium. Infected and normal erythrocytes were mixed in a proportion so that parasitaemia was between 2-4% at 0 hr.

**In vitro antiplasmodial activity of plant extracts:** The antiplasmodial activity of plant extracts was checked according to WHO method, which is based on assessing the inhibition of schizont maturation (WHO, 2001). The stock solution of extract was prepared by dissolving known quantity of extract in DMSO (20 mg/ml). The stock solution was further diluted with RPMI-1640, to make various concentrations (10, 20, 40, 80 and 100 μg) of extracts. Each concentration was added in triplicate in 24 well microtiter plate. Chloroquine (10 μM) has been used as a positive control. Negative control contained solvent alone (≤ 0.02%). 1 ml complete medium contained either 100 μl of extract (different concentrations in triplicate or standard drug in each well. The titer plate was shaken gently to mix contents. 0 hr smears were prepared and culture plate was incubated at 37°C in a candle jar (5% CO₂, 17% O₂, 78% N₂) according to the method of Trager and Jensen (1976). After 21 hrs of incubation, the smears from each well were prepared, fixed in methanol and stained with Giemsa’s stain. Inhibition of schizont development in comparison to the control wells was determined by following formula:

\[
100 \left[ \frac{(A-B)}{A} \right]
\]

Where, A is the average schizont maturation in the negative control well and B is the average schizont maturation in the extract or drug treated wells. IC₅₀ has been defined as concentration of
extract/drug corresponding to 50% schizont maturation as compared to control. It was obtained by plotting linear curve dose-response of extract (10-100 μg/ml) used and percentage of schizont maturation inhibition obtained.

**ACUTE TOXICITY**

The acute toxicity of the extracts was determined using BALB/c mice by administration of different concentration of extract by oral route using the limit test of Lorke (1983). Dried residue of ELEAB and ELEXS was dissolved in 0.05% Tween 80 and 0.03% ethanol, while the extract of *Berberis aristata* was dissolved in distilled water. 5g/kg body weight extract concentration was administered orally to 5 female mice that have been fasted for 4 hrs. This is the upper testing dose limit for any compound in the rodents. If the mice died, lower concentration of extracts were administrated, till LD$_{50}$ of extracts were determined. LD$_{50}$ has been defined as concentration of extract/drug corresponding to 50% mortality as compared to control. It was obtained by plotting linear curve dose-response of extract/drug used and percentage of mortality obtained. All the mice were monitored for 14 days after the administration of different concentrations of extracts for adverse side effects in the form of mortality. Histopathological examination of organs (liver, spleen and kidney) was done at the end of study.

**IN VIVO ANTIPLASMODIAL EFFICACY OF PLANT EXTRACTS**

The suppressive, preventive and curative activity of plant extracts was evaluated in *P. berghei* infected BALB/c mice to determine in vivo antiplasmodal efficacy of plant extracts by employing following methods.

**Evaluation of suppressive activity in early infection (4-day test)**

The suppressive activity was assessed by the method
described by Knight and Peters (1980). On day 0 (DO), all the mice were inoculated with $1 \times 10^7$ *P. berghei* infected erythrocytes and divided into different groups containing six mice in each group (Table 3.1).

**Table 3.1: Experimental design for the evaluation of in vivo suppressive activity of plant extracts**

<table>
<thead>
<tr>
<th>Groups (n=6)</th>
<th>ELEAB/CQ/Vehicle OD/Oral/mouse Dose D0-D3</th>
<th>ELEXS/CQ/Vehicle OD/Oral/mouse Dose D0-D3</th>
<th>AREBA/CQ/Vehicle OD/Oral/ mouse Dose D0-D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-1</td>
<td>250 mg/kg (ELEAB) 150 mg/kg (ELEXS) 150mg/kg (AREBA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-2</td>
<td>500 mg/kg (ELEAB) 250 mg/kg (ELEXS) 250mg/kg (AREBA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-3</td>
<td>750 mg/kg (ELEAB) 350 mg/kg (ELEXS) 350mg/kg (AREBA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-4</td>
<td>1000mg/kg (ELEAB) 500 mg/kg (ELEXS) 650mg/kg (AREBA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-5</td>
<td>5 mg/kg (CQ) 5 mg/kg (CQ) 5 mg/kg (CQ)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-6</td>
<td>0.2ml (vehicle) 0.2ml (vehicle) 0.2ml (D.W)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

