TO INVESTIGATE EFFECT OF SOME HEPATOPROTECTIVE AGENTS ON DRUG-INDUCED HEPATOTOXICITY IN ALBINO RATS

A Thesis submited to

SUMANDEEP VIDYAPEETH
AN INSTITUTION DEEMED TO BE UNIVERSITY
(Declared as Deemed to be University U/S 3 of UGC Act 1956)

In Partial Fulfillment of the Requirement for the Award of the Degree of

Doctor of Philosophy (Ph.D.)

In

Medical Pharmacology

(Faculty of Medicine)

By

Mrs. Ervilla Dass

(Registration No: Ph.D. 475A 2010)

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Febuary, 2018

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I declare that the thesis titled, **"TO INVESTIGATE EFFECT OF SOME HEPATOPROTECTIVE AGENTS ON DRUG-INDUCED HEPATOTOXICITY IN ALBINO RATS"** submitted for the degree **Doctor of Philosophy (Ph.D.)** in the subject **Pharmacology** by me is the record of original research work carried out during the period from **January-2010 to February-2018**, under the guidance and supervision of guide **Dr. (Mrs.) B. M. Sattigeri.**

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DECLARATION CERTIFICATE

I **Ervilla Dass**, declare that the thesis and titled **"TO INVESTIGATE EFFECT OF SOME HEPATOPROTECTIVE AGENTS ON DRUG-INDUCED HEPATOTOXICITY IN ALBINO RATS"** submitted by me for the degree Doctor of Philosophy (Ph.D.) in subject Pharmacology is the original research work carried out by me during the period from **January-2010 to February-2018,** under the guidance of **Dr. (Mrs.) B. M. Sattigeri** and has not formed the basis for the award of any degree, diploma associate ship, fellowship, titles in this or any other univeristy or other similar institution of higher learning.

Date: Signature of the Candidate

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INSTITUTIONAL ANIMAL ETHICS COMMITTEE

Ref. No. ...IAEC/ S.B.K.S.M.I.R.K.M./09-10 Date 18th October, 2010

To
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Department of Pharmacology,
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S.B.K.S. ... DEAN
Outward No. 7626
Date 18/10/10
Sign

We have received the application for animal experiment for the project entitled:
"To investigate effect of some hepatoprotective agents on drug-induced hepatotoxicity in albino rats" at the Institutional Animal Ethics Committee (IAEC) meeting held on **25th April, 2009** in the office of the Dean, S.B.K.S. Med. Inst. & Res. Centre, Piparia. The above mention protocol was examined and discussed. After considering the objective, relevant bibliography and experimental procedure, the committee members approved the above mentioned study to carry out under the supervision of your guide.
Further, in the IAEC meeting held on 26th March, 2010 progress report of the ongoing research proposal was presented and permission for use of 81 number of Albino Rats per year was unanimously accepted & approved. Further, in the IAEC meeting held on 21st August, 2010 the progress of ongoing research was discussed.

The member who attended this meeting held on **25th April, 2009** at which your proposal was discussed and approved is listed below.
The members of IAEC:-

1. Dr. G. V. Shah – Dean, Chairman - IAEC
2. Dr. Y. K. Agrawal – Nominee of CPCSEA
3. Dr. Rashmiben Vyas – Nonscientific socially aware member
4. Dr. H. A. Desai – Veterinary Officer
5. Dr. D. C. Sharma – Prof. and Head, Pharmacology
6. Dr. S. V. Desai – Prof. of Pharmacology
7. Dr. S. P. Pandya – Prof of Pathology
8. Ms. Ervilla Dass – Asst. Prof., Pharmacology, Member secretary - IAEC

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INSTITUTIONAL ANIMAL ETHICS COMMITTEE (IAEC)

PROJECT COMPLETION CERTIFICATE

Date: 10-10-2015

This is to certify that the Ph. D. research project of Mrs. Ervilla Dass entitled "To investigate effect of some hepatoprotective agents on drug-induced hepatotoxicity in albino rats" is completed under the guidance of Dr. Bhagya M. Sattigeri Professor & Head, Department of Pharmacology, S.B.K.S. Medical Institute and Research Centre, Sumandeep Vidyapeeth.

It was reviewed, accepted and approved by the committee in the IAEC meeting held on 10th October, 2015; on Saturday at 10:00 a.m. in the College Council Room, S.B.K.S. Medical Institute and Research Centre, Sumandeep Vidyapeeth Piparia.

8/4/16 648

DR. G. V. SHAH
DEAN
IAEC-Chairman

ACKNOWLEDGEMENT

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Last, but not least, I would like to dedicate this thesis to my family for their unceasing prayers; my parents, Mrs. & Mr. John David Parmar & Mrs. & Mr. Elliot Jacob Dass. Most important my husband Mr. Jagin Parmar and daughter Karol, for their love, patience, support and sacrifice, without them study would never have been possible. Their constant love and affection have always been a source of strength to me. This last word of acknowledgment I have saved for my dear husband, who has been with me all these years and has made them the best years of my life.

Ervilla Dass

ABSTRACT

Introduction: Drug induced hepatotoxicity is a potential adverse effect, contributing to the health burden, with several mechanisms involved in causing liver injury. Many drugs and ingested substances cause the problem, with few drugs available for the treatment. Hence, we aimed to evaluate the hepatoprotective effect of certain hepatoprotective agents.

Material and Methods: Diclofenac (72, 96 & 240 mg/kg) was administered orally to evaluate its per se effect. Further, DL-Methionine (700 & 1400 mg/kg) and N-Acetylcysteine (450 mg/kg), were also evaluated for per se effect, followed by evaluation of their hepatoprotective effect against the Diclofenac-induced hepatotoxicity (96 & 240 mg/Kg, single oral dose) in the albino rats.

Observations and Results: Diclofenac (96 & 240 mg/kg, single oral dose) per se, was found to be hepatotoxic, while DL-Methionine (700 &1400 mg/kg), and N-Acetylcysteine (450 mg/kg) though altered the liver enzymes levels it was not significant, hence they were found not to be hepatotoxic.

Both DL-Methionine and N-Acetylcysteine in above doses significantly protected the animals against the Diclofenac-induced hepatotoxicity. However, no statistical difference was found between the hepatoprotective effect of DL-Methionine and N-Acetylcysteine.

Conclusion: Both DL-Methionine and N-Acetylcysteine have been hepatoprotective against the Diclofenac-induced Hepatotoxicity

*Keywords:*Diclofenac, DL-Methionine, N-Acetylcysteine, Hepatoprotective agents

INDEX

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOS

GLOSSARY OF ABBREVIATIONS

%	-	percent
°C	-	Degree Celsius
4′-OHdic	-	4′-hydroxydiclofenac
ADR	-	Adverse drug reaction
ALP	-	alkaline phosphatase
ALT	-	alanine aminotransferase (formerly known as serum glutamic-pyruvic transaminase, SGPT)
ANOVA	-	analysis of variance
ANF	-	Antinuclear Factor
APAP	-	Paracetamol (Paracetamol)
AST	-	aspartate aminotransferase (formerly known as serum glutamic-oxaloacetic aminotransferases, SGOT)
ATP	-	Adenosine triphosphate
ATPase	-	Adenosine 5'-triphosphatase
BSEP	-	Bile salt efflux pump
CAM	-	Complementary and Alternative Medicine
CB	-	conjugated (direct) bilirubin
COX	-	cyclooxygenase
CPCSEA	-	Committee for the Purpose of Control and Supervision of Experiments on Animals
CYP450	-	cytochrome P450 mixed function oxidase
D-BIL	-	Direct bilirubin
DCLF	-	diclofenac
DDW-J	-	Digestive Disease Week-Japan
DILI	-	drug-induced liver injury
DILIN	-	Drug-Induced Liver Injury Network
DISC	-	Death-inducing signaling complex
DLFC	-	Diclofenac
DLST	-	Drug Lymphocyte Stimulation Test
EMA	-	European Medicines Agency
ER	-	Endoplasmic reticulum

FA	-	Free fatty acids
FDA	-	Food And Drug Administration
GGT	-	γ-glutamyltransferase
GGTP	-	gamma-glutamyl transpeptidase
GNMT	-	glycine-N-methyltransferase -
GPx	-	Glutathione peroxidase
GSH	-	Reduced glutathione
GSSG	-	Oxidized glutathione
GST	-	Glutathione S-transferase
h	-	hour
H&E	-	Haematoxylin and Eosin
HIV	-	Human Immunodeficiency Virus
HSD	-	Herbal Remedies and Dietary Supplements
HCC	-	Hepatocellular Carcinoma
IAEC	-	Institutional Animal Ethics Committee
I-BIL	-	Indirect bilirubin
ICH	-	International Conference on Harmonisation
IF	-	Interferon
IFCC	-	International Federation of Clinical Chemistry and Laboratory Medicine
IL	-	Interleukin
IM	-	Intramuscular
INN	-	International Non Proprietary Names
INR	-	International Normalized Ratio
IU	-	International Units
IV	-	Intravenous
JNK	-	C-jun N-terminal kinase
kg	-	kilogram
L	-	litre
LDH	-	Lactate dehydrogenase
LPO	-	Lipid peroxidation
LRC	-	Learning Resource Centre
LST	-	Lymphocyte Stimulation Test

MDA	-	malondialdehyde
MDH	-	Malate dehydrogenase
MET	-	DL-Methionine
mg	-	milligram
MHC	-	Major histocompatibility complex
min	-	minute
MARS	-	Molecular Adsorbent Recirculation System
MPT	-	Mitochondrial permeability transition (opening of the "MPT pore" in the inner mitochondrial membrane)
NAC	-	N-Acetylcysteine
NAD^+	-	Oxidised form of Nicotinamide adenine dinucleotide
NADH	-	Reduced form of Nicotinamide adenine dinucleotide
NADPH	-	Nicotinamide Adenine dinucleotide phosphate-reduced
NAFLD	-	Non-alcoholic fatty liver disease
NAPQI	-	N-acetyl-p-benzo quinone imine
NASH	-	Non Alcoholic Steato Hepatitis
NIH	-	National Institutes of Health
No.	-	Number
NSAIDS	-	Non steroidal anti-inflammatory drugs
OLT	-	Organ Liver Transplant
OTC	-	Over The Counter
P-5'-P	-	pyridoxal phosphate
PPARα	-	Peroxisome proliferator-activated receptor alpha
PGs	-	Prostaglandins
RBCs	-	Red Blood Cells
ROS	-	Reactive oxygen species
rpm	-	revolutions per minute
RUCAM	-	Roussel Uclaf Causality Assessment Method
SAMe	-	S-adenosylmethionine
sec	-	second
SEM	-	Standard error of mean
SOD	-	Superoxide dismutase
SOD_2	-	Manganese superoxide dismutase
SVIAEC	-	Sumandeep Vidyapeeth Institutional Animal Ethics Committee

T-BIL	-	Total bilirubin
TBL	-	Total Bilirubin (summation of conjugated and non-conjugated serum bilirubin)
TNFα	-	Tumor necrosis factor alpha
U	-	Units
UGT UDP	-	glucuronosyltransferase
ULN	-	Upper Limit of the Normal reference range (or N)
US	-	United States
WHO	-	World health organisation
μ	-	micro
μmol	-	micromole

CHAPTER :1

INTRODUCTION

The safety and efficacy of the drugs used in the treatment of various clinical conditions in any individual remains complex and multifactorial and difficult to analyse or identify the suspected drug that causes the Adverse Drug Reaction (ADR).

