CHAPTER : 3

MATERIALS AND METHODS

3.1 Drugs and chemicals:

The drugs and chemicals used in the study were; Diclofenac sodium 25 gm extrapure, DL-Methionine 100 gm extrapure and N-Acetylcysteine 10 gm extrapure of pure analytical grade were purchased from Aatur Instru Chem, Vadodara. Other materials included the Diagnostic kit reagents for the estimation of Liver Function Tests (LFTs), Distilled water & Ether. The chemicals used were 10% Formalin, Xylene, Hemotoxylin and Eosin stains, for preparation of histopathology slides.

3.2 Diagnostic Kit reagents used for estimation of Liver Function Tests:

In the present study, following liver enzymes were analyzed with the help of the diagnostic kits as mentioned below. Standard Erba estimation kit was used by using auto analyzer (Erba, Chem 7, Germany). Standard procedure as specified in the kit literature was followed.

1. Serum Glutamic-Pyruvic Transaminase (SGPT) - Erba diagnostics Manheim
2. Serum Glutamic-Oxaloacetic Aminotransferases (SGOT) - Erba diagnostics Manheim
3. Serum Alkaline Phosphatase - Erba diagnostics Manheim
4. Serum bilirubin – Direct and Indirect Bilirubin - Erba diagnostics Manheim
5. Total Bilirubin - Erba diagnostics Manheim
6. Serum Gamma-Glutamyl Transpeptidase (GGTP) - Erba diagnostics Manheim
3.3. **Materials & equipments:**

Equipments such as digital weighing balance (to weigh the experimental animals), digital weighing balance to weigh chemicals, MERCK UV-Visible spectrophotometer & cooling centrifuge machine. The wax blocks & glass slides were used for studying the histopathology studies.

Equipments such as glass beakers, glass measuring cylinders, pipettes, white paper, blood collecting tubes with closed cork, glass capillary tube, glass rod, specimen collecting jars with closed lid, sterile surgical cotton, hand gloves, white porcelain tray, aspiration needle or intragastric cannula /feeding needle for rats, cuticle scissors, German steel scissors, surgical blade, artery forceps, blunt forceps and dissection box, disposable syringe 5 ml &10 ml capacity.

3.4 **Use of small laboratory animals – Albino rats:**

The present research study was accepted & approved by the Institutional Animal Ethics Committee (IAEC), which is registered under Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of S.B.K.S.M.I. & R.C., Sumandeep Vidyapeeth Institute deemed to-be University, Piparia.

Albino rats of either sex weighing between 100 - 400g were used. All the animals used were housed separately in poly-propylene rat-cages and were allowed to acclimatize under controlled environmental conditions of temperature 24° ± 2°C and 55% ± 5%, relative humidity, in a 12-hour light-/dark cycle throughout the experiment. All animals were given free access to food and purified drinking water ad libitum.
3.5 Plan of Work and Methodology:

1. Demonstration of hepatotoxicity induced by Diclofenac sodium used in three different single oral dose.

2. Demonstration on the per se effect of DL-Methionine on liver.

3. Demonstration of the hepatoprotective effect of DL-Methionine on the liver injury caused by different doses of Diclofenac sodium.

4. Demonstration on the per se effect of N-Acetylcysteine on liver.

5. Demonstration of hepatoprotective effect of N-Acetylcysteine on the liver injury caused by different doses of Diclofenac sodium.

6. Comparison of the hepatoprotective effect of DL-Methionine and N-Acetylcysteine on the liver injury caused by different doses of Diclofenac sodium.
PHOTOGRAPHS:

Note: Photographs no. 1 – 12 shows the plan of work and methodology of the present study.

1. Weighing of experimental animal

2. Weighing of chemicals
3a. Preparation of drug solutions for drug administration

3b. Preparation of drug solutions for drug administration
4. Oral administration of the test drug
5. Collection of blood from retroorbital plexus
6. Collection of blood for serum analysis

7. Dissection for isolation of liver sample
8. Isolated liver samples

9. Wax-block preparation of liver
10. Tissue processing

11. Wax block slicing for slide preparation
12. Prepared histopathology slides
3.6 Experimental Design:

The albino rats were grouped for the experimental study, in various groups as shown in Table number 1.