intraperitoneal inoculum of $1 \times 10^7$ *P. berghei* infected red cells in citrate saline
Vehicle: 0.05% Tween 80 and 0.03% ethanol.

Treatment started 1 hr post inoculation of parasite on same day (D0) and continued for 4 consecutive days (D0 – D3). On fifth day (D4), 24 hr after the administration of last dose, thin blood smears were prepared from the tail of each mouse, fixed in methanol and stained with Giemsa’s stain. The percentage of parasite suppression was determined by using following formula:-

$$100 - \frac{\text{Number of infected erythrocytes}}{\text{Total number of erythrocytes}} \times 100$$

The ED$_{50}$, representing 50% suppression of parasite, when compared with untreated control was estimated by a common statistical procedure. These values were determined graphically by plotting logarithm of concentration of extracts versus percentage of parasite inhibition.
Evaluation of the preventive activity

The preventive (repository) activity was assessed using the method described by Peters (1965). Different groups, containing six mice each, were administered different concentrations of employed extracts/ pyrimethamine/Vehicle for 4 consecutive days (D0–D3) as explained in Table 3.2.

Table 3.2: Experimental design for the evaluation of in vivo preventive activity of plant extracts.

<table>
<thead>
<tr>
<th>Groups (n=6)</th>
<th>ELEAB/Pyr/Vehicle OD/Oral/mouse Dose (D0-D3) D4- i.p. infection*</th>
<th>ELEXS/Pyr/Vehicle OD/Oral/mouse Dose (D0-D3) D4- i.p. infection*</th>
<th>AREBA/Pyr/Vehicle OD/Oral/mouse Dose (D0-D3) D4- i.p. infection*</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-1</td>
<td>250 mg/kg (ELEAB)</td>
<td>150 mg/kg (ELEXS)</td>
<td>150 mg/kg (AREBA)</td>
</tr>
<tr>
<td>G-2</td>
<td>500 mg/kg (ELEAB)</td>
<td>250 mg/kg (ELEXS)</td>
<td>250 mg/kg (AREBA)</td>
</tr>
<tr>
<td>G-3</td>
<td>750 mg/kg (ELEAB)</td>
<td>350 mg/kg (ELEXS)</td>
<td>350 mg/kg (AREBA)</td>
</tr>
<tr>
<td>G-4</td>
<td>1.2 mg/kg (pyr)</td>
<td>1.2 mg/kg (pyr)</td>
<td>1.2 mg/kg (pyr)</td>
</tr>
<tr>
<td>G-5</td>
<td>0.2 ml (vehicle)</td>
<td>0.2 ml (vehicle)</td>
<td>0.2 ml (D.W.)</td>
</tr>
</tbody>
</table>

*intraperitoneal inoculum of 1 × 10⁷ P. berghei infected red cells in citrate saline

On day 5 (D-4), the mice were inoculated with 1 × 10⁷ P. berghei infected erythrocytes. Seventy-two hours later i.e. D7, the parasitaemia level was assessed by studying Giemsa stained blood smears by following same method as explained in schizontocidal activity in early infection.