1.1 Role of liver in drug-induced hepatotoxicity:

Liver being a principle organ for playing several vital roles in the body, is involved in several biochemical pathways, metabolism of nutritional factors, metabolising the administered drugs or any substance that is ingested, which could be either herbal or even natural chemicals. Thus, making it important to observe, for the drug-induced hepatotoxicity, at all phases of drug development that includes the pre-clinical toxicity studies, the different phases of clinical trial including the post-marketing surveillance.

The Drug Induced Liver Injury (DILI) is defined as the injury caused by exposure to a drug or non-infectious toxic agent and is associated with different levels of organ dysfunction [1]. Despite the advancement in research at molecular level, understanding and characterizing the mechanisms involved in causing the Drug induced Liver Injury, it is still difficult to diagnose and identify the suspected drug.

1.2 Types of drug induced liver injury:

The drug induced liver injury are mainly of two types**:(1)** D**ose-dependent**, which is also called as predictable, direct toxicity, reproducible and occurs after the consumption of the drug that exceeds a known toxic threshold level. In such cases, the **liver injury** that occurs is **proportional** to the **administered dose** [2], example

Paracetamol; **(2)** while the **Dose-independent** Drug induced Liver Injury is also called as unpredictable and idiosyncratic that occurs even at the therapeutic doses, and the **liver injury** caused is **not always proportional** to the **administered dose**, further, the time of damage, onset can also vary example Diclofenac, Sulindac, Trovafloxacin [3, 4].

1.3 An overview of drug induced liver injury:

Paracelsus stated that, "all substances (drugs) are poisons; there is none which is not a poison. The right dose differentiates a poison and a remedy." Any drug, therefore, despite of its trivial therapeutic action has a potential to harm. With the limitations on toxicity studies and clinical trials, in the process of a new drug development, the adverse drug effects that occur may not be in total are detected, before introduced into the market for the patient's use. Therefore, it becomes imperative to detect the infrequent yet significant adverse drug reactions that occur when the drug has entered the market. This can be achieved by the post-marketing surveillance.

The liver injury caused by the drug may vary with the extent of the damage, ranging from mild fatty liver to necrosis. Though uncommon and rare, it is contributing to the morbidity and mortality in the general population and remains as a potential complication for most of the prescribed drugs [5, 6]. Despite of the relative frequency, little information is available on the long-term outcome of drug induced liver injury. The reasons could include missed diagnosis, difficulty in establishing definite diagnosis, particularly in cases where the hepatotoxicity is reversible following the drug withdrawal with limited long term follow up (Dantrolene-induced chronic hepatitis or Flucloxacillin-induced cholestasis) [7, 8].

1.4 Incidence of drug induced liver injury:

Although, the incidence of drug induced liver injury is found to be low, the probability of it should always be considered in any case of the liver injury. According to the literature study, the incidence was between 1 in 10,000 and 1 in 100,000 which was found to be increased from the evidences of the recent study. The information from the recent registries show an annual incidence of 19.1 cases per 10,0000 inhabitants in Iceland, 13.9 cases per 100,000 inhabitants in France, with hospitalization of 5% and mortality 6% [4].

A prospective study conducted in US have shown that, 13% of the total cases were diagnosed as idiosyncratic hepatotoxicity; while 39% with acetaminophen-induced hepatotoxicity, however, it was interesting to know with the recent prevalence rates in south-east Asian registries, which revealed that 70% of the drug induced liver injury cases occurred due to Herbal And Dietary Supplements (HDS), which is surprisingly found to be increased in its prevalence; even through the Western registers, attributing to 16% of the total drug induced liver injury to be due to Herbal And Dietary Supplements [9]. The drug induced liver injury has been found as an important cause of hospital admissions, which are increased over the decades and is 45% in Spain [10].

In India, the drug induced liver injury contributes to 1.4% of the gastrointestinal admissions and 2.5% of hepatobiliary admissions, with gradual increase in the numbers over a period of years, of which 0.7% were found to be Idiosyncratic Drug induced Liver Injury (IDILI) [11, 12]. Although, there occurs geographical difference in the common drugs causing Drug induced Liver Injury, **worldwide antimicrobials** are considered the most common particularly in Europe, Amoxicillin and flucloxacillin are found to be the common drugs in the Europe, while in **India, Antituberculosis** drugs

are contributing more to the drug induced liver injury[11, 13]. As compared to the Western world, where Paracetamol or Acetaminophen was found to be the leading cause of Acute Liver Failure (ALF), followed by the antimicrobials. In India, both in adults and children, the antituberculosis drugs have been the leading cause of for drug induced liver injury, followed by the Non-Steroidal Anti Inflammatory Drugs (NSAIDs) 10% [14]. The incidence of liver injury caused by the Non-Steroidal Anti Inflammatory Drugs is ranging from 1 – 9 cases per 100,000 persons exposed, indicating an increased risk of these preparations which remains as a common drug used in the treatment of the most painful conditions. Diclofenac sodium, widely used among the Non-Steroidal Anti Inflammatory Drugs, across the world is known for its hepatotoxicity, where more than 60 cases were reported by Bank and co-workers in 1995 [15], indicating that small number of hospitalisation 0.023% is the strongest evidence for it to bear hepatotoxic effect.

1.5 Mechanisms of drug induced liver injury:

The exact mechanisms of the drug induced liver injury remains unclear and depends on the hepatotoxicity that could be either predictable (Paracetamol) or unpredictable (Diclofenac, Sulindac, and Flucloxacillin). The mechanism involved, in causing hepatic injury-induced hypersensitivity and metabolic aberration, in case of **predictable hepatotoxicity**, massive hepatocellular **necrosis**, when the Paracetamol is consumed in large doses. It is known to release a toxic **metabolite N-acetyl-p-benzoquinone imine** (NAPQI), which depletes the hepatoprotective glutathione, which in turns results in mitochondrial dysfunction, oxidative stress, that culminates into cellular damage, causing necrosis and death [16], while in case of **idiosyncratic**; the **inflammatory stress** hypothesis is considered, which results to conjugate with the drug

metabolite, that has a potential to precipitate Drug induced Liver Injury, with an evidence of important role of the innate and adaptive immune system through; involved in the pathogenesis of Drug induced Liver Injury [17].

1.6 Risk factors of drug induced liver injury:

With a wide range of drugs, including Antimicrobials, NSAIDs, Antiepileptic, Antipsychiatric drugs etc., causing the drug induced liver injury, several factors are known to influence the drug induced liver injury, and are hence considered as the **risk factors** these includes; the age, gender, alcohol, concomitant use of drugs, nutrition, HIV, genetic factors, the dose and the body mass of the individual.

1.7 Evaluation of drug induced liver injury:

Apart from the clinical evaluation, the diagnosis includes the causality assessment to identify the suspected drug; evaluation of the biochemical parameters which indicate the liver functioning status, and further; the histopathological studies to reveal and confirm the clinical diagnosis. Liver imaging can also remain the infiltrative hepatic diseases and fatty live diseases. The histopathological information could be drug-specific and would indicate the severity and latency of the biochemical pattern.

Although, 90% of recoveries have been registered on discontinuation of the drug, some may progress with the outcome as chronic liver disease [18]. The prognosis has been poor in women, elderly, individuals with pre-existing liver disease; those habituated to alcohol and individuals with genetic defect. Hence, it is always important to monitor the liver enzymes which are indicative of the hepatotoxicity.

1.8 Treatment of drug induced liver injury:

The treatment for Drug induced Liver Injury mainly consists of discontinuation of the involved drug, followed by treatment with specific drugs. The specific drugs for the treatment of Drug induced Liver Injury are very scarce. However, N-Acetylcysteine (NAC) remains as a specific antidote for Paracetamol or Acetaminophen-induced toxicity, where it is known to benefit by replenishing the Glutathione stores. Similarly, as symptomatic treatment, drugs like Corticosteroids, Antihistamines, Cholestyramine, L-Carnitine, Folic acid, Methionine and Ursodeoxycholic acid have been used in the treatment of Drug induced Liver Injury [19, 20,].

1.9 Aim and Objectives of the Study:

1.9.1 AIM:

The present research was conducted to explore the hepatoprotective action of DL-Methionine and N-Acetylcysteine on the albino rats on dose-related hepatotoxicity of the hepatotoxic drug Diclofenac sodium.

1.9.2 OBJECTIVES:

1) To evaluate dose-dependent hepatic injury by orally administered Diclofenac sodium.

2) To evaluate the hepatic changes due to the dose dependent hepatic injury caused by Diclofenac sodium.

3) To demonstrate the hepatoprotective effect of DL-Methionine against the hepatotoxic drug Diclofenac sodium by oral route of administration in small animals.

4) To demonstrate the hepatoprotective effect of N-Acetylcysteine against the hepatotoxic drug Diclofenac sodium by oral route of administration in small animals.

5) To compare the hepatoprotective effect of DL-Methionine with N-Acetylcysteine.

6) To demonstrate the hepatoprotective effect of N-Acetylcysteine on hepatotoxic drug other than Paracetamol.

CHAPTER: 2

REVIEW OF LITERATURE

2.1 Introduction of Liver:

Liver is an essential organ of the body and is involved in the vital functions, to maintain the internal homeostasis. The main function of which is synthesis and secretion of bile into the gallbladder and second part of the duodenum, where it is playing an important role of metabolizing all the ingested substances, which may be in the form of food, nutrients, drugs or chemicals. Liver synthesizes many essential proteins, stores the nutrients that are released into circulation at the time of starvation and detoxify the ingested harmful substances in the process of metabolism, hence, considered as a vital organ of the body.

