CHAPTER 4

MATERIALS AND METHODOLOGY
# CHAPTER 4

## MATERIALS AND METHODOLOGY

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of the Sub-Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>PLANTS SELECTED FOR INVESTIGATION</td>
<td>40</td>
</tr>
<tr>
<td>4.1.1</td>
<td><em>Canavalia Gladiata</em></td>
<td>40</td>
</tr>
<tr>
<td>4.1.2</td>
<td><em>Berberis Vulgaris</em></td>
<td>42</td>
</tr>
<tr>
<td>4.1.3</td>
<td><em>Zizyphus Jujuba</em></td>
<td>44</td>
</tr>
<tr>
<td>4.2</td>
<td>METHOD OF PREPARATION &amp; EVALUATION</td>
<td>47</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Collection of plant material</td>
<td>47</td>
</tr>
<tr>
<td>4.2.2</td>
<td>Preparation of Extract</td>
<td>47</td>
</tr>
<tr>
<td>4.2.3</td>
<td>Phytochemical screening</td>
<td>48</td>
</tr>
<tr>
<td>4.3</td>
<td>EXPERIMENTAL ANIMALS &amp; TOXICITY STUDIES</td>
<td>52</td>
</tr>
<tr>
<td>4.4</td>
<td>MATERIALS &amp; METHODS</td>
<td>53</td>
</tr>
<tr>
<td>4.4.1</td>
<td>Drugs &amp; chemicals</td>
<td>53</td>
</tr>
<tr>
<td>4.4.2</td>
<td>Induction of Hepatoprotective activity &amp; Study Design</td>
<td>54</td>
</tr>
<tr>
<td>4.4.3</td>
<td>Study Design of Immunomodulatory activity</td>
<td>65</td>
</tr>
</tbody>
</table>
4. MATERIALS AND METHODOLOGY

4.1. PLANTS SELECTED FOR THE INVESTIGATION.

4.1.1. CANAVALIA GLADIATA (Fabaceae)

- **Botanical name:** Canavalia gladiata
- **Synonyms:** Clementea, Cryptophaseolus Kuntze
- **Kingdom:** Plantae
- **Class:** Magnoliopsida
- **Order:** fabales
- **Family:** Fabaceae
- **Genus:** Canavalia
- **Species:** Gladiata
- **Telugu name:** Chammakaya
- **Sanskrit name:** Mahasimbi, Assisimbi.
- **Hindi name:** Lalkududumpal
- **English name:** Sword bean

*Figure 4.1 Canavalia Gladiata fruit*
The legume is a used as a vegetable in interiors of central and south central India, though not commercially farmed. The fruits are eaten as a vegetable in Africa and Asia. Sword Bean is a twining, nearly erect annual herb, growing to 6 ft. Flowers are pink and white, 2.5-4 cm across. Flowering is in April-October. Flowers bisexual, papilionaceous, often resupinate; calyx up to 1.5 cm long, 2-lipped with a large 2-fid upper lip and a much smaller 3-fid lower lip; corolla white, standard c. 3.5 cm long; stamens 10, all joined; ovary superior, style slender, curved, stigma small. Seeds are 2-3.5 cm × 1.5-2 cm, red or red-brown, rarely black, pink or white; hilum 1.5-2.0 cm long.

Fruit a linear-oblong pod, slightly compressed, sometimes curved, 20-40 (-60) cm × 3.5-5 cm. widest near the apex. 8-16-seeded, spirally dehiscent; each valve with ventral rib and extra rib spaced c. 4 mm. Seeds 2-3.5 cm × 1.5-2 cm, red or red-brown, rarely black, pink or white; hilum 1.5-2.0 cm long. Seedling with epigeal germination, first 2 leaves simple, opposite, with stipules connate.

The chemical constituents from Canavalia Gladiata were isolated by chromatography on silica gel, and identified on the basis of physic-chemical constants and spectral analysis. Six compounds were isolated and their structures were identified Gallic acid, Methyl gallate, 1,6-di-o- galloyl-β-D-glucopyranoside, B-sitosterolm, Lupeol and δ-tocopherol. 45.

Plant pacifies vitiated kapha, pitta, burning sensation, over perspiration, anorexia, wounds, ulcers and general debility, kidney
stones, jaundice. Several species are valued legume crops, including Common Jack-bean (*C. ensiformis*), Sword Bean (*C. gladiata*) and *C. cathartica*. The Common Jack-bean is also known as the plant from which the lectin concanavalin A, con A is produced. This lectin is of major commercial and scientific importance as a reagent in glycoprotein biochemistry and immunology. Also, the Jack-bean is a common source of purified urease enzyme for scientific research. Bay Bean (*Canavalia rosea*) supposedly is mildly psychoactive when smoked; it is used in tobacco substitutes.

4.1.2. **BERBERIS VULGARIS** (*Berberidaceae*)

- **Botanical name:** *Berberis Vulgaris*
- **Synonyms:** Berberry
- **Kingdom:** Plantae
- **Class:** Magnoliophyta
- **Order:** Ranunculales
- **Family:** Berberidaceae
- **Genus:** *Berberis*
- **Species:** *vulgaris*  
  ![Figure.4.2.BerberisVulgaris root](image)
- **Telugu name:** Mani pasupa
- **Sanskrit name:** Daruharidra
- **Hindi name:** Daruhaldi
- **English name:** Indian barberry
Berberis vulgaris leaves have oval shapes and are grouped in rosettes. It has spiny stems, yellow flowers, and fruits are ellipsoidal, with sour, astringent taste, and red color. Lance shaped leaves measure 0.4-2.2 inches and margins are finely serrate. Barberry flowers occur in a drooping 0.8-2.4 inch long raceme. Fruits are egg shaped 1-3 seeded that measure up to 0.5 inches and seeds are about 6mm long. Root growth is extensive with a thick mass of fibrous roots or root crown extending up to 3-4.5 m and rhizome produced from the root crown grow few inches below ground and penetrate up to 2-3 feet.