Evaluation of curative activity of extracts in established infection

A modified method similar to that described by Ryley and Peters (1970) was employed to evaluate curative (schizontocidal) activity of extracts in established infection of P. berghei. All the mice were divided into different groups as described in the Table 3.3.
Table 3.3: Experimental design for the evaluation of in vivo curative activity of plant extracts

<table>
<thead>
<tr>
<th>Groups (n=6)</th>
<th>ELEAB/CQ/Vehicle OD/Oral/mouse D0- i.p. infection* Dose (D3-D6)</th>
<th>ELEXS/CQ/Vehicle OD/Oral/mouse D0- i.p. infection* Dose (D3-D6)</th>
<th>AREBA/CQ/Vehicle OD/Oral/mouse D0- i.p. infection* Dose (D3-D6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-1</td>
<td>250 mg/kg (ELEAB)</td>
<td>150 mg/kg (ELEXS)</td>
<td>150 mg/kg (AREBA)</td>
</tr>
<tr>
<td>G-2</td>
<td>500 mg/kg (ELEAB)</td>
<td>250 mg/kg (ELEXS)</td>
<td>250 mg/kg (AREBA)</td>
</tr>
<tr>
<td>G-3</td>
<td>750 mg/kg (ELEAB)</td>
<td>350 mg/kg (ELEXS)</td>
<td>350 mg/kg (AREBA)</td>
</tr>
<tr>
<td>G-4</td>
<td>5 mg/kg (CQ)</td>
<td>5 mg/kg (CQ)</td>
<td>5 mg/kg (CQ)</td>
</tr>
<tr>
<td>G-5</td>
<td>0.2 ml (vehicle)</td>
<td>0.2 ml (vehicle)</td>
<td>0.2 ml (D.W.)</td>
</tr>
</tbody>
</table>

*intraperitoneal inoculum of $1 \times 10^7$ *Plasmodium berghei* infected red cells in citrate saline

On the first day (D0), all experimental mice were injected intraperitoneally with standard inoculum of $1 \times 10^7$ *Plasmodium berghei* infected erythrocytes. Seventy-two hours later, the mice were administered different concentrations of the extracts as explained in the Table 3.3. Chloroquine (5 mg/kg/day) was given to the positive control group and 0.2 ml vehicle to the negative control group. The drug/extract/vehicle was given once daily for 5 days. Thin blood films stained with Giemsa were prepared from tail blood of each mouse daily for 5 days from day 3 (D3) onward to monitor the parasitaemia level. The mean survival time for each group was determined arithmetically by finding the average survival time (days) in each group over a period of 30 days (D0–D29).

**COMPARATIVE STUDY OF IN VIVO ANTIMALARIAL EFFICACY OF PLANT EXTRACTS/HOMEOPATHIC MEDICINES/STANDARD ANTIMALARIAL DRUGS**

Concentrations of plant extracts showing maximum schizontocidal activity during early infection, i.e. 750 mg/kg/mouse (ELEAB), 250 mg/kg/mouse (ELEXS) and 350 mg/kg/mouse
A COMPARATIVE STUDY OF SOME ANTIMALARIALS ON CLEARANCE OF BLOOD STAGE PLASMODIUM BERGHEI INFECTION

(AREBA), were compared with standard allopathic drug i.e. quinine (10 mg/kg/mouse) and artesunate (100 mg/kg/mouse) along with homeopathic mother tinctures diluted in distilled water of china (1 : 2 φ) and Eucalyptus/mouse for their \textit{in vivo} antiplasmodial activity against \textit{Plasmodium berghei}.

\textbf{Experimental Design}

White Swiss BALB/c mice (22-25g) were divided into nine groups containing ten mice in each group. Mice were inoculated with $1 \times 10^7 \ P. \ berghei$ infection on D0 except G-1. The experimental groups were treated with different concentrations of extracts/drugs once a day (OD) orally for 6 consecutive days starting from D0-D5 as given below:-

\textbf{G-1}: Normal mice were administered (0.2ml/mouse/OD/Oral) distilled water for six days from D0-D5.

\textbf{G-2}: Mice of this group were also administered 0.2ml distilled water/mouse/OD/oral for six days from D0-D5.

\textbf{G-3}: Mice of this group were administered 750mg/kg ELEAB/mouse/OD/oral for six days from D0-D5.

\textbf{G-4}: Mice of this group were given 250mg/kg ELEXS/mouse/OD/oral for six days from D0-D5.