Liver is involved in the metabolism of the major nutrients such as carbohydrate, fats, proteins and both the fat soluble and water soluble vitamins. It plays a pivot role in the metabolism of urea, iron, and alcohol. Further, it is involved in the synthesis of several proteins including the clotting factors, those mediated in the process of inflammation, the hormone binding proteins, lipids, carbohydrates, vitamins and bile salts. It is considered as a storage site for glucose, proteins, fats and vitamins which are released for utilization during the scarcity, while they are stored when they are in excess. It is considered as a major organ involved in detoxifying the chemicals and the toxins released from the infecting organisms which are neutralized in the liver. It is known to degrade/metabolize the drugs, enzymes, hormones, cytokines and various other chemicals.

The kupffer cells of liver are considered as the part of reticuloendothelial system, which forms the nonspecific defences of the body that helps in cell phagocytosis and killing of the microorganisms. It thus, contributes in maintenance of body immunity.

It helps in converting vitamin D3 to 25-hydroxyvitamin D3. It is also a major site for conversion of T4 to T3 and is known to secrete insulin-like growth factor (Somatomedin) that mediates important functions of growth factors. It is also known to play an important role in degradation of many hormones such as insulin, glucagon, growth hormone, gastrointestinal hormones, etc **[25].**

It lies in the upper quadrant of abdomen, which consists of two main lobes delineated by connective tissue. Each lobule has a central vein, as a centre from which, the plates of liver cell radiate like the spokes of bicycle of the wheel, to the periphery of the lobule and the liver cells have the capacity to regenerate; although the process of renewal is slow. The loss of hepatic tissue by either surgical excision; injury or the effect of toxins; all trigger the mechanism by which the hepatocytes begin to divide and grow, till the normal size is attained (regenerate).

Each lobule consists of the sinusoids that carry blood from the portal vein and hepatic artery of the portal tract to drain into the central vein. The walls of these sinusoids are generally made up of endothelial cells, but at some places they are the macrophages which are called as kupffer cells. There is a space between the sinusoid and the hepatocytes called as **"Space of Disse"** (perisinusoidal space), which serves as a root for the removal of certain substances, from blood and to discharge certain products into the blood.

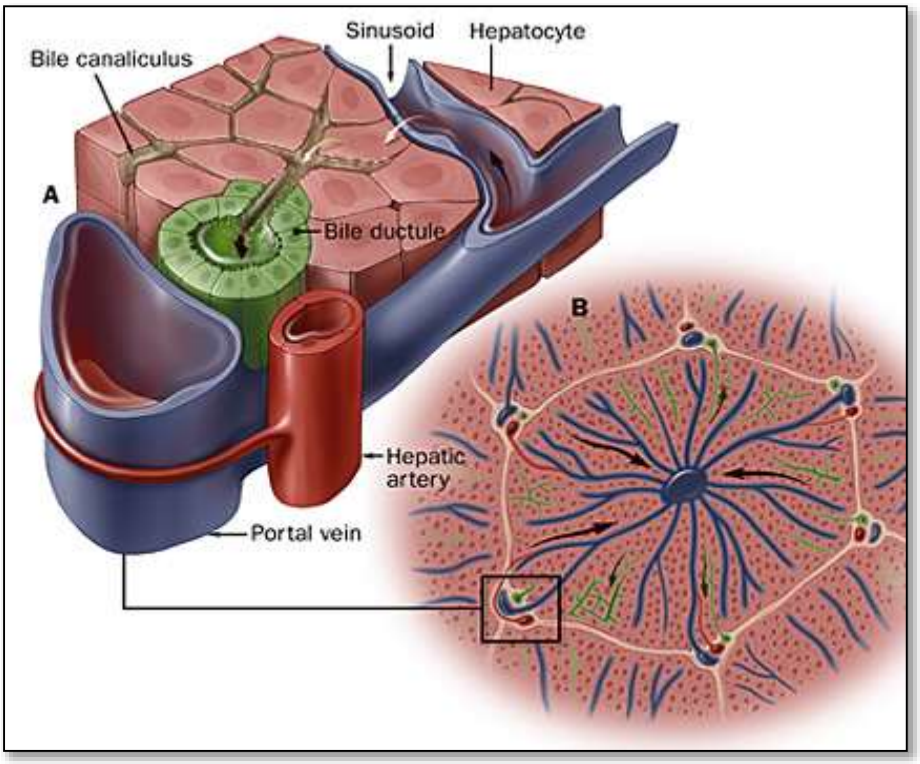


Figure 1: Cross section of normal liver and liver lobule

Along with the liver, lies the gall bladder, into which the hepatic duct transports the bile produced by the liver cells. It is in this organ that the administered drugs get structurally altered, and may either result in releasing the metabolites which may be either, active, inactive, or toxic in nature. Hence, it is considered as a vulnerable target for injury from the administered drugs, chemicals and may thus be responsible for the altered liver function [25].

Liver is involved in almost all biochemical pathways to growth, immunity, nutrition and reproduction [26]. With the vital role of metabolism liver is involved in removing the toxic waste from the body, which is mainly done by the smooth endoplasmic reticulum of the liver, which is a principle "**metabolic clearing house**" for both **endogenous chemicals** like cholesterol, steroid hormones, fatty acids and proteins, and **exogenous substances** such as drugs and alcohols, thus playing a central

role of transformation and clearance of the exposed chemicals that may lead to toxic injury to liver. The functional reserve of the liver is often known to mask the clinical impact of early liver damage that occurs, since, it is a center for metabolic disposition of almost all the administered drugs, hence, the drug induced hepatic injury is a potential problem that eventually follows.

Generally the process of metabolism occurs in two phases:

i. **Phase I** includes oxidation, reduction and hydrolysis and

ii. **Phase II** which involves the reactions catalyzed by transferase enzymes in which the CYP450 system plays a major role which explain possibility of hepatotoxicity.

Hence, it is crucial to maintain a healthy liver for overall health and wellbeing of an individual **[26, 27].**

2.2 Drug-Induced Hepatotoxicity:

Hepatotoxicity refers to the liver dysfunction, liver damage or to the chemical-driven liver damage associated with an overload of medicinal agents or xenobiotic. The chemical substances which cause liver injury are called hepatotoxins or hepatotoxicants. Hepatotoxicants are exogenous compounds of clinical relevance and may include overdoses of certain medicinal drugs, industrial chemicals, natural chemicals (microcystins), herbal remedies and dietary supplements **[28, 29].**

The hepatotoxicity caused by the administered drugs may sometimes occur even when they are used within therapeutic ranges, while minorities of the drugs have predictable dose-dependent liver injury. The toxicity may be due to primary compound itself or may result from a reactive metabolite or from an immunologically-mediated response ultimately affecting the hepatocytes, the biliary epithelial cells and/or liver

vasculature which may be evident on histopathological examinations. The hepatotoxic effect depends on the concentration of the hepatotoxicants which could include either the parent toxic substance or the toxic metabolite, or the differential expression of the enzymes or the concentration gradient of cofactors in blood [30, 3].

2.2.1 Herbal and Dietary Supplement (HDS) induced Liver toxicity:

Apart from the medicines, the current trend of using therapeutic agents is observed to be with the herbal preparations, which amounts to 80% of the world's population who prefer the herbal preparations for therapeutic purposes, as shown by the World Health Organization (WHO) estimate in 1998 [31].

This has been a traditional practice in some parts of the world, mainly in the east or Africa. The herbal preparations may have no pharmacological properties even if recognized as medicines, therefore, although they could be beneficial, they would also have toxic and adverse effects. Similar to other medicines, the herbal preparations are likely to cause the liver toxicity which can be either direct or idiosyncratic. Therefore, with the use of herbal medicines, one has to pay attention for the herb-herb, herb-drug interactions and also the toxic effects of co-administered preparations. The Herbal and dietary supplements includes the Vitamins, minerals, amino acids, proteins, enzymes, gland or organic tissues, chemically synthesized molecules and anabolic steroids. Since, there is no regulation for the use of such preparations, pertaining to their composition, dose, and quality there is no ensured safety and effectiveness thus leading to toxicity which is not rare.

It has been observed through the Drug-Induced Liver Injury Network (DILIN) registry, that the herbal and dietary supplements are responsible for the 16% of the liver

injury, while 76% was attributed to the mixed-active principles or the single active principle that was responsible [32,33, 34].

2.2.2 Epidemiology:

Most of the data pertaining to the hepatotoxicity induced by the drug is retrieved from the retrospective studies from database of the Pharmacovigilance center or the Pharmaceutical companies. On account of which many events remain ignored and all the information available is to be only the tip of an iceberg [35]. They are found to be commonly seen in the adults than in children, with females being at higher risk than males with the genetic factors also contributing to it. The risk of hepatotoxicity for majority of the drugs ranges from one in 10,000 to one in 100,000 patients [36]. For some drugs such as Antihistamines, Penicillins, and Minocycline the hepatotoxicity is exceedingly low.

However, such retrospective studies, yield information which exhibit the limitation pertaining to the information, with more than 900 drugs, toxins, and herbs that have been reported to cause the liver injury. The drugs account 20-40% of all instances of fulminant hepatic failure, while the drug induced liver injury may account for 10% of all cases of acute hepatitis, 5% of all hospital admissions and 50% of all liver failures.

More than 70% of the case of idiosyncratic reactions results in liver transplantation or death. However, the Drug Induced Liver Injury which is estimated to be 14% -19% per 100,000 people is probably underestimated which may be due to uncertain diagnosis or underreporting [37]. The drug induced liver injury occurring due to prescription and non-prescription drugs medicines, herbal or dietary substances show a variation in regional distribution of the offending drugs. About, 5% - 33% of the

patients on treatment for tuberculosis (TB) are reported to complicate with Drug Induced Liver Injury.

The drug induced liver injury may be associated with considerable morbidity and mortality, thus contributing to the resultant hospitalization and associated costs [38, 39]. As an adverse drug reaction, the drug induced liver injury attributes to 18% of the deaths in hospitalized patients worldwide [40, 41, 42]. Duh et al (1999) [43], have shown that 41 cases per 40.6 cases per 100,000 persons had acute liver failure attributed to the drugs in general population. While it was indicated by Meier et al (2005) [44] in the International Classification of Disease (9^{th} version) (ICD-9), that 57/4209 (1.4%) of in-patients had developed hepatotoxicity (ICD-10), and Hussaini et al (2007) [13] showed that out of 1, 636, 792 persons who were followed for 5 years; 2.4 per 100,000 per year developed non-fatal Drug Induced Liver Injury.