The main bioactive compounds from B. vulgaris are alkaloids (berberine, berbamine, jatrorrhizine, columbamine, berberubine, oxicanthine, palmatine;), vitamin C, resin, and tannins, but also flavonoids like quercetin and kaempferol46.

The most used plant parts in phytotherapy are bark (from stem and root); also leaves and fruits are used. The most important pharmacological uses of B. vulgaris are in kidney, bile, and liver diseases, and also in ocular and mouth problems and have properties of tonic, antimicrobial, antiemetic, antipyretic, antipruritic, and cholagogue properties, and has also been used in some cases in cholecystitis, cholelitisias, jaundice, dysentery, leishmaniaisiasis, malaria, and gall stones. Most of these properties are due to the presence of some alkaloids: berberine and palmatine have the capacity to inhibit MAO enzyme, jatrorrhizine has the same pharmacologic properties like berberine, but it has lower toxicity; oxicanthine acts as
vasodilator and hypotensive, as well as berbamine; the latest has also anti-inflammatory and antioxidative properties. Most of bioactive properties of *B. vulgaris* are due to the overall antioxidant activity generated by the presence of some flavonoid compounds, alkaloids or other compounds with phenolic hydroxyl groups\textsuperscript{46}.

4.1.3 **ZIZYPHUS JUJUBA** (Rhamnaceae)

- **Botanical name:** *Zizyphus Jujuba*
- **Synonyms:** Jujube Dates
- **Kingdom:** Plantae
- **Class:** Magnoliopsida
- **Order:** Rosales
- **Family:** Rhamnaceae
- **Genus:** *Zizyphus*
- **Species:** *Jujuba*
- **Telugu name:** Regu
- **Sanskrit name:** Udbhayakantaka
- **Hindi name:** Ber
- **English name:** Indian jujube
Zizyphus jujuba are erect trees or small to large shrubs or climbers, species may be spiny or not but more commonly are so and species may be glabrous or relatively hairy. Leaves are alternate or rarely sub opposite, the leaf base is slightly asymmetrical or symmetrical. Flowers are 5-merous, actinomorphic. Flowers are borne sometimes solitary or 2-3 together in axillary cymes or in umbels or racemes arranged in terminal panicles. Inflorescences may be pedunculate or sessile. Calyx with triangular acute lobes up to 2 mm long, dentate, calyx valvate, keeled on the inside. The ovary is 2-4 celled with 2-4 styles (usually 2) which are distinct but can be somewhat connate.

Fruits are sub globose, ovoid or oblong, usually drupes which are 1-4 celled and 1-4 seeded but drupes mostly contain 1 seed. The flesh of the drupe is usually juicy pulp but may rarely be relatively dry. Seeds contain large embryos with endosperm sparse or absent. Fruiting branch lets may or may not be deciduous. Fruit is a
glabrous globose or oval edible drupe varying greatly in size from (1-2) cm diameter but some oval varieties can reach 5 x 3 cm. The pulp is acidic and sweet, the fruit greenish, yellow or sometimes reddish. Species of *Zizyphus* are considered to be multipurpose plants although use of the fruits is the major focus of interest. The pulp of the fruits is of most importance in relation to nutrition recorded fresh mature with 81-97 % pulp and considered the range 91.6-92.9 %. The richness of the pulp in nutritive compounds has been widely recognised protein, phosphorus, calcium, carotene and Vitamin C and oranges in phosphorus, iron, vitamin C and carbohydrates and exceeds them in calorific value\(^49\).

The amino acids asparagine, aspartic acid, glycine, glutamic acid, serine, áserine and threonine, are found in the pulp. Pulp contains about 70 IU Vitamin A /100 g and the β-carotene content ranges from 75 to more than 80 mg/100 g. *Zizyphus jujuba* fruits are very rich in vitamins C and B1 (thiamine) and B2 (riboflavin), bioflavonoids and pectin A, Pectin has a number of pharmaceutical properties such as binding bile acid, lowering plasma cholesterol and anti diarrhoeal properties. The glycoside, saponin, Triterpenoic acids, present in the plant are Betulinic acid, Oleanolic acid, jujubasaponins II, III, IV, V and VI and jujuboside B from the leaves and seeds\(^49\). Jujube fruits have usefulness in Neurological properties like Cognitive activities, Antifertility/contraception, Hypotensive and antinephritic
effect, Antifungal activity, Antiallergic, Anti-inflammatory effect, Antispastic effect, Antibacterial.

4.2. METHOD OF PREPARATION AND EVALUATION

4.2.1 Collection of Plant material

The seeds of *Canavalia Gladiata*, roots of *Berberis vulgaris* and fruits of *Zizyphus jujuba* were collected and authenticated by taxonomists. These were made free from the adherent foreign material, air-dried, cut into small pieces and coarsely powdered mechanically.