\textbf{G-5}: Mice of this group were administered with 350mg/kg AREBA /mouse/OD/oral for six days from D0-D5.

\textbf{G-6}: Mice of this group were administered 0.2 ml (1 : 2 dilution) of china mother tincture/mouse/OD/oral for six days from D0-D5. Mother tincture (φ) of china was obtained from a recognized homeopathic company (Schwabe India Pvt. Ltd). Mother tincture contains 1 part of drug and 6 part of nascent alcohol and 1 : 2 dilution of mother tincture was prepared by diluting 1 part of mother tincture (φ) of china with 2 parts of distilled water.
MATERIALS AND METHODS

G-7: Mice of this group were administered 0.2 ml (1:2 dilution) of mother tincture/mouse/OD/oral for six days from D0-D5. Mother tincture of *Eucalyptus* was obtained from a recognized homeopathic company (Schwabe India Pvt. Ltd.) and 1:2 dilution of mother tincture was prepared by diluting 1 part of mother tincture (ϕ) of *Eucalyptus* with 2 parts of distilled water.

G-8: Mice of this group were given 10mg/kg quinine sulphate base/mouse/OD/oral for six days from D0-D5. Quinine was obtained from a recognized allopathic company (Skymax, Laboratory Pvt. Ltd., India). Quinine was dissolved in the distilled water before its administration to the mice.

G-9: Mice of this group were administered 100mg/kg artesunate/mouse/OD/oral for six days from D0-D5. Artesunate (Falcigo) was obtained from Cadilla pharmaceutical company, India. Each tablet of Falcigo contained 50mg base of artesunate, which was suspended in 1 ml NaHCO$_3$ 5% (w/v) and 5 ml NaCl 0.9% (w/v) before its administration to the mice.

Thin blood smears were prepared from each mouse on alternate days to monitor the percentage of parasitaemia inhibition. On day 7, all mice were weighed and five mice from each group were sacrificed after anesthetizing with diethyl ether, while other mice were kept to observe the mean survival time period up to one month. Blood from each group of mice was aspirated by jugular vein incision and subjected to double density centrifugation for isolation of mononuclear (MN) and polymorphonuclear (PMN) cells. Isolated MN and PMN cells were subjected to AO/EB staining for morphological assessment of apoptosis (Kashibhatla, 1998) and scanning electron microscopy, whereas, tissues - liver, spleen and kidney were taken out for histopathological and biochemical examinations.
SEPARATION OF WBCs BY DOUBLE DENSITY GRADIENT CENTRIFUGATION

The pooled blood of mice obtained from same group was subjected to double density gradient centrifugation using Histopaque-1119 (Sigma) and Histopaque-1077 (Sigma) to separate MN cells from PMN cells (Czuprynski and Brown, 1998). Pooled blood from different experimental groups was centrifuged at 700g for 30 min in REMI, 8C centrifuge at room temperature.

According to Sigma procedure, a double gradient is formed by layering 2 ml of Histopaque-1077 over Histopaque-1119 (Fig. 3.2).

The blood was slowly poured over it along the walls of the tube without disturbing the two layers. The tube was centrifuged at 700g for 20 to 30 min. Two separate white layers of WBCs suspension were formed. The upper white layer of mononuclear WBCs was aspirated out from junction of plasma layer and histopaque-1077 and second layer of PMN cells from junction of histopaque 1077 and 1119. 2-3 ml of PBS pH 7.2 (Phosphate Buffer Saline) (Potassium dihydrogen orthophosphate, 0.043% w/v; disodium hydrogen orthophosphate, 0.148% w/v; sodium chloride, 0.08% w/v) was added.
to each aspirated WBC layer. It was centrifuged at 600g for 5 min. Supernatant was discarded. The above procedure was repeated twice and finally the pellet of WBCs was suspended in minimum volume of PBS, pH 7.2 and subjected to AO/EB staining and scanning electron microscopy.