Despite of low incidence, the probability of Drug Induced Liver Injury should always be considered when there is an acute liver injury. Several registries in both the Western and Asian countries have provided the useful information on the etiopathology, presentation, diagnosis and its management. In the global scenario, of the drug induced liver injury; the incidence was 19.1 cases per 100,000 inhabitants in Iceland, and of 13.9 cases per 100,000 inhabitants in France, with hospitalization of 12% and mortality of 6%.

In a prospective US based study, of 308 acute liver failure cases; idiosyncratic hepatotoxicity was confirmed in a total of 40 cases (13%), while Paracetamol overdose accounted for 120 cases (39%). In another study conducted at US, it was found that 46% of the drug induced liver injury that occurred involved antibiotics, the results of which were found to be similar from Spanish and Icelandic registries [44, 33, 45]. However,

in an Italian case-control study, the annual incidence was 4.1 cases per 100,000 inhabitants and about half of the patients received Non Steroidal Anti Inflammatory Drugs, which are similar to the Swedish and English studies [112], while a database of general practitioners from U.K. revealed that, on assessment of large population between 1994 and 1999, the rise of Drug Induced Liver Injury was more than 100 of 100,000 cases for INH and Chlorpromazine, more than 10 cases of 100,000 for Amoxicillin-Clavulanic acid and Cimetidine, and fewer than 10 cases per 100,000 for other drugs [10]. Sgro C. et al (2002) [4], in a prospective study reported an incidence of 13.9 ± 2.4 per 100,000 individuals between 1997 and 2000 [4]; in which they detected 34 cases in a population of 81301; which was 16 times higher than the one that was spontaneously reported to the regulatory authorities and which proves the gross underreporting of the cases of Drug Induced Liver Injury [4, 46].

A single center study from **India** showed that 1.4% of the all the gastrointestinal admissions attributed to the drug induced liver injury, 2.5% of hepatobiliary admissions with the gradual increase in the number of cases over years. All these patients admitted with jaundice 0.7% were considered to be due to idiosyncratic drug-induced liver injury (IDILI) [11, 12].

Although, antimicrobials and the Paracetamol-induced injury are considered to be commonest in the Western part of the world, in India, the hepatotoxicity has been greatly attributed to the antitubercular drugs, followed by the Non Steroidal Anti Inflammatory Drugs, affecting mainly the adults and the children and contributing to 5.7% - 22%, of all cases of acute liver failure [47, 48]. Rathi C, et al, (2017) [49] in their focused study, on Drug Induced Liver Injury, at a tertiary hospital in **India**, have shown that of the overall mortality of 15% - 85%, 8 deaths were related to liver injury, which

was found to be higher when compared from the study of Iceland, where only one death was considered as a consequence of Drug Induced Liver Injury of the total 11 deaths that occurred. Of 70% of the death, that occurred in the study conducted by Rathi C et al, 2017 **[49],** were attributed to the antituberculosis drug induced liver injury. However, they have reported antimicrobial agents to be the commonest cause followed by the Non Steroidal Anti Inflammatory Drugs, which is found to be similar in several other parts of the world. However, Rathi C et al **[49]** have reported that the medium age for drug induced liver injury in India was 38 years, with equal gender ratio. Further, a prospective cohort study in a tertiary healthcare center, which evaluated 185 patients in time period between January 2000 to December 2016, found that mean average age was 53 years. Only 2% had previous chronic liver disease, while 57.8% showed hepatocellular pattern and 18.3% shows a cholestatic pattern; 23.2% with mixed pattern of liver injury. They have reported with the involvement of several classes of drugs to cause the liver injury which included the Antibiotics (23.4%), NSAIDs (35.5%), Immunosuppressant (10.9%), Statins (4.3%), Antiepileptic and Antipsychiatric drugs (7.6%) and others (9%). However, they have reported that in 25% of the cases two or more drugs were simultaneously involved (Licata et al, 2017) **[50]**.

It is however interesting to see that in south East Asian registries, the high prevalence for drug induced liver injury is related to the use of Herbal Medicines (70%), which seems to be totally different when compared with the Western Registries, while in recent years it is observed for an increase in herbal and dietary supplement induced hepatotoxicity (16%) **[9]**.

Therefore, the drug induced liver injury represents the leading cause of the drug withdrawal or prevention of the drug marketing across the globe.

2.3 Classification of Drug Induced Liver Injury:

2.3.1 According to causative agents:

The drug induced liver injury can be classified according to the **causative agents, as medications, herbs, health foods or dietary supplements, folk remedies, combined and others**. Further, the herbs can be sub-categorized as **herbal preparations, herbal medications, or medicinal herbs or plants**.

2.3.2 According to prescription:

They can also be classified as either **prescription medications or non-prescription medications** caused drug induced liver injury.

2.3.3 Folk remedies:

They can also be categorized as those caused by the **folk remedies, the traditional remedies** which do not fit into herbal medications and herbal preparations but cause the liver injury are categorized as folk remedies; while the preparations which are intended to supplement the diet and provide the nutrients in the form of **vitamins, minerals, fibers, fatty acids, amino acids, etc.,** which may be either deficient or may not be consumed in sufficient quantities, such supplement-induced liver injury, can be categorized as health foods or dietary supplements injury **[51].**

2.3.4 Based on Adverse Drug Reaction:

However, as an adverse drug reaction, the drug induced liver injury may be further divided into **idiosyncratic reactions** and **non-idiosyncratic** reactions (predictable) and they tend to be dose-related for e.g. hepatotoxicity due to Paracetamol overdose. However, the unpredictable reactions, occurring in less than 1% of the exposed, are generally considered to be independent of the dose administered.

2.3.4.i Idiosyncratic reactions:

The idiosyncratic reactions are further being classified as **allergic** and **non-allergic**. The allergic reactions involve the immune system which is identified by **Lymphocyte Stimulation Test**. However, it depends on the characteristic presentation with fever, rash, eosinophilia, presence of auto antibodies, and rapid recurrence of hepatotoxicity on re-exposure to the drug [52]. The allergic bases in the pathogenesis of idiosyncratic hepatotoxic reaction are supported by all these features. However, only some of which in a variable proportions may be present in the affected individuals. Therefore, the drug administered can cause either **allergic** (Allopurinol, Diclofenac, Dihydroxydiazine, Halothane, Methyldopa, Minocycline, Phenytoin, ACE inhibitors, Erythromycin) or **non-allergic** toxicity (Bromfenac, Troglitazone, Tolcapone, Neviparine, Pyrazinamide, Rifampicin, Terbinafine, Pyrazinamide, Rifampicin, Valproic Acid, Zafirlucast).

However, it is believed that the allergic hepatotoxicity is not related to the dose administered but this could not be the fact because, these reactions are rare, when the dose of any of the drug is less than 10 mg/kg and they occur more frequently at higher doses or due to frequent exposure (e.g. Halothane) or the immunological phenomenon points to the required of dose threshold [53].

The **non-allergic** idiosyncratic reactions may not present with the features of hypersensitivity but it is not possible to entirely exclude the allergic mechanism. The long latency period for these reactions to occur is an important feature of non allergic idiosyncratic hepatotoxic reactions.

Patients may show normal findings on investigating the Liver Function Tests (LFTs) for the period of six months, but then may suddenly develop the hepatotoxicity.

This sounds more dangerous particularly for the drugs, which are known to accumulate, for e.g. Amiodarone [54], which shows an accumulation related hepatic injury. Those reactions may be **dose-related** (Statins) [55] or may be **dose-unrelated** (Troglitazone) [56].

However, in case of non-allergic idiosyncratic reactions, rechallenge might not reproduce the injury, indicating that the factors present at the time of original injury are no longer present further, indicating development of adaptation.

2.4 Pathophysiology of Drug Induced Liver Injury:

The number of drugs associated with adverse reactions in the form of liver injury is extensive [57]. Although several steps or mechanisms are involved in causing hepatic injury and the process may involve either **direct injury** or the subsequent **activation of inflammatory pathways**, it also depends on the environmental factors, individual's genetic susceptibility.

The initial trigger is considered to be the administered drug, or the drug metabolite which are the resultants of **Phase I** drug metabolism and the polymorphic cytochrome P450 (CYP450) family of enzyme proteins. However, this does not eliminate the toxic compounds arising from the conjugative **Phase II** metabolism.

2.4.1 Apoptosis and necrosis:

The liver injury from the administered drug may be through several pathways such as the **cell-stress, mitochondrial inhibition** or **immune activation**; all of which ultimately lead to **Mitochondrial Permeability Transition** (MTP). The direct cell stress may be exerted through various mechanisms that include glutathione depletion or binding of metabolites to enzymes, lipids, nucleic acids, or other structures; while the mitochondrial inhibition occurs through either uncoupling or inhibition of

mitochondrial respiratory chain which results in the depletion of ATP and accumulation of Reactive Oxygen Species (ROS) **[58]**.

The Mitochondrial Permeability Transition (MPT) disrupts the mitochondrial membranes by increasing the permeability and proton influx and disturbing synthesis of ATP. The increased permeability of the outer mitochondrial membrane with release of cytochrome C and other pro-apoptotic proteins into the cell, which ultimately results in cell **apoptosis** or **necrosis** as indicated in **figure no.2.**