4.2.2 Preparation of Extract

The plant materials were cleaned, shade dried and coarsely powdered mechanically. The powdered material was soaked in 70% aqueous-methanol/ethanol for 3 days with occasional shaking. It was filtered through a muslin cloth and then through a filter paper. This procedure was repeated thrice and the combined filtrate was evaporated on a rotary evaporator under reduced pressure to a thick, semi-solid mass of dark black colour, i.e. the crude extract. The obtained extract is fractionized, one part is homogenized with ethyl acetate which gives ethyl acetate fractions and other is homogenized with 2M Sulphuric acid followed by extraction with chloroform three times gives the fractions of chloroform extracts. The material is also used to prepare aqueous extracts by soaking in the water for 3 days with occasional shaking and filtered. The obtained extracts were kept in desiccators to remove moisture and stored properly until used.
**Fig: 4.4. Preparation of extract and fraction from plant parts**

### 4.2.3. Phytochemical screening

The methanolic extract of *Canavalia gladiate*, *Zizyphus Jujuba* and ethanolic extract of *Berberis Vulgaris* was screened for the presence of various Phytochemical constituents like Carbohydrates, alkaloids, Tannins, steroids, Glycosides, Saponins and Flavanoids and later ethyl acetate and chloroform fractional extracts of *Canavalia gladiate*, *Berberis Vulgaris*, *Zizyphus Jujuba* is used for further evaluation to determine Flavonoids fraction\(^{26}\).
Test for Carbohydrates

Molisch test is performed for identification of carbohydrates where the purple color ring formation is characteristic identification of the carbohydrates.

Test for alkaloids

About 50mg of the extract was stirred with few ml of dilute hydrochloric acid and filtered. The filtrate was tested with various alkaloidal reagents as follows

Mayer’s Test: To a few ml of filtrate, 2-3 drops of Mayer’s reagent was added along the sides of the test tube. A white or creamy precipitate indicates presence of alkaloids.

Wagner’s Test: To a few ml of filtrate, 2-3 drops of Wagner’s reagent was added along the sides of the test tube. A reddish-brown precipitate indicates presence of alkaloids.

Dragendorff’s Test: To a few ml of filtrate, 2-3 drops of Dragendorff’s reagent was added along the sides of the test tube. A brick-red precipitate indicates presence of alkaloids.

Test for tannins

About 0.5 g of extract was added was in 10 ml of water in a test tube and filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or blue-black color.

Glycosides

Keller-kiliani test was performed to assess the presence of cardiac glycosides. The crude dry powder of each plant was treated
with 1 ml of FeCl3 reagent (mixture of 1 volume of 5% FeCl3 solution and 99 volumes of glacial acetic acid). To this solution a few drops of concentrated H$_2$SO$_4$ was added. Appearance of greenish blue color within a few minutes indicated the presence of cardiac glycosides$^{33}$. 

**Steroids**

Liebermann-Burchard reaction was performed to assess the presence of steroids. A chloroform solution of the crude dry powder of each plant was treated with acetic anhydride and a few drops of concentrated H$_2$SO$_4$ were added down the sides of the test tube. A blue green ring indicated the presence of terpenoids.

**Saponins**

The presence of saponins was determined by Frothing test. The crude dry powder of each plant was vigorously shaken with distilled water and was allowed to stand for 10 minutes and classified for saponin content as follows: no froth indicate absence of saponins and stable froth more than 1.5 cm indicated the presence of saponins.

The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites that possess an aromatic ring bearing one or more hydroxyl constituents. Phenolic compounds are widely found in the secondary products of medicinal plants, as well as in many edible plants. A number of studies have focused on the biological activities of phenolic compounds, which are potential antioxidants and free radical-scavengers. Several studies have
described the antioxidant properties of medicinal plants, foods, and beverages which are rich in phenolic compounds\textsuperscript{1}.

Flavonoids are a broad class of plant phenolics that are known to possess a well established protective ability against membrane lipoperoxidative damages.

**Test for Flavonoids**

To aqueous extract 10% lead acetate solution is added to form yellow precipitate shows Flavonoids. The presence of Flavonoids was estimated by Shinoda test. The alcoholic extract of the crude dry powder of each plant was treated with a few drops of concentrated HCl and magnesium ribbon. The appearance of pink or tomato red color within a few minutes indicated the presence of flavonoids\textsuperscript{53}.

**Flavonoid determination:**

Aluminium chloride colorimetric method was used to determine Flavonoid content. Plant extract (0.5ml of 1:10g/ml) in methanol was mixed with 1ml of methanol, 0.1 ml aluminium chloride (10 %) and 0.1 ml potassium acetate (120 mM). The mixture was allowed to stand for 30 min at room temperature; then the absorbance was measured at 415 nm. Quercetin was used as standard. Flavonoid content is expressed in terms of quercetin equivalent (mg/ml of extracted compound). Total phenolic content was higher in methanol extract followed by chloroform than in aqueous acidic component of the extracts\textsuperscript{1}.
The confirmation of the Flavonoid is done by TLC on silica gel in Butanol-acetic acid-water (4:1:5)\textsuperscript{50}.