**Table 1**: All the experimental animals were grouped into a total of thirteen groups with each group containing 6 rats (n=6).

**Table 1: Experimental Design.**

<table>
<thead>
<tr>
<th>Group I</th>
<th>Control - Distilled Water 10 ml/kg p.o.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group II</td>
<td>Diclofenac sodium 72 mg/kg p.o.</td>
</tr>
<tr>
<td>Group III</td>
<td>Diclofenac sodium 96 mg/kg p.o.</td>
</tr>
<tr>
<td>Group IV</td>
<td>Diclofenac sodium 240 mg/kg p.o.</td>
</tr>
<tr>
<td>Group V</td>
<td>DL-Methionine per se 700 mg/kg p.o.</td>
</tr>
<tr>
<td>Group VI</td>
<td>DL-Methionine per se 1400 mg/kg p.o.</td>
</tr>
<tr>
<td>Group VII</td>
<td>N-Acetylcysteine per se 450 mg/kg p.o.</td>
</tr>
<tr>
<td>Group VIII</td>
<td>Diclofenac sodium 96 mg/kg p.o. + DL-Methionine 700 mg/kg p.o.</td>
</tr>
<tr>
<td>Group IX</td>
<td>Diclofenac sodium 240 mg/kg + DL-Methionine 700 mg/kg p.o.</td>
</tr>
<tr>
<td>Group X</td>
<td>Diclofenac sodium 96 mg/kg + N-Acetylcysteine 450 mg/kg p.o.</td>
</tr>
<tr>
<td>Group XI</td>
<td>Diclofenac Sodium 240 mg/kg + N-Acetylcysteine 450 mg/kg p.o.</td>
</tr>
<tr>
<td>Group XII</td>
<td>Diclofenac Sodium 96 mg/kg + DL-Methionine 1400 mg/kg p.o.</td>
</tr>
<tr>
<td>Group XIII</td>
<td>Diclofenac Sodium 240 mg/kg + DL-Methionine 1400 mg/kg p.o.</td>
</tr>
</tbody>
</table>
3.7 Demonstration of hepatotoxicity:

After overnight fasting, the albino rats belonging to group I (control group) were treated with the distilled water of 10 ml/kg orally, while, the albino rats that belonged to the group II, III and IV were administered with single oral dose of Diclofenac sodium in the doses of 72 mg/kg, 96 mg/kg and 240 mg/kg body weight (n=6), respectively. All the drugs and control vehicle were administered by per oral (p.o.) and the volume administered was maintained constant in all the albino rats at 10 ml/kg.

After 24 hour of post-treatment with positive control drug Diclofenac sodium in different groups as indicated in table no. 1; whole blood was collected in labeled collecting glass tubes; from retro-orbital plexus of eye, with the help of glass capillary tube, for the estimation of haemato-biochemical parameters in serum. Serum was stored at −20°C until analyzed and were assessed to determine the extent of liver injury at the end of 24 hours of exposure of the drug. Serum was separated immediately through centrifugation at 3000 r.p.m. for the determination of liver enzymes, alanine amino transferase, aspartate amino transferase, alkaline phosphatase, Gamma-Glutamyl Transpeptidase (GGTP) or Gamma-Glutamyl Transferase (GGT), & total bilirubin.

3.7.1 Histopathological examination

Liver from each animal was immediately dissected out and washed with normal saline in glass petridish and preserved in 10% formalin for fixation for histopathological studies in separately labeled specimen collection jars. The livers were excised quickly and fixed in 10% formalin and paraffin embedded. Sections of about 4- 6 μm were stained with haemotoxylin and eosin (H&E) for histopathological evaluation. In brief, 4-6 μm thick section of paraffin embedded rat liver was dewaxed with distilled water for 2 min. Then the section was stained with haemotoxylin for 5 min at room
temperature. After 15 min, the section was counterstained with eosin for 2 min, dehydrated with alcohol, washed with xylene and blocked by eosin. Hemotoxylin and eosin stained studies were observed under microscope. The sections were observed and desired areas were photographed in photomicroscope. The sections were viewed under 40x or 100 x magnifications \[^{134}\].