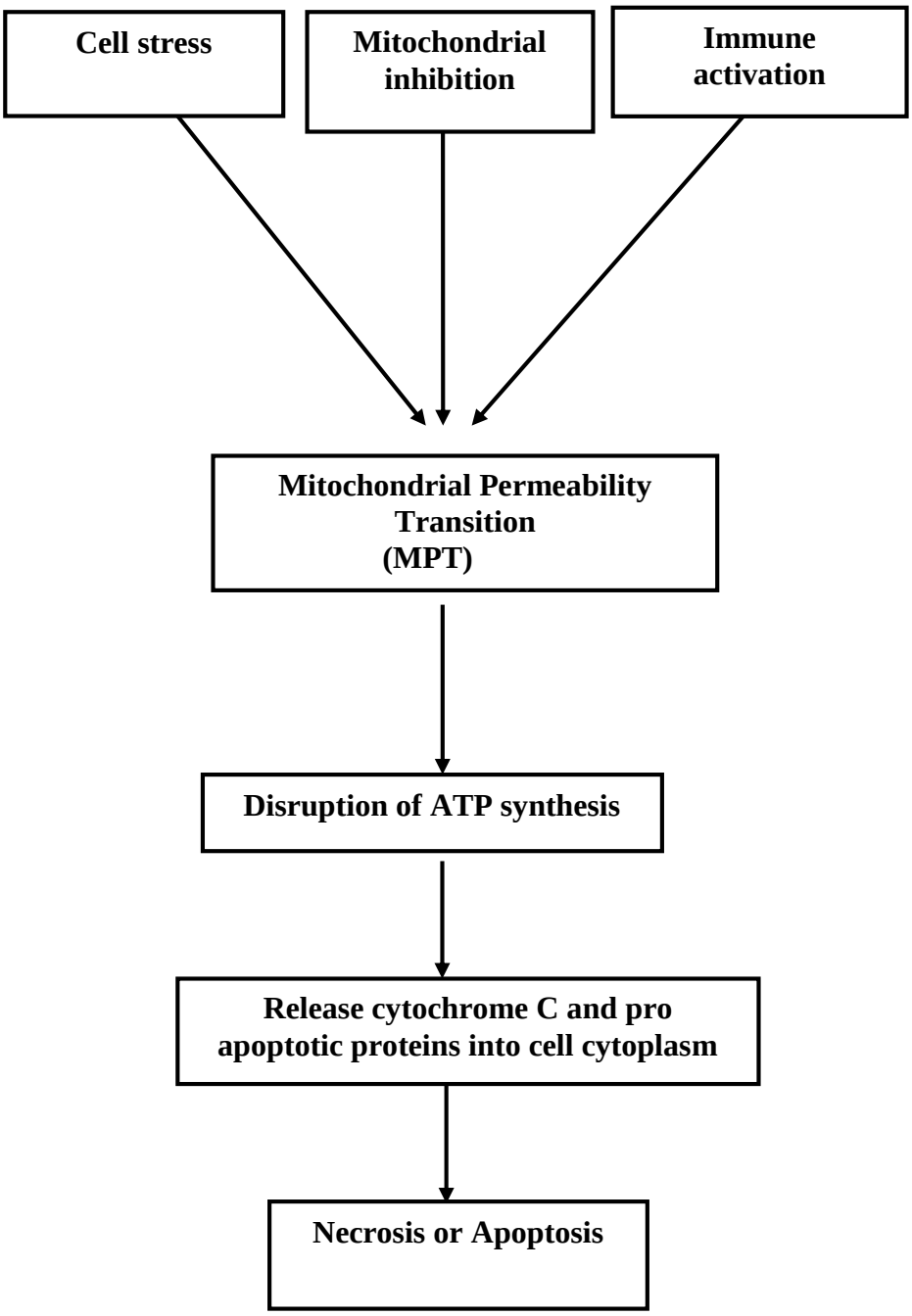


Figure 2: Shows the steps involved in hepatocytes injury

Apoptosis is an ATP-dependent, in presence of which the cytochrome C will bind a cytoplasmic scaffold protein and pro-caspase 9 to form an apoptosome that results in cytoplasmic and nuclear condensation and fragmentation. The process of apoptosis occurs without loss of plasma membrane integrity.

Necrosis is due to compromised mitochondrial function by **Mitochondrial Permeability Transition** and depletion of **ATP**, however, the result is severe disruption of cell processes, followed by bleb formation, actin oxidation, microfilament breakage, cellular swelling and eventually plasma membrane rupture [59].

Several other mechanisms involved in causing hepatotoxicity includes; role of intracellular antioxidant reduced Glutathione (GSH), direct effect of toxicants, formation of reactive metabolites, role of transporters and altered calcium homeostasis. The mechanisms involved may have either direct effect on organelles or indirect effect through the activation and inhibition of signaling kinases, transcription factors and gene-expression profiles, with the ultimate resultant effect leading to **cell death** caused by either cell shrinkage or nuclear disassembly (**apoptosis**) or swelling and lysis (**necrosis**).

2.4.2 Other mechanisms involved in hepatotoxicity:

Other mechanisms involved in hepatotoxicity includes; **zonal necrosis, hepatitis, cholestasis, steatosis, granuloma, vascular lesions, neoplasm and veno-occlusive diseases**.

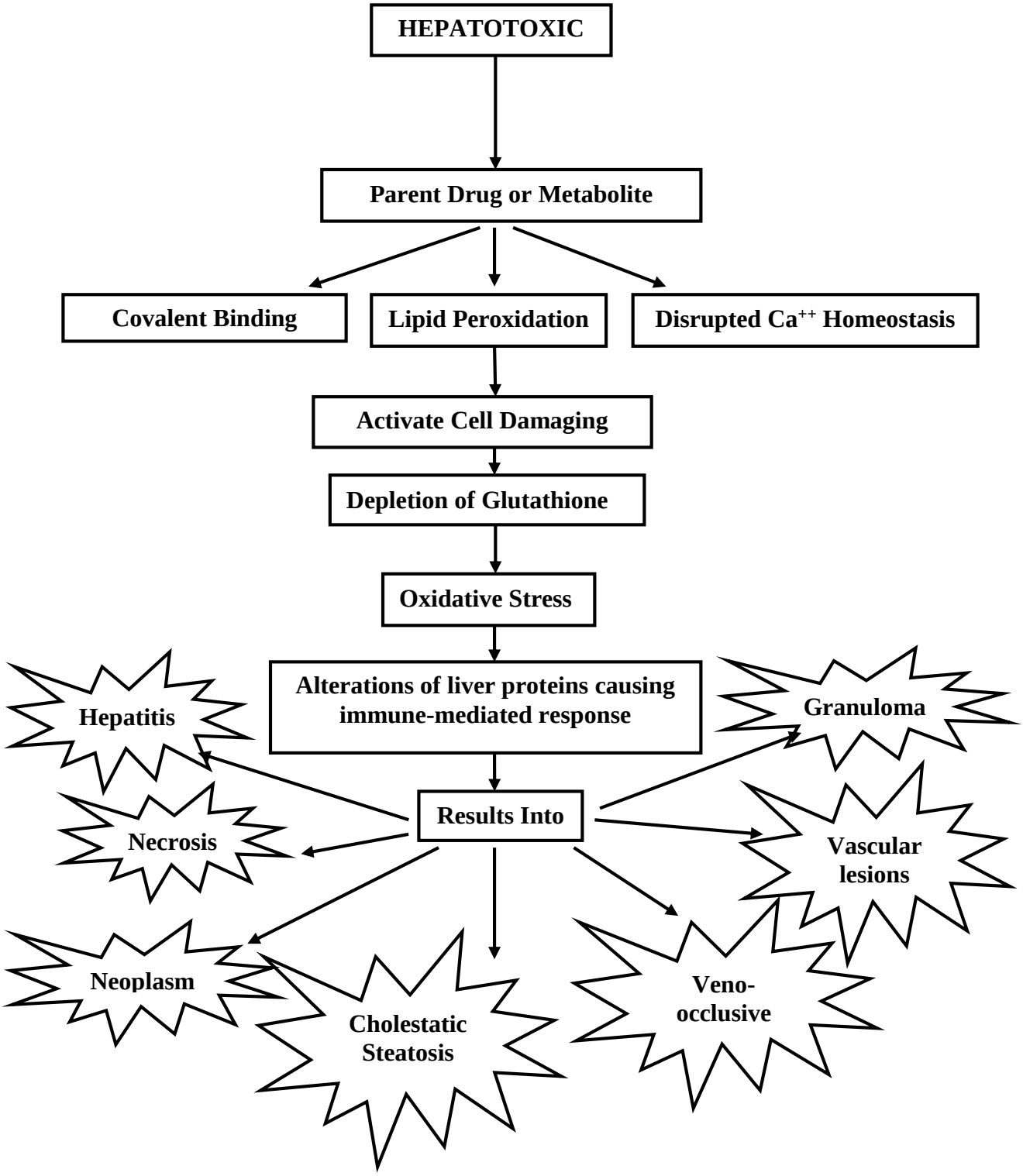


Figure 3: Mechanisms involved in causing hepatotoxicity

2.4.2.a Zonal necrosis: This type of injury generally occurs due to exogenous substances such as Paracetamol [60] and Carbon tetrachloride [61, 62], which is confined to a particular zone of the liver lobule and manifest with very high level of alanine aminotransferase (SGPT) along with severe disturbance of liver function leading to acute liver failure.

2.4.2.b Hepatitis: In this type of liver injury there occurs hepatocellular necrosis associated with infiltration of inflammatory cells. It may be of viral, focal and chronic. In viral hepatitis, the features are similar to that of acute viral hepatitis, which may be caused by Halothane, Isoniazid, Acetaminophen, Bromfenac, Nevirapine, Ritonavir, Troglitazone and Phenytoin [63, 64, 65]. The focal hepatitis accompanies with lymphocytic infiltration as caused by Aspirin, while the chronic hepatitis resembles the autoimmune hepatitis in clinical, serological and histological findings, which may be seen in the hepatotoxicity caused by Methyldopa, Diclofenac, Dantrolene, Minocycline and Nitrofurantoin.

2.4.2.c Cholestasis: In this type of liver injury there occurs impairment of the bile flow, with resultant itching and jaundice (as seen in case of Angiotensin-Converting Enzyme Inhibitors, Amoxicillin, Chlorpromazine, Erythromycins and Sulindac). It may be of **inflammatory** cholestasis (Allopurinol, Co-Amoxiclav, Carbamazepine); **bland** (steroids and androgens) or **ductal** (Chlorpromazine and Flucloxacillin) [66].

2.4.2.d Steatosis: In this type of liver injury there occurs triglyceride accumulation with resultant small droplet (**microvesicular** as seen in Aspirin, Ketoprofen, Tetracycline, Nucleoside Reverse Transcriptase Inhibitors and Valproic acid) or large droplet (**macrovesicular** fatty liver as seen in Paracetamol and Methotrexate). Drugs like Amiodarone, Chlorpheniramine may cause phospholipidosis, while Nucleoside

Reverse Transcriptase Inhibitors may be associated with a life threatening condition called lactic acidosis [67, 68].

2.4.2.e Granuloma: These are generally located in periportal or portal areas and show features of systemic vasculitis and hypersensitivity. Drugs like Allopurinol, Sulfonamides, Pyrazinamide, Phenytoin, Isoniazid, Penicillin and Quinidine are some of the hepatotoxic drugs which are found to cause such injury [30, 69].

2.4.2.f Vascular lesions: The liver injury damaging the vascular endothelium is generally caused by chemotherapeutic agents and anabolic steroids [70, 71].

2.4.2.g Neoplasm: On long term exposure of some hepatotoxicants and toxins like vinyl chloride, anabolic steroids, and arsenic may cause Hepatocellular Carcinoma (HCC), angiosarcoma and or liver adenomas [66].

2.4.2.h Veno-occlusive: The hepatic vein becomes occluded, thus blocking the blood supply to the liver. The occlusion is non-thrombotic but associated with congestion and potentially fatal necrosis of centrilobular hepatocytes. Drugs like Pyrrolizidine alkaloids, Busulfan and Cyclophosphamide have been associated with this type of hepatic injury [62, 70, 72].