4.3. EXPERIMENTAL ANIMALS & TOXICITY STUDIES

Adult male albino Wister rats weighing 140-200g (4-8 weeks) were used for the study acquired from MAHAVEER ENTERPRISES. They were housed in polypropylene cages and were maintained at room temperature of 23\textdegree{}C ± 2\textdegree{}C and relative humidity 50\%. They were maintained in 12hr: 12hr light:dark cycle throughout the period of acclimatization and experimental study. All the study protocols were reviewed and approved by Institutional Animal Ethical Committee (IAEC).

Acute Toxicity Study

The Acute Toxicity Studies was performed using rats as per OECD Guideline No.423 (short term toxicity). The animals are selected of both sex and acclamatised to the conditions by housing and feeding at 22\textdegree{}C (± 3\textdegree{}C) humidity 30-70 % with 12:12 dark and light cycle. After this the animals are randomly selected, marked to permit individual identification and caged. The doses are prepared and administered using intubation cannula to animals which are prior fasted overnight and after administration fasted for 3-4 hrs. The dose level to be used as starting dose is selected as per Global Hormonised System from 5,50,300,2000 mg/kg b.wt. The animals are observed for
signs of toxicity from 30 minutes after observation up to 24 hrs with a time interval of 4 hr and animals are sacrificed with euthansia.\textsuperscript{51}

\textbf{4.4. MATERIALS & METHODS}

\textbf{4.4.1. Drugs, Chemicals}

The drugs, chemicals and solvents used in the study were of analytical grade.

\textit{Azathioprine} (AZA)

6-\{(1-methyl-4-nitro-1H-imidazol-5-yl)sulfanyl\}-7H-purine, C\textsubscript{9}H\textsubscript{7}N\textsubscript{7}O\textsubscript{2}S

Azathioprine is an immunosuppressive pro-drug. It is converted into 6-mercaptopurine in the body where it blocks purine metabolism and DNA synthesis.

6-Mercaptopurine (6-MP) and its prodrug azathioprine are thiopurine analogues and are immuno-modulatory agents of the AZA compound, 88\% is converted \textit{via} nonenzymatic process to 6-MP.

\textbf{Adverse effects}

- Azathioprine has been complicated by a high incidence of serious adverse drug reactions including hepatotoxicity. The hepatotoxicity has already been documented in rats with elevation of reactive oxygen species (ROS) which caused the mitochondrial disruption leading to cell necrosis.

- Side effects that are probably hypersensitivity reactions include dizziness, diarrhea, fatigue, and skin rashes\textsuperscript{55}. 

**Silymarin**

Silymarin, an antioxidant flavonoid complex derived from the herb milk thistle (Silybum marianum), has long been used as a liver tonic. Two new studies show that it can reduce insulin resistance (the underpinning of adult-onset diabetes, hypertension, and hypercholesterolemia) and diabetic complications. A third study reports that it may have some anti-cancer benefits as well.

*Silymarin Adverse Reactions*

Occasional laxative effects, Abdominal bloating, diarrhoea, flatulence, loss of appetite, anorexia, nausea, stomach upset\(^5^6\).

**Levamisole**

Levamisole is a synthetic imidazothiazole derivative that has been widely used in treatment of worm infestations in both humans and animals. As an antihelmintic, it probably works by targeting the nematode nicotinergic acetylcholine receptor. As an immunomodulator, it appears that Levamisole is an immunostimulant which has been shown to increase NK cells and activated T-cells in patients receiving this adjuvantly along with 5FU for Stage III colon cancer\(^5^7\).

**4.4.2. Induction of Hepatotoxicity & Study Design**

1mg/ml of Azathioprine solution was given through intraperitonially to all the group of animals and the samples were collected from the animals through retro-orbital plexus root and the
liver bio marker parameters were estimated like SGOT, SGPT, ALP and Bilirubin.

**Study Design for Hepatoprotective Activity**

The rats were randomly divided into 5 groups each group consisting of 6 animals.

**Normal control:** Rats received vehicle in oral route.

**Toxic control:** Azathioprine intoxicated rats received 15mg/kg I.P with a single dose of Azathioprine.

**Treatment group 1:** Azathioprine intoxicated Rats were treated with methanolic extracts of *Canavalia gladiate* (MECG), *Zizyphus Jujuba*, (MEZJ) and ethanolic extract *Berberis Vulgaris* (EEBV) at a dose 100mg/kg/day.

**Treatment group 2:** Azathioprine intoxicated Rats were treated with methanolic extracts of *Canavalia gladiate* (MECG), *Zizyphus Jujuba*, (MEZJ) and ethanolic extract *Berberis Vulgaris* (EEBV) at a dose 200mg/kg/day.

**Treatment group 3:** Azathioprine intoxicated Rats were treated with Chloroform fraction of methanolic extracts of *Canavalia gladiate* (MECG), *Zizyphus Jujuba*, (MEZJ) and ethanolic extract *Berberis Vulgaris* (EEBV) at a dose 100mg/kg/day.

**Treatment group 4:** Azathioprine intoxicated Rats were treated with Chloroform fraction of methanolic extracts of *Canavalia gladiate*
(MECG), *Zizyphus Jujuba*, (MEZJ) and ethanolic extract *Berberis Vulgaris* (EEBV) at a dose 200mg/kg/day.

**Treatment group 5:** Azathioprine intoxicated Rats were treated with Ethyl acetate fraction of methanolic extracts of *Canavalia gladiate* (MECG), *Zizyphus Jujuba*, (MEZJ) and ethanolic extract *Berberis Vulgaris* (EEBV) at a dose 100mg/kg/day.