### 3.8 Demonstration of hepatoprotective effect of DL-methionine:

After overnight fasting, the albino rats belonged to group V and VI were treated with DL-Methionine \[^{132}\] in the doses of 700 mg/kg and 1400 mg/kg body weight, p.o., (n=6 & n=6), respectively. The volume administered was maintained at 10 ml/kg.

After 24 hour of post-treatment with DL-Methionine in different groups as indicated in table no. 1; whole blood was collected in labeled collecting tubes from retro-orbital plexus of eye with the help of glass capillary tube, for estimation of haematobiochemical alterations in serum to record the observations of the value of liver function tests (LFTs) with various parameters as described below. Hence, the serum was used for the estimation of biochemical parameters. Serum was stored at \(-20^\circ\)C until analyzed and were assessed to determine the extent of liver injury at the end of 24 hours of exposure of the drug. Serum was separated immediately through centrifugation at 3000 r.p.m. for the determination of liver enzymes, alanine amino transferase, aspartate amino transferase, alkaline phosphatase, Gamma-Glutamyl Transpeptidase (GGTP) or Gamma-Glutamyl Transferase (GGT), & total bilirubin.

### 3.8.1 Histopathological examination

Liver from each animal was immediately dissected out and washed with normal saline in glass petridish and preserved in 10% formalin for fixation for histopathological studies in separately labeled specimen collection jars. The livers were excised quickly.
and fixed in 10% formalin and paraffin embedded. Sections of about 4-6 µm were stained with haemotoxylin and eosin (H&E) for histopathological evaluation. In brief, 4-6 µm thick section of paraffin embedded rat liver was dewaxed with distilled water for 2 min. Then the section was stained with haemotoxylin for 5 min at room temperature. After 15 min, the section was counterstained with eosin for 2 min, dehydrated with alcohol, washed with xylene and blocked by eosin. Hemotoxylin and eosin stained studies were observed under microscope. The sections were observed and desired areas were photographed in photomicroscope. The sections were viewed under 40x or 100 x magnifications. 

3.9 Demonstration of hepatoprotective effect of N-Acetylcysteine

After overnight fasting, the albino rats belonging to group VII was administered with N-Acetylcysteine orally in the dose of 450 mg/kg body weight, p.o. (n=6). The volume administered was maintained at 10 ml/kg. All the drugs and control vehicle were administered by per oral (p.o.) and the volume administered was maintained constant in all the albino rats at 10 ml/kg.

After 24 hour of post-treatment with N-Acetylcysteine; whole blood was collected in labeled collecting tubes from retro-orbital plexus of eye with the help of glass capillary tube, for estimation of haemato-biochemical parameters in serum. Serum was stored at −20°C until analyzed and were assessed to determine the extent of liver injury at the end of 24 hours of exposure of the drug. Serum was separated immediately through centrifugation at 3000 r.p.m. for the determination of liver enzymes, alanine amino transferase, aspartate amino transferase, alkaline phosphatase, Gamma-Glutamyl Transeptidase (GGTP) or Gamma-Glutamyl Transferase (GGT), & total bilirubin.
3.9.1 Histopathological examination

Liver from each animal was immediately dissected out and washed with normal saline in glass petridish and preserved in 10% formalin for fixation for histopathological studies in separately labeled specimen collection jars. The livers were excised quickly and fixed in 10% formalin and paraffin embedded. Sections of about 4-6 μm were stained with haemotoxylin and eosin (H&E) for histopathological evaluation. In brief, 4-6 μm thick section of paraffin embedded rat liver was dewaxed with distilled water for 2 min. Then the section was stained with haemotoxyline for 5 min at room temperature. After 15 min, the section was counterstained with eosin for 2 min, dehydrated with alcohol, washed with xylene and blocked by eosin. Hemotoxylin and eosin stained studies were observed under microscope. The sections were observed and desired areas were photographed in photomicroscope. The sections were viewed under 40x or 100 x magnifications [134].