2.4.3 The other mechanisms involved in causing hepatotoxicity include:

2.4.3.a Role of intracellular antioxidant reduced glutathione:

Major causes of the hepatotoxic reactions are the drug-induced intrahepatic cholestasis, which are often noticed during the process of the new drug development. It has been suggested that a covalent binding of Reactive Oxygen Species (ROS) as well as reactive intermediates, to be likely contributors of several harmful drug reactions [73].

It has also been suggested that the membrane lipid peroxidation, is directly related to the depletion of an intracellular antioxidant that is reduced glutathione (GSH), thus leading to altered functional integrity of hepatic structure and in case of severe damage it could be fatal to the hepatocytes. Thus, the concentration of intracellular glutathione is a key determinant of membrane integrity and extent of hepatic cell injury [74].

The lipid peroxy radicals lead to increased cell membrane permeability, decreased cell membrane fluidity, inactivation of membrane proteins and loss of polarity of mitochondrial membranes. The metal ions like iron and copper participate in redox cycling which lead to the formation of reactive oxygen free radical which can deplete glutathione, through oxidation or they oxidize the critical protein sulfhydryl groups involved in cellular enzymatic regulation to initiate lipid peroxidation.

Excess of ethanol, contributes to generation of free radicals, lipid peroxidation and glutathione depletion, all causing hepatotoxicity. Similarly, the halogenated hydrocarbon, hyperperoxides, acrylonitrile, cadmium, chloroacetamide are all known to exhibit the hepatotoxic effect due to lipid peroxidation [75, 76].

2.4.3.b Formation of reactive metabolites:

Many of the hepatotoxicants drugs like Carbon tetrachloride, Amodiaquine, Paracetamol, Halothane, Isoniazid, Allyl Alcohol and Bromobenzene are metabolically converted into reactive metabolites which are known to covalently bind to the cellular macromolecules with resultant inactivation of cellular functions. Glutathione is an efficient detoxifying pathway for most of these reactive metabolites. However, in case of alkylating agents, oxidative stress and the excess substrates for conjugation deplete the glutathione and thus rendering the cells more susceptible to the toxic effects of

reactive metabolites. Further, the reactive metabolites alter the liver proteins leading to an immune response and immune-mediated liver injury.

2.4.3.c Calcium homeostasis:

Calcium is an important ion involved in a wide variety of the critical, Physiological functions which makes it important to maintain the calcium homeostasis in the cell. The concentration of the calcium between inside to outside the cell is maintained by an active membrane associated Calcium and Magnesium effluxing, Adenosine Triphosphatase (ATPase) which is an important and potential target for the hepatotoxicants. The drug-induced hepatotoxicity disrupts the calcium homeostasis which is occurring due to increase permeability of plasma membrane, mitochondrial membrane and the membranes of smooth endoplasmic reticulum by the intracellular calcium levels that result in activation of membrane damaging enzymes such as ATPases, Phospholipases, Proteases and Endonucleases which damage the microfilaments that support the cell structure. Drugs like Quinines, Peroxides, Paracetamol, Iron and Cadmium cause hepatotoxicity involving this mechanism.

2.4.3.d Direct effect of toxicants:

Some of the hepatotoxic drugs act directly on the cellular targets such as the plasma membrane, mitochondria, endoplasmic reticulum, nucleus and lysosomes to cause disruption of their activity [77]. Several toxic chemicals and metal ions bind to the mitochondrial membranes and enzymes causing cellular toxicity. Many of those drugs act as direct inhibitors and uncouplers of mitochondrial electron transport. For example, Chlorpromazine, Phenothiazines, Erythromycin, have direct effect on surfactants on the hepatocytes [78, 79].

2.5 Risk Factors of Drug Induced Liver Injury:

There are several risk factors which are involved in hepatotoxicity that include; **Age, gender, race, alcohol ingestion, concomitant medication or polypharmacy, nutrition, HIV, pre-existing liver disease, genetic factors, drug formulations, Dose and several other factors**.

2.5.i Age: Studies have shown that no age is an exempt for the drug induced liver injury. Interestingly, the drug induced acute liver failure is common in relatively young individuals in India [47, 48]. However, the studies conducted at India, shows that both children and adult are at risk, with 8.7% of Drug Induced Liver Injury occurring in children, of which the combined antituberculosis drugs and antiepileptic drugs were found to be the leading causes of Drug Induced Liver Injury in children. Some drug specifically are known to cause liver injury that is specific to the age group (Old age - Acetaminophen, Halothane, INH, Amoxicillin-Clavulanic Acid; Young age - Salicylates, Valproic acid).

2.5.ii Gender: Women are generally considered to be more at risk for Drug Induced Liver Injury. However the liver injury caused could be gender specific (Female - Halothane, Nitrofurantoin, Sulindac; Male - Amoxicillin-Clavulanic Acid)

2.5.iii Race: Several drugs are known to have different types of hepatotoxicity which are different based on the race. For example, blacks may be more susceptible to Isoniazid (INH) toxicity. The rate of metabolism is under the control of the enzyme cytochrome P-450 enzymes thus it can vary from one individual to another individual.

2.5.iv Alcohol ingestion: The chronic use of alcohol particularly in under-nourished individuals depletes glutathione stores to increase the risk of alcohol-induced liver injury.

Hepatotoxicity induced by alcohol ingestion is a most common type of Drug Induced Liver Injury. Alcohol depletes the glutathione stores which are hepatoprotective in nature and makes the individual more susceptible to the toxicity effect of the drug.

2.5.v Concomitant Medication or Polypharmacy:

The causality assessment of Drug Induced Liver Injury becomes more complicated and challenging with concomitant administered drugs. Many drugs may cause liver injury by their reciprocal interaction such as one drug may increase the potentiality of hepatotoxicity for the other drug that is co-administered, for example, Carbamazepine and INH cause inhibition of metabolism of either drug thereby increasing the serum levels of the drug and increasing the probability of hepatotoxicity [49].

Similarly, the combined preparations used in the treatment of tuberculosis, have shown an increase in propensity of hepatotoxicity, when Pyrazinamide, Isoniazid and Rifampicin all are found to be hepatotoxic drugs [50].

2.5.vi Nutrition: The nutritional deficiency in an individual makes him more susceptible to the Drug Induced Liver Injury; this tendency is mainly attributed to the reduced glutathione levels, which is also supported by the hypoalbuminic status [51, 52].Liver injury commonly is known to be caused by (Paracetamol), in individuals with fasting or malnutrition.

2.5.vii Human Immunodeficiency Virus (HIV): Generally individuals suffering with vulnerable HIV infections are prone for the opportunistic infections, such as Tuberculosis, carinii infection and concomitant Hepatitis B and C infections [53]. Further, these individuals being subjected to the drug-drug and drug-disease interaction becomes more susceptible to the Drug Induced Liver Injury, which is further making them more prone with their diminished stores of glutathione that act as the predisposing

factor. The common drugs associated with the risk for Drug Induced Liver Injury includes Zidovudine, Stavudine, Neviparine, Efavirenz, Abacavir, etc [53, 54].

2.5.viii Liver disease: Individual with the pre-existing liver disease are more susceptible to drug induced liver injury which may be due to the consequence of the low and diminished reserve that could worsen the hepatotoxicity.

2.5.ix Genetic factors: The genetic differences in the enzyme CYP450 may result in abnormal reactions to the administered drugs, including the idiosyncratic reactions. The polymorphism of the enzymes and the proteins are considered as predisposing factors for the Drug Induced Liver Injury. The acetylator status of N-Acetyltransferase-2 (NAT-2) and other such as CYP and GST M1 and T1 genetic polymorphism have shown an extensive influence on the drug induced liver injury; particularly those caused by the Antituberculosis drugs, therefore, the slow acetylators [55] show increased tendency to develop severe INH-induced hepatotoxicity. Similarly, it was that, there is an association of CYP 2E1 genetic polymorphism and GST M1 "null" mutation and GST T1 "NULL" mutation with hepatotoxicity to antituberculosis drug [56, 57].

Some studies have linked the human leukocyte antigen polymorphism with the drug induced liver injury. Particularly, it has been observed for the risk of the antituberculosis drugs caused hepatotoxicity with HLA-DQB1*0201. The HLA class one and two gene polymorphism has shown to play a major role in the pathogenesis and the expression of the biochemical parameters in the individuals, who suffer with Drug Induced Liver Injury [58, 59].

2.5.x Drug formulation: The long-acting hepatotoxic drugs are known to cause more injury than those drugs with shorter duration of action.

2.5.xi Dose: Most of the idiosyncratic Drug Induced Liver Injury (IDILI) are unique characteristics of the host and not to the drug, however Lammert et al, 2008 [24], have shown the association of the dose of the exposed drug and hepatotoxicity, who observed that the drugs administered in the doses more than 50 mg increases the risk of Drug Induced Liver Injury. Further, those drugs metabolized by the liver and excreted in the biliary canaliculi had increased risk of causing Drug Induced Liver Injury [60].

2.5.xii Other factors: Several other host factors enhance susceptibility to drug induced liver injury, for example; Large Body Mass Index/obesity (Halothane); Diabetes mellitus (Methotrexate, Niacin) and Renal failure (Tetracycline, Allopurinol) [61, 62].

2.6 Diagnosis of Drug Induced Liver Injury:

It is difficult to diagnose the drug induced liver injury due to the lack of specific signs & symptoms and tests. The manifestations vary to range from a asymptomatic elevation of liver enzymes to fulminant hepatic failure. Hence, the causality assessment of drug induced liver injury becomes difficult. However, the clinical history and the pattern of liver injury is characteristic to the drug administered and is helpful in diagnosis.

The common ways of diagnosing the liver injury include:

2.6.A. Causality Assessment:

The different approaches which help in determination of causality for drug induced liver injury includes:

i. Positive Rechallenge: This fulfils the Koch's postulate and is regarded as the gold standard. However, a positive rechallenge can often be unacceptably dangerous.

ii. Ad Hoc approach: It is a second method, considered for causality assessment, which appears to be reasonable but carries no logic justification.

iii. Roussel Uclaf Causality Assessment Method (RUCAM) scale: This is widely used method and has been validated to provide objective and consistent assessment, but though, cumbersome for routine clinical use. It provides a semi-quantitative evaluation of causality through assigning a score ranging between -3 and +3 points to each of its seven domains, that includes, time to onset of the reaction, risk factors, concomitant medications; non-medication related causes; previous information on the medication; and response to readministration if any.