**Treatment group 6:** Azathioprine intoxicated Rats were treated with Ethyl acetate fraction of methanolic extracts of *Canavalia gladiate* (MECG), *Zizyphus Jujuba*, (MEZJ) and ethanolic extract *Berberis Vulgaris* (MEBV) at a dose 200mg/kg/day.

**Silymarin group:** Azathioprine intoxicated Rats treated with Silymarin which is a reported standard drug at 100mg/kg/day per oral day 1-22.

*Collection of blood sample:*

Blood samples were collected from all the groups of animals at 0, 7, 14, 22 day intervals through puncture of retro orbital plexus and were centrifuged at 3000 revolutions per minute (RPM) for 15min. Serum was separated and stored at -20°C and used for estimating SGOT, SGPT, ALP and Bilirubin levels.

*Preparation of Liver Tissue Homogenate*
The rats are sacrificed and liver are isolated apart of it is dissolved with ice cold buffer, minced and homogenized. The other part of tissue is used for histopathological studies

*Histopathological examination*

The animals were dissected; the livers were from all groups carefully removed, washed with 0.9% saline solution and preserved in formalin solution (10% formaldehyde) for histopathological studies.57

**Estimation of Biochemical Parameters**

The following are the biochemical parameters estimated to evaluate the effect of the test materials against the experimentally induced hepatotoxicity in rats. They are SGPT, SGOT Alkaline phosphatise (ALP), Bilirubin.

**SGPT (Serum Glutamate Pyruvate Transaminase)25**

*Clinical significance*

Alanine transaminase is present large amounts in liver, kidney, heart and skeletal tissues. It is also present in spleen, lungs, pancreas, brain and erythrocytes at lower concentration. Primary to liver damages and secondary to other causes result in elevated levels of GPT.

*Principle*

SGPT converts L- Alanine and α- ketoglutarate to pyruvate and Glutamate. The pyruvate formed reacts with 2,4, Dinitrophenyl
hydrazine to procedure a hydrazone derivative, which in an alkaline medium produces a brown coloured complex whose intensity is measured. The reaction does not obey Beer’s law and hence a calibration curve is plotted using a pyruvate standard. The activity of SGPT (ALAT) is read off this calibration curve.

\[
\begin{align*}
\text{L-Alanine} & \quad \text{SGPT} & \quad \text{Pyruvate} \\
+ & \quad \text{pH 7.4} & \\
\alpha\text{-Ketoglutarate} & \quad \text{L-Glutamate} & \\
\text{Pyruvate} & \quad \text{Alkaline} & \quad 2,4\text{, Dinitrophenyl Hydrazone} \\
+ & \quad \text{Medium} & \quad \text{(Brown coloured complex)} \\
\end{align*}
\]

2,4DNPH

*Reagents used:*

- Enzyme Reagent
- Buffer Solution

*Preparation and stability of working reagent:* Reconstitute one vial of Enzyme reagent with 10 ML Buffer solution, this working reagent is stable upto 30 days at 2-8°C.

*Sample:* Serum free hemolysis. SGPT is reported to be stable in serum for 3 days at 2-8°C

*Procedure:*

Mix working reagent 1ml and Sample 0.1ml and incubate it for 1 minute, measure the change of optical density per minute (ΔOD/min) for 3 minutes. The wavelength observed at is 340nm.

*Calculation:* Activity (U/L) = ΔOD/min × 1768
Serum Glutamate Oxaloacetic Transaminase (SGOT)\textsuperscript{25}

\textit{Clinical significance}

Aspartate transaminase is present in all human tissues of the body. It also presents large amounts in liver, kidneys, heart and skeletal muscles. Elevated levels are associated with liver disease or damage, myocardial infraction, muscular dystrophy. In myocardial infraction GOT levels increase after 3-8 hours of onset of attack and returns to normal in 4-6 weeks. The duration and extent of increase in levels is proportional to the severity of attack.

\textit{Principle}

SGOT converts L- Aspartate and α- ketoglutarate and Glutamate. The oxaloacetate formed reacts with 2,4, Dinitrophenyl hydrazine to procedure a hydrazone derivative, which in an alkaline medium produces a brown coloured complex whose intensity is measured. The reaction does not obey Beer’s law and hence a calibration curve is plotted using a pyruvate standard. The activity of SGOT (AST) is read off this calibration curve.

\[
\begin{array}{ccc}
\text{L-aspartate} & \text{SGOT} & \text{Oxaloacetate} \\
+ & \text{pH 7.4} & + \\
\alpha\text{-ketoglutarate} & & \text{L-glutamate}
\end{array}
\]

\[
\begin{array}{ccc}
\text{Oxaloacetate} & \text{Alkaline} & 2,4 \text{ Dinitrophenyl Hydrazone} \\
+ & \text{Medium} & \text{(Brown coloured complex)}
\end{array}
\]

2, 4, DNPH
Reagents used:

- Enzyme reagent
- Buffer reagent

Preparation and stability of working reagent: Reconstitute one vial of Enzyme reagent with 10 ML Buffer solution, this working reagent is stable upto 30 days at 2-8°C.

Sample: Serum free hemolysis. SGOT is reported to be stable in serum for 3days at 2-8°C

Mix working reagent 1ml and sample 0.1ml and after 1 minute incubation, measure the change of optical density per minute (ΔOD/min) for 3 minutes. The Wave length observed at is 340nm.