3.10 Demonstration of hepatoprotective effects of DL-Methionine by concomitant administration of positive control group:

After overnight fasting, the albino rats belonging to group VIII, IX, XII and XIII were treated with DL-Methionine and Diclofenac sodium concomitantly. The volume administered was maintained at 10 ml/kg in all the albino rats. Following this, 24 hours later the blood samples were collected by glass capillary method from retro orbital plexus of eye and the serum was separated after centrifugation method at 3000 rpm and was preserved at −20° C temperature till further analysis. The serum samples were then analyzed for the estimation of the liver enzymes.
3.10.1 Histopathological examination:

Liver from each animal was immediately dissected out and washed with normal saline in glass petridish and preserved in 10% formalin for fixation for histopathological studies in separately labeled specimen collection jars. The livers were excised quickly and fixed in 10% formalin and paraffin embedded. Sections of about 4-6 μm were stained with haematoxylin and eosin (H&E) for histopathological evaluation. In brief, 4-6 μm thick section of paraffin embedded rat liver was dewaxed with distilled water for 2 min. Then the section was stained with haematoxylin for 5 min at room temperature. After 15 min, the section was counterstained with eosin for 2 min, dehydrated with alcohol, washed with xylene and blocked by eosin. Hemotoxylin and eosin stained studies were observed under microscope. The sections were observed and desired areas were photographed in photomicroscope. The sections were viewed under 40x or 100 x magnifications [134].

3.11 Demonstration of hepatoprotective effects of N-Acetylcysteine by concomitant administration of positive control group:

After overnight fasting, the albino rats belonging to group X and XI was treated with N-Acetylcysteine and Diclofenac sodium concomitantly. The volume administered was maintained at 10 ml/kg in all the albino rats. Following this, 24 hours later the blood samples were collected by glass capillary method from retro orbital plexus of eye and the serum was separated after centrifugation method at 3000 rpm and was preserved at −20° C temperature till further analysis. The serum samples were then analyzed for the estimation of the liver enzymes.
3.11.1 Histopathological examination:

Liver from each animal was immediately dissected out and washed with normal saline in glass petridish and preserved in 10% formalin for fixation for histopathological studies in separately labeled specimen collection jars. The livers were excised quickly and fixed in 10% formalin and paraffin embedded. Sections of about 4-6 μm were stained with haematoxylin and eosin (H&E) for histopathological evaluation. In brief, 4-6 μm thick section of paraffin embedded rat liver was dewaxed with distilled water for 2 min. Then the section was stained with haematoxylin for 5 min at room temperature. After 15 min, the section was counterstained with eosin for 2 min, dehydrated with alcohol, washed with xylene and blocked by eosin. Hemotoxylin and eosin stained studies were observed under microscope. The sections were observed and desired areas were photographed in photomicroscope. The sections were viewed under 40x or 100 x magnifications [134].

3.12 Clinical Evaluation of Liver Injury:

Method of assessment of drug-induced liver injury included the following parameters:

1) Determination of serum SGPT (Serum Glutamic-Pyruvic Transaminase) [135]
2) Determination of serum SGOT (Serum Glutamic-Oxaloacetic Aminotransferases) [135]
3) Determination of serum Alkaline phosphatase [136]
4) Determination of serum bilirubin – direct and indirect Bilirubin. [137]
5) Determination of total bilirubin [137]
6) Determination of serum Gamma-Glutamyl transpeptidase (GGTP) levels [138, 139]
7) Gross appearance of liver after each drug administration & liver removed after dissection.

8) Determination of liver morphology changes.