Theoretically, the overall score may range anywhere from -5 to +14, but based on the final score, a causal relationship between the implicated agent and the liver injury was established as follows; **highly probable (>8), probable (6-8), possible (3-5), unlikely (1-2),** or **excluded (<0)** [63, 64].

iv. Clinical Diagnostic Scale or the M&V scale: This was developed to overcome the complexity of the RUCAM scale. However, this scale is developed to overcome the features of RUCAM scale, with less focus on its components [65]. With the overall score corresponding to five probability degrees, that includes **definite, probable, possible, unlikely,** and **exclude**. However, limitations of the M & V scale includes positive rechallenge, poor performance in atypical cases, poor description of the excluded alternative causes [65, 66].

v. Naranjo Scale: Apart from RUCAM scale and M &V scale the Naranjo scale is also used for the assessment of the adverse drug reactions, but this scale is not found to be specific to the hepatic adverse drug reaction [67, 68].

vi. Digestive Disease Week-Japan (DDW-J) scale: This is a recently proposed causality assessment scale in Japan, derived from the RUCAM scale, with modifications in chronological criteria, concomitant drug, and extrahepatic manifestations. This is considered to be superior to the RUCAM and M&V scale. It also includes an in vitro **Drug Lymphocyte Stimulation Test** (DLST) evaluation criteria [69, 70, 71].

vii. R values: Based on the pattern of serum enzymes, the R values helps in the diagnosis of the type of liver injury. The ratio of serum Alanine Aminotransferase (ALT) to Alkaline Phosphatase (ALP) is designated as the R value. The **hepatocellular drug induced liver injury is defined as R ≥ 5; cholestatic as R ≤ 2; and mixed as 2<R<5** [72, 73].

viii. Hy's rule: This rule states that for monitoring Drug Induced Liver Injury, which states that elevation of liver enzymes (AST or ALT more than 3 × ULN or ALP more than 1.5 × ULN) in combination with elevated bilirubin (more than 3 × ULN) at any time after starting a new drug suggests serious liver injury [74].

ix. Food and Drug Administration (FDA) guideline: It is recently proposed by FDA, that ALT greater than 8×ULN, ALT greater than 5×ULN for two weeks, ALT greater than 3×ULN in association with serum bilirubin greater than 2×ULN, more than 1.5×PT-INR, or symptoms of liver injury should be used to predict severe hepatotoxicity [75].

2.6.B. Laboratory Investigations:

In case of hepatotoxicity the serum alanine aminotransferase [ALT] increases abnormally. The increased serum ALT is a consequence of release by the dead or the dying hepatocytes and thus is considered as a sensitive semi quantitative measure of

liver injury. As a general rule, rise in the serum levels when found to be three times greater than the upper limit of normal (ULN) are identified as sensitive specific signal for liver toxicity. However, the three times rise in the serum ALT than the ULN in the absence of an increase in the serum bilirubin levels, reflects very mild injury.

Hence, the biochemical markers such as Alanine Aminotransferase [ALT] or Serum Glutamic-Pyruvic Transaminase (SGPT); Aspartate Aminotransferase [AST] or Serum Glutamic-Oxaloacetic Aminotransferases [SGOT]; Alkaline Phosphatase [ALP] and Total Bilirubin Levels [TBL] are relevant markers of hepatotoxicity; the increased bilirubin levels indicate overall liver function [76].

The levels of serum liver enzymes, such as, transaminases, Alkaline Phosphatase, Gamma-Glutamyl Transpeptidase [GGTP] or Gamma-Glutamyl Transferase [GGT]; help in detecting the injury to the hepatocytes.

i. **Alanine Aminotransferase [ALT] or Serum Glutamic-Pyruvic Transaminase [SGPT]:**

   It is considered as a standard biomarker for studying the hepatotoxicity induced by any drug or chemical substance. It plays an important role in gluconeogenesis and amino acid metabolism. It catalyzes the reductive transfer of an amino group from alanine to α-ketoglutarate, in order to yield glutamate and pyruvate. The normal serum levels are in the range of 5-50 U/L, which are elevated during the hepatotoxic state. Hence, estimation of this enzyme is a more specific test to detect the hepatic damage or hepatocellular necrosis [26].

ii. **Aspartate Aminotransferase [AST] or Serum Glutamic-Oxaloacetic Aminotransferases [SGOT]:**

Serum Glutamic Oxaloacetate Transaminase [SGOT] catalyzes the reductive transfer of an amino group from aspartate to α-ketoglutarate to yield oxaloacetate and glutamate. The normal levels of which range from 7-40 U/L are elevated in case of hepatocellular injury, yet it is considered as a less specific marker to indicate hepatotoxicity because, since the enzyme is also found in other organs like heart, muscle, brain and kidneys apart from the liver; damage to any of these organs may also elevate the SGOT levels. However, the ratio between serum AST and serum ALT can be made use of to differentiate the liver damage from that of the other organ damage [26].

iii. Alkaline Phosphatase [ALP]:

Alkaline phosphatase is eliminated in the bile that hydrolyzes monophosphates at an alkaline pH and is particularly present in the biliary ducts of the liver. The normal levels of the enzyme ranges from 20-120U/L, the level of which may be elevated when bile excretion is inhibited, as seen in the cases of hepatotoxicity, due to either congestion or obstruction of the biliary tract. Hence, it is considered as a biomarker for the hepatobiliary effects [26, 77, 78].

iv. Gamma-Glutamyl Transpeptidase [GGTP] or Gamma-Glutamyl Transferase [GGT]:

γ-Glutamyl transferase [GGT] or transpeptidase [GGTP] is found in liver, the normal levels of which range from 0-51 U/L. The enzyme catalyzes the transfer of γ-glutamyl groups to amino acids and short peptides, and is more useful to indicate hepatic injury as compared to ALP. However, the comparison of the two enzymes, GGT and ALP, helps in determining the hepatotoxicity. The normal GGT level with an elevated ALP level is suggestive of bone disease as

GGT is not found in bone; while an elevated level of both the enzymes i.e. GGT and ALP are suggestive of the liver or bile duct disease. Hence, GGT is considered as a specific biomarker of hepatobiliary injury, in human beings. It was also reported as a specific indicator of bile duct lesions in the rat liver.

v. Total Bilirubin Levels [TBL]:

Bilirubin is derived by the degradation of haemoglobin from the red blood cells (RBCs) and is excreted from the liver. The normal levels of it range from 0.2 to 1.2 mg/dL. In case of hepatotoxicity or liver damage, the excretion of bilirubin does not occur in the normal manner, thus causing an increase in the bilirubin levels in the blood and extracellular fluid. The increased serum bilirubin is mainly due to decreased hepatic clearance, thus an increase in bilirubin level with little or no increase in ALT indicates cholestasis, as in case of hepatic injury, however, total bilirubin can be a better indicator of liver disease and severity as compared to ALT **[26]**.

2.6.C Histopathological examination:

In addition to the biochemical patterns series of possible histopathological patterns were found to be specific in patients with suspected drug induced liver injury that includes**: acute hepatitis, chronic hepatitis, acute cholestasis, chronic cholestasis, cholestatic hepatitis, granulomatous changes, steatosis, steatohepatitis, coaugulative/ confluent necrosis, massive / sub-massive necrosis, vascular injury, hepatocellular alteration, nodular regenerative hyperplasia, mixed injury unclassifiable injury, minimal nonspecific changes,** and **normality.**

To understand about the liver injury causing hepatotoxicity, histopathological findings and their correlation with the biochemical markers is important. The

histopathology reveals the histopathological features, the degree of cholestasis, duct injury, duct paucity, microvesicular changes, inflammation and the necrosis. The pattern of classification is critical to define the pathological differential diagnosis.

The histological findings may range from minor fatty change, hepatocytes anisonucleus, mild portal based inflammation and focal necrosis to more severe hepatocellular necrosis, fibrosis and cirrhosis. Further, the hepatic injury based on the histopathological findings may be divided in two forms, such as, **pure (bland) cholestasis** and **cholestatic hepatitis** as commonly seen with anabolic steroids [30].

2.7 Treatment of Drug Induced Liver Injury:

On confirming the patient to be suffering with the liver damage based on comprehensive histopathological evaluation, clinical signs and symptoms, it is essential to begin the treatment at an earliest.

Many agents including **N-Acetylcysteine (NAC), Silymarin, Antioxidants, S-Adenosine Methionine, Ursodeoxycholic acid,** or a **combination of these** have been used in the treatment of drug induced liver injury and other forms of liver toxicity [79, 80].

i. It is suggested by the **Food and Drug Administration (FDA)** to perform the laboratory monitoring of ALT or AST (> 3 x ULN) helps in distinguishing the drug induced liver injury, from that of adaptive response and tolerance.

The suspected drug should be discontinued when ALT or AST > 8 x ULN, ALT or AST > 5 x ULN for more than two weeks, and ALT or AST > 3 x ULN with TB > 2 x ULN or INR > 1.5 and ALT or AST > 3 x ULN with hypersensitivity symptoms and signs [81].

ii. **N-Acetylcysteine (NAC)** is considered as the **s**pecific antidote in case of Paracetamol overdose. Studies have shown that administration of N-Acetylcysteine would increase the probability of transplant-free survival in those adults diagnosed with idiosyncratic drug induced liver injury due to other causes [2, 82, 83, 84].

iv. **Corticosteroids** can be used when the clinical and laboratory findings of hypersensitivity is obvious and in those cases where the drug induced liver injury induces autoimmune hepatitis [20].

v. **Antihistamines** and **bile acid sequestrants** such as Hydroxyzine and Diphenhydramine along with Cholestyramine have been useful as a symptomatic treatment for the treatment of itching in cholestatic drug induced liver injury [21]. However, Cholestyramine has a specific indication for treatment of Leflunomide-induced liver injury [85], where the drug metabolites undergo extensive enterohepatic circulation resulting in a long half-life with continued liver injury, despite discontinuation of the drug [86].

vi. **L-Carnitine and Folic acid,** Intravenous Carnitine is a recommended drug for the treatment of Valproate-induced direct hepatotoxicity [87, 22] while, folic acid is used to reduce the Methotrexate toxicity [23]. Valproate inhibits the biosynthesis of Carnitine, by affecting the beta-oxidation of fatty acids. Therefore, supplementation of Carnitine increases the beta-oxidation of Valproate and benefits the liver injury that has been caused [88].

vii. **Bile acid sequestrants** Ursodeoxycholic acid (UDCA), irrespective of its efficacy and uncertainty, has been widely used along with Cholestyramine in the treatment of Cholestatic- drug induced liver injury, where its **membrane stabilizing action** protects the hepatocytes and cholangiocytes by replacing the

endogenous, cytotoxic bile salts and also by enhancing the function of transporters [89] .

viii. **Organ Liver Transplant (OLT),** although in cases of acute liver failure, OLT is considered as the rescue treatment, availability of the organ in time remains a critical limitation [18].

ix. **Molecular Adsorbent Recirculation System (MARS)**, This kind of a theory and approach along with other **extracorporeal detoxification system** have been proposed as supportive therapy in patients with who are suggested for liver transplantation. However, with an unclear information on its efficacy and cost-benefit this approach remains debatable [90, 91].