Calculation:

Activity (U/L) = \( \frac{\Delta OD \times 1768}{\text{Min}} \)

Alkaline phosphatase (ALP)

Clinical significance

Alkaline phosphatase measurements are used in the diagnosis and treatment of liver, bone, parathyroid, and intestinal diseases.

Principle

Serum ALP hydrolyzes phenyl phosphate disodium hydrogen phosphatase at pH 10.0. The phenol is so formed reacts with 4-
aminoantipyrine in alkaline medium in presence of oxidizing agent potassium ferricyanide to form a red coloured complex whose absorbance is proportional to the enzyme activity.

**Reagents:**

1. P-NPP substrate
2. Buffer Solution

**Reagent preparation:**

Reconstitute one vial of P-NPP substrate with 5 ml of buffer solution.

**Procedure:**

- Pipette into clean dry tubes Reconstituted reagent 1 ml Bring to essay temperature & add sample 0.02 ml.
- Mix well and read and record initial absorbance $A_0$ at 30 seconds and repeat reading after every minute.

$$\Delta A/\text{min.} = \frac{(A_3-A_0)}{3}$$

- If the $\Delta A$/minute is greater than 0.35 dilute one part of sample with 9 parts of isotonic saline and reassay. Multiply the result by 10 to compensate for the dilution.

**Wave length:** 405nm

**Calculations:**

Serum ALP activity in U/L = $\Delta A/\text{min} \times 2742$
**Bilirubin Test**

*Clinical Significance:*

The abnormal retention of bilirubin usually results in jaundice, a condition is characterised by increased bilirubin in blood and deposition of a brownish yellow pigment in the skin, sclera, and mucous membrane.

*Principle:*

Bilirubin reacts with diazotised sulfanilic acid in acidic medium to form azobilirubin, a pink colored complex whose absorbence is proportional to bilirubin concentration. Direct bilirubin, being water soluble is allowed to react with diazotised sulfanilic acid in the absence of an activator, while for total bilirubin (direct & indirect) the diazotisation is carried out in the presence of an activator.

*Reagents used:*

- Diazo A
- Diazo B
- Activator
- Artificial standard (100mg %)

*Sample: serum/ plasma*

- Hemolysis should be avoided, because hemoglobin produces falsely low values with diazo methods.
• Both conjugated and unconjugated bilirubin are photo oxidized on exposure to white or ultra violet light. Specimens should be protected from direct exposure to either artificial or sunlight as soon as they are drawn.

• Bilirubin in serum is stable for 3 days at 2-8°C in the dark.

Procedure:

• Blood was collected in a clean dry container use of plastic (or) siliconized container was a voided since it may prolong clotting time.

• For plasma separation, sodium citrate (4:1ratio) was used as anticoagulant.

Clean dry test tubes were taken and labelled as T1&T2 (for total bilirubin), D1&D2 (for direct bilirubin). The contents were mixed well. The absorbances of D1 &D2 were read exactly after 1min at 540nm. T1 &T2 were kept in dark at room temperature for 5min and then the absorbances were measured at 540nm.

Calculation:

Total bilirubin in mg% = \( \frac{\text{Absorbance of T1} - \text{Absorbance T2} \times 10}{\text{Absorbance of standard}} \)

Direct bilirubin in mg% = \( \frac{\text{Absorbance of D1} - \text{Absorbance of D2}\times10}{\text{Absorbance of standard}} \)

Super Oxide Dismutase

Superoxide dismutases are the enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. So they
are an important antioxidant defense in nearly all cells exposed to oxygen.

The liver tissue homogenate is prepared using normal saline and ice cold buffer and SOD activity was measured by xanthine oxidase method using purified bovine erythrocyte SOD (5000 units/mg solid) as a standard. The reaction between 50 mM xanthine, 50 mM xanthine oxidase 1000 U and 0.1 mM EDTA was used to generate superoxide radicals (O$_2$$^-$$^-$$^-$$^-$) and uric acid at pH 7.8. The superoxide radicals produced react with 50 mM nitroblue tetrazolium (NBT) to give a red formazan dye that was measured spectrophotometrically at 250 nm. SOD present in the sample competes with the NBT for superoxide radicals and so inhibits the production of formazan dye. SOD was measured by the degree of inhibition of formazan dye formation. The SOD activity was expressed as U/mg protein$^{43}$.

**Glutathione**

It is an antioxidant, preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides. Glutathione is found almost exclusively in its reduced form, since the enzyme that reverses it from its oxidized form, glutathione reductase is constitutively active and inducible upon oxidative stress. Glutathione forms a coloured complex with DTNB, which is measured spectrophotometrically.
In order to estimate the hepatic GSH, an aliquot of liver homogenate (0.1 ml) was mixed with 1.7 ml 0.1M potassium phosphate buffer (pH 8) followed by the addition of 0.1 ml Ellman’s reagent [5,5-dithiobis(2-nitrobenzoic acid)] (DTNB) dissolved in 1% sodium citrate to give a concentration of 1mM. After 5 min, the absorbance was measured spectrophotometrically at 412 nm against a blank.