3.12.1 Biomarkers of hepatotoxicity:

The measurement of levels of substances that may be present in the blood helps in the initial detection of hepatotoxicity. Several enzymes that trigger important chemical reactions in the body are produced in the liver and are normally found within the cells of the liver. However, if the liver is damaged or injured, the liver enzymes spill into the blood, causing elevated liver enzyme levels. The levels of the liver enzymes like transaminases, alkaline phosphatase, \( \gamma \)-glutamyl transpeptidase, in the blood can be measured to know the normal functioning of liver. These enzymes help in detecting injury to hepatocytes.

1) Liver injury can be diagnosed by certain biochemical markers like Alanine Aminotransferase [ALT] or SGPT (Serum Glutamic-Pyruvic Transaminase); Aspartate Aminotransferase [AST] or SGOT (Serum Glutamic-Oxaloacetic Aminotransferases; Alkaline Phosphatase [ALP], Alkaline phosphatase, Bilirubin and GGT. Elevations in serum enzyme levels were taken as the relevant indicators of liver toxicity. Macroscopic and in particular histopathological observations and investigation of additional clinical biochemistry parameters allows confirmation of hepatotoxicity \[133\].
Estimation of Bio-Chemical Parameters:

**Transaminases**

It is a process in which an amino group is transfers from an amino acid to an alpha-keto acid. It is an important step in the metabolism of amino acids. The enzymes responsible for transamination are called transaminases (amino-transferases) \[^{[135]}\].

Two diagnostically useful transaminases are glutamate oxaloacetate transaminase or SGOT and glutamate pyruvate transaminase or SGPT.

**3.12.1 a Determination of Serum Glutamate Oxaloacetate Transaminase (SGOT):**

**Principle**

This reagent is based on International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) recommendations, without pyridoxal phosphate \[^{[135]}\]. The series of reactions involved in the assay system is as follows:

1. L-Aspartate + 2-oxoglutarate \( \text{SGOT/AST} \) Oxaloacetate + L-Glutamate
2. Oxaloacetate + NADH MD Malate + NAD\(^+\)
3. Sample pyruvate + NADH LDH L-lactate + NAD

**Methodology:** NADH without pyridoxal phosphate (P-5'-P)

1. SGOT / ASAT present in the sample catalyses the transfer of the amino group from L-aspartate to 2-oxoglutarate forming oxaloacetate and L-glutamate.
2. Oxaloacetate in the presence of NADH and Malate dehydrogenase (MDH) is reduced to L-malate. In this reaction NADH is oxidized to NAD. The reaction is
monitored by measuring the rate of decrease in absorbance at 340 nm due to the oxidation of NADH to NAD.

3. Addition of Lactate dehydrogenase (LDH) to the reagent is necessary to achieve rapid and complete reduction of endogenous pyruvate so that it does not interfere with the assay

Procedure:
1. 100 μl of serum was taken in a clean eppendorf tube.
2. 1000 μl of reagent – 1 (TRIS, L-Aspartate, Malate dehydrogenase (MDH) and Lactate dehydrogenase (LDH) was added to the tube.
3. The tube was mixed well and incubated for 5 min at 37°C
4. 250 μl of reagent – 2 (2-Oxoglutarate and NADH) was added, mixed and incubated for 1 min at 37°C.
5. After 1 min, decrease in absorbance was read every minute.
6. Activity of the enzyme was calculated by using the following formula

ASAT activity (U/I) = _A/min x factor.

3.12.1b Determination of Serum Glutamic-Pyruvic Transaminase (SGPT):

Principle

This ALT/GPT reagent is based on the recommendations of the IFCC without pyridoxal phosphate \[^{135}\]. The series of reactions involved in the assay system is as follows:

L-Alanine + 2-oxoglutarate  \[\text{ALT/GPT}\]  Pyruvate + L-Glutamate

Pyruvate + NADH  \[\text{LDH}\]  L-Lactate + NAD^+

Sample pyruvate + NADH  \[\text{LDH}\]  L-Lactate + NAD
1. The amino group is enzymatically transferred by SGPT / ALAT present in the sample from alanine to the carbon atom of 2-oxoglutarate yielding pyruvate and L-glutamate.