**FLUORESCENT STAINING USING ACRIDINE ORANGE (AO)/ETHIDIUM BROMIDE (EB)**

A suspension of $2 \times 10^6$ WBCs was made in 25 μl PBS, pH 7.2. Cell suspension was incubated with 10 μl each of AO/EB solution (1 part of 100 μg/ml AO in PBS; 1 part of 100 μg/ml EB in PBS). The suspension was mixed gently. Each sample was prepared just prior to its examination under fluorescent microscope (Leica, Germany) and its quantification, thereafter (Kashibhatla, 1998).

10 μl of above suspension was placed on a microscope slide and covered with a glass cover slip. At least 300 cells were examined under fluorescent light microscope using fluorescent filter before calculating the number of apoptotic cells. Live cells fluoresce green, whereas dead/apoptotic cells appear orange under U.V. light. Percentage of apoptotic cells was calculated as:-

$$\frac{\text{Number of live / apoptotic / dead cells}}{\text{Total number of white blood cells}} \times 100$$

**SCANNING ELECTRON MICROSCOPY OF BLOOD CELLS**

Scanning electron microscopy of red blood cells and white blood cells of experimental mice from each group (G-1 to G-9) were carried out by the method of Clearance *et al.* (1974). The following procedure was adopted:

One drop of red blood cell and aspirated white blood cell suspension as mentioned earlier was fixed in 4% glutaraldehyde in PBS, pH 7.2 for 20 min at room temperature. After fixation, drop of
A COMPARATIVE STUDY OF SOME ANTIMALARIALS ON CLEARANCE OF BLOOD STAGE PLASMODIUM BERGHEI INFECTION

respective suspension was centrifuged at 600 g in REMI, 8C centrifuge at room temperature.

Supernatant was discarded and pellet thus obtained was washed thrice using 10 ml PBS, pH 7.2 each time at 600 g for 5 min. Finally pellet was washed in distilled water. It was placed over silver foil attached over an iron stub. The stubs were placed inside the sputter for gold coating (15 min). These were then viewed under scanning electron microscope (JEOL 2601) at Electron Microscopy Unit, Regional Sophisticated Instrumentation Center, Central Instrumentation Laboratory, Panjab University, Chandigarh, India.

HISTOLOGICAL PREPARATION OF TISSUE

For histopathological studies, small tissue samples from experimental mice of G-1 to G-9 on day 7 were fixed in Bouin’s fixative for overnight (Pearse, 1980). The fixed tissues were processed for Hematoxylin and Eosin (H&E) staining using the conventional laboratory procedure. Following an overnight fixation, the tissues were dehydrated through ascending grades of alcohol, cleared in benzene and embedded in low melting point paraffin (58-60°C). Blocks were prepared and sections of 5 μm thickness were cut using microtome. Sections were placed serially on clean glass slides and then de-paraffinised in xylene, followed by washing in descending grades of alcohol. Sections from each liver, spleen and kidney tissue were stained with Hematoxylin and then counterstained with Eosin by the method of Baker (1945) for studying histology under light microscope.

BIOCHEMICAL PARAMETERS

Mice were sacrificed after light anesthesia with diethyl ether. The required organs (liver and spleen) were extracted out on ice. They were washed properly in 0.9% saline solution, dried and then
weighed. The tissues were then homogenized in cold tris-HCl buffer (0.2M, pH 7.0) to obtain 10% homogenate (w/v). The homogenate was subjected to cold centrifugation at 1000 g for 30 min in REMI (8C). The pellet was discarded and supernatant was termed as enzyme extract, which was used for various biochemical estimations.

**Estimation of Protein Content**

Protein contents of different samples were estimated employing slightly modified method of Lowry *et al.* (1951).