**Catalase**

Catalase measurement was done based on the ability of Catalase to oxidize hydrogen peroxide.

\[
2 \text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} 2\text{H}_2\text{O} + \text{O}_2
\]

The hepatic CAT activity was measured using \(\text{H}_2\text{O}_2\) as a substrate that is decomposed by CAT enzyme. A mixture of 50 mM phosphate buffer (pH 7.0), 20 mM H2O2 and 0.1 ml liver homogenate in a final volume of 3 ml was incubated at temp 25°C for 30 min. After transferring in to a cuvette the absorbance was measured at 240nm. The change in absorbance was measured for 3 minutes. The average change in absorbance per mminute for each assay was calculated and the results were expressed in terms of Units per mg protein.

### 4.4.3 Study Design for Immunomodulatory Activity

**In-vitro methods:**

**Neutrophils adhesion test**

- Vehicle control group: Rats served as normal control group for 14 days.
• Toxic control group: Azathioprine intoxicated rats

• Treated with aqueous extract: Azathioprine intoxicated Rats were treated with aqueous extracts of *Canavalia gladiate*, *Zizyphus Jujuba*, and *Berberis Vulgaris* 100 mg/kg/day daily for 14 days respectively.

• Treated with aqueous extract: Azathioprine intoxicated Rats were treated with aqueous extracts of *Canavalia gladiate*, *Zizyphus Jujuba*, and *Berberis Vulgaris* 100 mg/kg/day daily for 14 days respectively.

• Treated with Alcoholic extract: Azathioprine intoxicated Rats were treated methanolic extracts of *Canavalia gladiate*, *Zizyphus Jujuba*, and ethanolic extract of *Berberis Vulgaris* 100 mg/kg/day daily for 14 days respectively.

• Treated with Alcoholic extract: Azathioprine intoxicated Rats were treated methanolic extracts of *Canavalia gladiate*, *Zizyphus Jujuba*, and ethanolic extract of *Berberis Vulgaris* 200 mg/kg/day daily for 14 days respectively.

• Treated with Alcoholic extract: Azathioprine intoxicated Rats were treated chloroform fraction of methanolic extracts of *Canavalia gladiate*, *Zizyphus Jujuba*, and ethanolic extract of *Berberis Vulgaris* 100 mg/kg/day daily for 14 days respectively.

• Treated with Alcoholic extract: Azathioprine intoxicated Rats were treated Chloroform fraction of methanolic extracts of *Canavalia gladiate*, *Zizyphus Jujuba*, and ethanolic extract of *Berberis Vulgaris* 200 mg/kg/day daily for 14 days respectively.
• Treated with Alcoholic extract: - Azathioprine intoxicated Rats were treated Ethyl acetate fraction of methanolic extracts of *Canavalia gladiate*, *Zizyphus Jujuba*, and ethanolic extract of *Berberis Vulgaris* 100 mg/kg/day daily for 14 days respectively.

• Treated with Alcoholic extract: - Azathioprine intoxicated Rats were treated Ethyl acetate fraction of methanolic extracts of *Canavalia gladiate*, *Zizyphus Jujuba*, and ethanolic extract of *Berberis Vulgaris* 200 mg/kg/day daily for 14 days respectively.

• Standard drug treated: - Azathioprine intoxicated rats treated with Levamisole (50 mg/kg/per oral) for 14 days.

• On the 14th day of the treatment, blood samples from all the groups were collected by puncturing retro-orbital plexus under mild ether anaesthesia.

• Blood was collected and analyzed for total leukocyte count (TLC) and differential leukocyte count (DLC) by fixing blood smears and staining with Leishman’s stain.

• After initial counts, blood samples were incubated with nylon fiber (80 mg/ml of blood sample) for 15 min at 37°C. The incubated blood samples were again analyzed for TLC and DLC.

• The product of TLC and % neutrophil gives neutrophil index (NI) of blood sample. Percentage of neutrophil adhesion was calculated as follows,

\[
\text{Neutrophil adhesion} = \frac{\text{NIU} - \text{NIT} \times 100}{\text{NIU}}
\]
Where, NIU: Neutrophil Index before incubation with nylon fiber.

NIT: Neutrophil Index after incubation with nylon fiber

**Phagocytosis Using Carbon Clearance Method**\(^{60}\)

Azathioprine intoxicated rats was treated with the extracts and fractions for 15 days. On 15th day of treatment, rats were injected with 0.1 ml of carbon suspension intravenously through tail vein. Blood samples (25 µl) were collected from retro-orbital plexus just before and at 4, 8, 12 and 16 min after injection. Blood samples were lysed with 2 ml of 0.1% acetic acid and absorbance of samples recorded at 675 nm. The graph for absorbance versus time was plotted for each animal in respective test group and phagocytic index was calculated using the formula

\[
\text{Phagocytic Index (PI)} = \frac{K_{\text{sample}}}{K_{\text{standard}}}
\]

\(K_{\text{sample}}\) - represents the slope of absorbance versus time curve for extract-treated samples \(K_{\text{standard}}\)-represents the slope of absorbance versus time curve for blood sample collected before treatment.

**In-vivo methods**

**Delayed type hypersensitivity (DTH) response**\(^{16}\)

- Vehicle control group: - Rats served as normal control group for 21 days.
- Toxic control group:- Rats with AZA treated served as toxic control group for 21 days.
- Treated with aqueous extract: - Azathioprine intoxicated Rats were treated with aqueous extracts of *Canavalia gladiate*,
Zizyphus Jujuba, and Berberis Vulgaris 100 mg/kg/day daily for 21 days respectively.