2. Pyruvate is reduced to lactate by LDH present in the reagent with the simultaneous oxidation of NADH to NAD. The reaction is monitored by measuring the rate of decrease in absorbance at 340 nm due the oxidation of NADH.

3. Endogenous sample pyruvate is rapidly and completely reduced by LDH during initial incubation period to avoid interference during the assay.

Methodology: NADH without pyridoxal phosphate (P-5'-P)

Procedure:

1. 100 μl of serum was taken in a clean eppendorf tube.

2. 1000 μl of reagent – 1 (TRIS, L-Alanine and Lactate dehydrogenase (LDH) was added to the tube.

3. The tube was mixed well and incubated for 5 min at 37°C.

4. 250 μl of reagent – 2 (2-Oxoglutarate and NADH) was added, mixed and incubated for 1 min at 37°C.

5. After 1 min, decrease in absorbance was read every minute for 3 min at 334 nm, 340 nm and 365 nm.

6. Activity of the enzyme was calculated by using the following formula

ALAT activity (U/I) = \_A/min x factor
3.12.1c Determination of serum Alkaline Phosphatase (ALP):

**PRINCIPLE**

The method according to IFCC recommendation. This method utilises 4-nitrophenyl phosphate as the substrate. Under optimised conditions ALP present in the sample catalyses the following reaction \[^{[136]}\].

\[
\text{AMP + 4-NPP + H}_2\text{O} \xrightarrow{\text{ALP}} \text{4-nitrophenol + phosphate} \\
\text{Mg}^{2+}/\text{Alkaline pH}
\]

At the pH of the reaction, 4-nitrophenol has an intense yellow colour. The reagent also contains a metal ion buffer system to ensure that optimal concentrations of Zinc and Magnesium are maintained. The metal ion buffer can also chelate other potentially inhibitory ions which may be present. The reaction is monitored by measuring the rate of increase in absorbance at 405 or 415 nm which is proportional to the activity of ALP in the serum.

3.12.1d Determination of serum Total Bilirubin levels (Serum TBL):

**Principle**

Modified method of Pearlman & Lee \[^{[137]}\] in which a surfactant is used as a solubilizer. Bilirubin glucuronate reacts directly with sulphodiazonium salt and forms coloured derivative azobilirubin. The colour intensity of formed azobilirubin measured at 540 - 550 nm is proportional to direct bilirubin concentration in the sample. Total Bilirubin = Indirect Bilirubin + Direct Bilirubin.

3.12.1e Determination of serum Gamma glutamyltransferase (Serum GGT):

**Principle**

Kinetic colorimetric method according to Persijn & Van Der Silk \[^{[138]}\]. Standardized against recommended IFCC method. GGT present in the sample catalyzes the transfer
of the glutamyl group from the substrate $\gamma$-glutamyl-3-carboxy-4-nitroanilide to glycylglycine forming glutamyl glycylglycine and 5-amino-2-nitrobenzoate.

$$L-\gamma$-glutamyl-3-carboxy-4-nitroanilide + glycylglycine \xrightarrow{GGT} L-\gamma$-glutamylglycylglycine + 5-amino-2nitrobenzoate$$

The rate of formation of 5-amino-2-nitrobenzoate is proportional to the activity of GGT present in the sample and can be measured kinetically at (400-420) nm.
3.13 Statistical analysis:

All the observed data were collected and entered in the Microsoft excel sheet. Values to be compared were analyzed statistically. All results were expressed as Mean ± SEM. All calculations were performed using statistical software SPSS version 21.0 computer-based. Results were compared and analyzed by using repeated measures Analysis of Variance (ANOVA) and post hoc and values were considered to be significant when P values were less than or equal to 0.05 ($p \leq 0.05$).