To prepare a sample for protein estimation, 0.9 ml of cold 10% w/v TCA (Tricarboxylic acid) was added to 0.1 ml of tissue extract. The supernatant was discarded after centrifugation at 800g for 15 min and the pellet was dissolved in 5ml of 0.1N of sodium hydroxide. To 0.1 ml of sample, 0.9 ml of distilled water was added. Solution D was prepared by mixing 8% w/v sodium carbonate solution in 1% w/v sodium hydroxide (Solution A), 0.06% (w/v) sodium potassium tartrate (Solution C) and 0.1 (w/v) copper sulphate (Solution D). 5 ml of solution D was added to each sample. After 15 min, 0.5 ml of Folins reagent (1N) was added and after 30 min, the sample was read at 680 nm in spectrophotometer against proper blank. Various concentrations of Bovine Serum Albumin (BSA), ranging from 10 to 100 µg/ml were used as standard to calculate concentration of protein content in unknown sample.

**Calculation of protein concentration**

\[
\frac{\text{O.D. of test} - \text{O.D. of black}}{\text{O.D. of standard} - \text{O.D. of blank}} \times \text{Concentration of standard} \times \text{dilution factor}
\]

**Alkaline Phosphatase (ALP) Estimation (E.C.-3.1.3.1)**

ALP activity was assayed according to method of Bergmeyer (1963). 1 ml of buffered substrate (5.5 mM p-nitrophenol phosphate in 0.5 M glycine-sodium hydroxide buffer, pH 10.5) was incubated at 37°C for 5 min to pre equilibrate. After this 0.1 ml of enzyme was
added and incubated again for 15 min at 37°C. Then 5 ml of 0.1 N sodium hydroxide was added to stop the reaction and the liberation of p-nitrophenol was measured at 420 nm. 0.04 μmol/ml p-nitrophenol was used as working standard and the optical density of all the tubes was read at 420 nm after 10 min.

**Calculations**

\[
\text{Activity of Enzyme (Units/min/mg protein)} = \frac{\text{O.D. of test} - \text{O.D. of blank} \times \text{Vol. of std.} \times \text{Conc. of std.}}{\text{O.D. of std.} - \text{O.D. of blank} \times \text{Incubation time} \times \text{Protein conc.}}
\]

**Units**

The enzyme activity was expressed as nmol p-nitrophenol formed/ml/min/mg protein.

**Acid Phosphatase (ACP) Estimation (E.C.-3.1.3.2)**

ACP activity was assayed according to method of Bergmeyer (1963). In this method, p-nitrophenol phosphate was used as a substrate to yield p-nitrophenol having \( V_{\text{max}} \) at 420 nm. The activity of enzyme is directly proportional to the amount of p-nitrophenol liberated per unit time. 1 ml of buffered substrate containing equal volume of stock substrate (5.5 mM p-nitrophenol phosphate in 0.5 M glycine buffer and tartarate (0.09M), citrate (0.04M), pH 4.9 was incubated at 37°C for 5 min to pre equilibrate. After this, 0.1 ml of enzyme was added and incubated again for 15 min at 37°C. Then 5 ml of 0.1 N sodium hydroxide was added to stop the reaction and the liberation of p-nitrophenol was measured. 0.04 μM/ml p-nitrophenol was used as working standard and the optical density of all the tubes was read at 420 nm after 10 min.

**Calculations**

\[
\text{Activity of Enzyme (Units/min/mg protein)} = \frac{\text{O.D. of test} - \text{O.D. of blank} \times \text{Vol. of std.} \times \text{Conc. of std.}}{\text{O.D. of std.} - \text{O.D. of blank} \times \text{Incubation time} \times \text{Protein conc.}}
\]
Units

The enzyme activity was expressed as nmol p-nitrophenol formed/ml/min/mg protein.

Asparate Aminotransferase (AST) Estimation (E.C.- 2.6.1.1)

Hepatic AST was estimated by Reitman and Frankel method (1957). The reaction mixture contained 0.5 ml solution of buffered substrate (200 mM DL-aspartic acid and 0.2 mM α-ketoglutaric acid in 0.2 M phosphate buffer, pH 7.4). To this, 100 µl of enzyme was added followed by a brief incubation at 37°C for 60 min. The reaction was terminated by the addition of dinitrophenyl hydrazine (DNPH) (1 mM/l) and the tubes were kept at room temperature for 20 min. Finally, 5 ml of 0.4 N sodium hydroxide was added. Control tubes were also run simultaneously for each test sample. In the control tubes, the reaction was inhibited by the addition of 0.5 ml of (1 mM/l) DNPH prior to the enzyme (AST) source. Sodium pyruvate (4 mM) was used as a standard and the optical density of all the tubes was read at 510 nm after 10 min.