- Treated with aqueous extract: - Azathioprine intoxicated Rats were treated with aqueous extracts of Canavalia gladiate, Zizyphus Jujuba, and Berberis Vulgaris 100 mg/kg/day daily for 21 days respectively.

- Treated with Alcoholic extract: - Azathioprine intoxicated Rats were treated methanolic extracts of Canavalia gladiate, Zizyphus Jujuba, and ethanolic extract of Berberis Vulgaris 100 mg/kg/day daily for 21 days respectively.

- Treated with Alcoholic extract: - Azathioprine intoxicated Rats were treated methanolic extracts of Canavalia gladiate, Zizyphus Jujuba, and ethanolic extract of Berberis Vulgaris 200 mg/kg/day daily for 21 days respectively.

- Treated with Alcoholic extract: - Azathioprine intoxicated Rats were treated chloroform fraction of methanolic extracts of Canavalia gladiate, Zizyphus Jujuba, and ethanolic extract of Berberis Vulgaris 100 mg/kg/day daily for 21 days respectively.

- Treated with Alcoholic extract: - Azathioprine intoxicated Rats were treated Chloroform fraction of methanolic extracts of Canavalia gladiate, Zizyphus Jujuba, and ethanolic extract of Berberis Vulgaris 200 mg/kg/day daily for 21 days respectively.

- Treated with Alcoholic extract: - Azathioprine intoxicated Rats were treated Ethyl acetate fraction of methanolic extracts of
Canavalia gladiate, Zizyphus Jujuba, and ethanolic extract of Berberis Vulgaris 100 mg/kg/day daily for 21 days respectively.

- Treated with Alcoholic extract: - Azathioprine intoxicated Rats were treated Ethyl acetate fraction of methanolic extracts of Canavalia gladiate, Zizyphus Jujuba, and ethanolic extract of Berberis Vulgaris 200 mg/kg/day daily for 21 days respectively.

- Standard drug treated: - Azathioprine intoxicated rats treated with Levamisole (50 mg/kg/per oral) for 21 days.

- On day 21st all animals from all the groups were challenged with 0.03 ml of 20% Sheep RBCs in sub plantar region of right hind paw Foot pad edema in rat was used for detection of cellular immune response.

- On 21st day, injection of 0.1ml of 20% SRBCs in the sub plantar region of right hind paw in the volume of 0.03 ml and normal saline in left hind paw in same volume.

- Foot pad reaction was assessed after 24hours on 22nd day, in terms of increase in the thickness of footpad as a result of hypersensitivity reaction due to oedema; the thickness of the right hind footpad was measured using Plethysmometer.

- The footpad reaction was expressed as the difference in the thickness (millimetre) between the right foot pad injected with Sheep RBC and the left footpad injected with normal saline.

**Evaluation of immunoprophylactic effect**

- Group I (vehicle):- Rats served as normal control group for 15 days.
• Toxic control group: - Rats with AZA treated served as toxic control group for 15 days.

• Treated with aqueous extract: - Azathioprine intoxicated Rats were treated with aqueous extracts of *Canavalia gladiate*, *Zizyphus Jujuba*, and *Berberis Vulgaris* 100 mg/kg/day daily for 15 days respectively.

• Treated with aqueous extract: - Azathioprine intoxicated Rats were treated with aqueous extracts of *Canavalia gladiate*, *Zizyphus Jujuba*, and *Berberis Vulgaris* 100 mg/kg/day daily for 15 days respectively.

• Treated with Alcoholic extract: - Azathioprine intoxicated Rats were treated methanolic extracts of *Canavalia gladiate*, *Zizyphus Jujuba*, and ethanolic extract of *Berberis Vulgaris* 100 mg/kg/day daily for 15 days respectively.

• Treated with Alcoholic extract: - Azathioprine intoxicated Rats were treated methanolic extracts of *Canavalia gladiate*, *Zizyphus Jujuba*, and ethanolic extract of *Berberis Vulgaris* 200 mg/kg/day daily for 15 days respectively.

• Treated with Alcoholic extract: - Azathioprine intoxicated Rats were treated chloroform fraction of methanolic extracts of *Canavalia gladiate*, *Zizyphus Jujuba*, and ethanolic extract of *Berberis Vulgaris* 100 mg/kg/day daily for 15 days respectively.

• Treated with Alcoholic extract: - Azathioprine intoxicated Rats were treated Chloroform fraction of methanolic extracts of
Canavalia gladiate, Zizyphus Jujuba, and ethanolic extract of Berberis Vulgaris 200 mg/kg/day daily for 15 days respectively.

- Treated with Alcoholic extract: Azathioprine intoxicated Rats were treated Ethyl acetate fraction of methanolic extracts of Canavalia gladiate, Zizyphus Jujuba, and ethanolic extract of Berberis Vulgaris 100 mg/kg/day daily for 15 days respectively.

- Treated with Alcoholic extract: Azathioprine intoxicated Rats were treated Ethyl acetate fraction of methanolic extracts of Canavalia gladiate, Zizyphus Jujuba, and ethanolic extract of Berberis Vulgaris 200 mg/kg/day daily for 15 days respectively.

- Standard drug treated: Azathioprine intoxicated rats treated with Levamisole (50 mg/kg/per oral) for 15 days.

On 15th day after 3hr of the last dose of aqueous extract and methanolic extract, Escherichia coli (0.5ml/100kg body weight, intraperitoneal) was injected to control, aqueous and methanolic groups of rats and percentage of mortality was observed after 24hours.