Calculations

\[
\text{Activity of Enzyme (Units/min/mg protein)} = \frac{\text{O.D. of test} - \text{O.D. of blank} \times \text{Vol. of std.} \times \text{Conc. of std.}}{\text{O.D. of std.} - \text{O.D. of blank} \times \text{Incubation time} \times \text{Protein conc.}}
\]

Units

The enzyme activity was expressed as nmol pyruvate formed/min/mg protein.

Alanine Amino Transferase (ALT) Estimation (E.C.-2.6.1.2)

Hepatic ALT was assayed according to method of Reitman and Frankel (1957). Assay procedure was essentially the same as that for AST, except for the use of different substrate, i.e. DL-alanine (200 mM/l) instead of DL-aspartic acid. Moreover, the incubation time also varied, which was 30 min as against 60 min in AST. Sodium
pyruvate (4mM) was used as a standard and the optical density of all the tubes was read at 510 nm after 10 min.

**Calculations**

\[
\text{Activity of Enzyme (Units/min/mg protein)} = \frac{\text{O.D. of test} - \text{O.D. of blank} \times \text{Vol. of std.} \times \text{Conc. of std.}}{\text{O.D. of std.} - \text{O.D. of blank} \times \text{Incubation time} \times \text{Protein conc.}}
\]

**Units**

The enzyme activity was expressed as nmol pyruvate formed/min/mg protein.

**Lactate Dehydrogenase (LDH) Estimation (E.C.-1.1.1.27)**

Lactate dehydrogenase activity was measured according to the method of King (1965). The enzyme lactate dehydrogenase catalyses the conversion of lactate in the buffer substrate into pyruvate in the presence of nicotine adenine dinucleotide (NAD). The increased concentration of pyruvate is measured colorimetrically using 2,4-dinitrophenyl-hydrazine (2,4-DNPH) as chromogen in alkaline medium. The reaction mixture contained 0.1 ml buffered substrate containing (0.1 M glycine buffer, 0.1 N sodium hydroxide, 35% (w/v) sodium lactate (pH 10.0) and 0.2ml NAD solution (5 mg/ml). To this 100 μl of five fold diluted enzyme was added followed by a brief incubation at 37°C for 15 min. The reaction was terminated by the addition of 1 ml dinitrophenyl hydrazine (DNPH) and the tubes were kept at room temperature for 15 min. Finally, 10.0 ml of 0.4 N sodium hydroxide was added. Control tubes were also run simultaneously for each test sample. In the control tubes, the reaction was inhibited by the addition of 10.0 ml of DNPH prior to the enzyme LDH source. Sodium pyruvate (4mM) was used as a standard and the optical density of all the tubes was read at 510 nm after 10 min.

**Calculations**

\[
\text{Activity of Enzyme (Units/min/mg protein)} = \frac{\text{O.D. of test} - \text{O.D. of blank} \times \text{Vol. of std.} \times \text{Conc. of std.}}{\text{O.D. of std.} - \text{O.D. of blank} \times \text{Incubation time} \times \text{Protein conc.}}
\]
MATERIALS AND METHODS

Units

The enzyme activity was expressed as nmol pyruvate formed/min/mg protein.

STATISTICAL ANALYSIS

• Statistical analysis was carried out employing Graph pad software 3 and data has been expressed as mean ± S.E.M. for each group.

• The statistical significance of inter group difference of various parameters were determined by unpaired Student’s t test.

• Comparison was made between the test groups and control group of mice and the p value < 0.05 has been termed as statistically significant value.