2.8 Prevention of Drug Induced Liver Injury:

With the idiosyncratic nature of the many drugs used in the treatment of several clinical conditions, it would be extremely difficult for anybody to identify which drug during the course of treatment causes the hepatotoxicity. However, considering several points, while making use of drugs in treatment of any conditions probably would guide us, in identifying the culprit drug to take precautions and prevent the liver injury that can be caused by the administered drug [59].

2.8.A Rational drug prescription:

This remains the central point of consideration, while using the drugs, to treat any clinical condition, which helps in minimizing the drug induced liver injury. Particularly, when patients is associated with several risk factors such as old age, co-morbid diseased condition, HIV status, daily dose of the drugs more than 50 mg and in those with polypharmacy.

2.8.B Caution in treatment of Tuberculosis (TB):

Caution administered while treating the TB patients, where most of them are known to have hepatotoxic effect, as an adverse effect, would help in lowering the incidence of drug induced liver injury including Acute Liver Failure (ALF).

2.8.C Drug-interaction:

It is an essential for every treating physician to update his knowledge while treating an individual with more than two drugs. This will help to be cautious with the probable drug–drug interaction and drug-disease interactions which are very common to cause the drug induced liver injury, e.g. Methotrexate.

2.8.D Education:

A simple measure of educating the patients and caregivers on the signs and symptoms of liver injury such as nausea, vomiting, anorexia, dark urine or jaundice will help in early diagnosing of the liver injury so that that the progression can be prevented.

2.9. Non-Steroidal Anti-Inflammatory Drugs:

The drug induced liver injury, though uncommon is found to be a major cause of liver failure and mortality [92]. Further, they are considered as an important cause for terminating the clinical trials and withdrawing the drug from the market [93]. Although many drugs have hepatotoxicity as an adverse effect, the common culprit drugs includes Antibiotics, Antiepileptics, Complementary and Alternative Medicine (CAM) and the most important of all, Non Steroidal Anti Inflammatory Drugs [94, 11].

Non Steroidal Anti-Inflammatory Drugs, are considered as the pivot in the management of chronic painful conditions along with the acute pains that include rheumatological disorders, fever and other common painful conditions (71.6% in cancer pain) [94], for which they are used as both prescriptive and Over The Counter (OTC) medications [95], causing a risk of 1-8 cases per 100000 patients per year use of Non Steroidal Anti Inflammatory Drugs causing liver injury [94, 95].

These Non-Steroidal Anti-Inflammatory Drugs act by inhibiting cyclo-oxygenase (COX) enzyme, which regulates the synthesis of prostaglandins (PGs) [97], and are generally associated with gastrointestinal, cardiovascular, and renal adverse events [96]. The duration and dose of the drug used, contributes to the severity of the complications [98]. The structural component of **aryl acetic acids** (Indomethacin), **aryl propionic acids** (Ibuprofen, Ketoprofen and Flurbiprofen) and **anthranilates** (Meclofenamic acid and analogues) are responsible to inhibit both isoforms of the cyclooxygenase enzyme Type 1 and Type 2, which are involved in the synthesis of gastroprotective prostaglandins. Thus, they contribute to the severe gastrointestinal toxicities [99]. It was reported that the Gastro-intestinal related adverse events were observed more in those who used Diclofenac as compared with Aceclofenac [100].

The hepatotoxicity due to the administered drug may occur at any time after the drug administered, but often occurs within 6-12 weeks of initiating the therapy [101], which may present with **two main clinical patterns**:

i. An acute hepatitis with jaundice, fever, nausea, greatly elevated transaminases and sometimes eosinophilia.

ii. The alternative pattern could be serological Antinuclear Factor (ANF) positive; and histological, periportal inmmation with plasma and lymphocyte infiltration and fibrosis extending into the lobule, which are features of chronic active hepatitis.

Additionally, Non-Steroidal Anti-Inflammatory Drugs-induced idiosyncratic hepatotoxicity includes the risk factors such as feminine gender, age above 50 years and underlying autoimmune disease and concomitant exposure to other hepatotoxic drugs.

It has been observed that patient who suffers with hepatotoxicity due to one Non-Steroidal Anti-Inflammatory Drugs, often is susceptible to the same type of reaction on rechallange or on administering the drug belonging to the same group (sister-drug) e.g. Diclofenac and Tiaprofenic acid [102]. The metabolic aberrations are mainly due to genetic polymorphism accounting for the incidence of 1-8 per 100,000 prescriptions of Non Steroidal Anti Inflammatory Drugs.

2.9.1 Mechanism of hepatotoxicity induced by Non-Steroidal Anti-Inflammatory Drugs:

The **hepatic injury** caused by the Non Steroidal Anti Inflammatory Drugs involves mainly **two mechanisms,** which include **hypersensitivity and metabolic**

aberrations. The in vitro animal models used to investigate the possible mechanisms of NSAID-related hepatotoxicity, using rat liver mitochondria and freshly isolated rat hepatocytes showed that Diphenylamine, a common structure in the Non-Steroidal Anti-Inflammatory Drugs uncoupled the oxidative phosphorylation and decrease the hepatic ATP content and induce hepatocyte injury [103, 104].

Further, incubation of mitochondria with Diphenylamine and Mefenamic acid or Diclofenac sodium caused mitochondrial swelling. A spectral shift of safranine-binding spectra to mitochondria occurred, which indicated the loss of mitochondrial membrane potentials (characteristics uncoupling of oxidative phosphorylation). Additionally, it was found that on adding oligomycin, which blocked the ATPase, had shown protection against the cell injury [103].

Since, it is reported by Pareek and Chandurkar [100], that the gastrointestinal-related adverse effects, induced by the non steroidal anti-inflammatory drugs are severe and generally found on prolonged use of these preparations with significantly higher ($p=0.053$) number of gastrointestinal-related adverse effects observed among the patients using Diclofenac [100].

2.10 Diclofenac sodium:

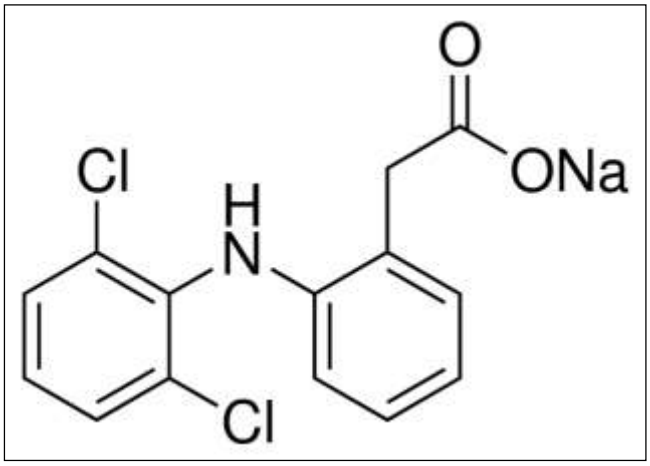


Figure 4: Chemical structure of Diclofenac sodium

Diclofenac sodium is a sodium salt form of Diclofenac, a benzene acetic acid derivative, sodium; 2'-[2-(2,6-dichloroanilino) phenyl acetate. It is also one of the commonly used over the counter preparations and is available in the form of enteric coated tablets, suppositories, injections for intravenous or intramuscular use and as ointments, gels and sprays for topical use.

Diclofenac is a non-selective reversible and competitive inhibitor of cyclooxygenase (COX), which acts subsequently by blocking the conversion of arachidonic acid into prostaglandin precursors. It leads to the inhibition of formation of prostaglandins that are involved in pain, inflammation and fever.

2.10.1. Diclofenac Sodium - Hepatotoxic potential:

Potentially all the administered drugs being metabolized in the liver can be involved in causing damage, either directly or with the metabolites released with the varying degree of severity. Of all the drugs used for the treatment of various therapeutic conditions, the most common hepatotoxic drug includes antibiotics, NSAIDs, Statins, Antiplatelets, Immunosuppresants and Herbal Remedies and the Dietary supplements

(HSD). Of all the Non Steroidal Anti Inflammatory Drugs, Diclofenac has been found to cause more of the gastrointestinal adverse effects than Aceclofenac.

Diclofenac is mainly used as an analgesic and anti-inflammatory preparation in the treatment of chronic painful conditions. It is available as the sodium salt and it remains the most extensively used non steroidal anti-inflammatory drug with similarity in its efficacy to that of Naproxen.

The non steroidal anti-inflammatory drugs being the leading group of drugs causing drug induced liver injury with hepatocellular pattern, Diclofenac is identified to cause rare but potentially serious hepatotoxicity with the frequency of 3.6 per 100000 users [4, 105].

2.10.2 Common adverse reactions of Diclofenac sodium:

Diclofenac sodium generally causes mild adverse effects such as epigastric pain, nausea, headache, dizziness or rashes; less common adverse effects such as gastric ulceration and bleeding; whereas, rarely but at times severely may cause renal and hepatic injury. The hepatic injury may reflect with reversible inhibition of serum aminotransferases [106, 107].

2.10.3. Mechanism of Diclofenac-induced hepatotoxicity:

The **major pathway** involved in the metabolism of Diclofenac is through 4'-hydroxylation by CYP 450 [108, 109], while, the **minor pathway** includes the formation of **5' hydroxyl-diclofenac** catalyzed by the number of cytochrome including CYP 3 A4, CYP2C8, CYP2 C18, and CYP 2C19 and **3' hydroxy-diclofenac** catalyzed by CYP 2C9 [110,111, 112].

Further, Diclofenac and its metabolites undergo glucuronic acid and sulphate conjugation mediated by UDP-Glucuronosyl Transferase (UGT) 2B7 **[113]**. This acyl glucuronyl can form adduct (a compound formed by an addition reaction with hepatocellular proteins) ultimately resulting in immune mediated destruction of the hepatocytes **[114, 115]**.

The impairment of 4- hydroxylation or an increased metabolism through the minor pathways result with the formation of the reactive metabolites for Diclofenac and adduct formation. It is presumed that polymorphism of CYP 2C9, that is responsible for the major pathway of metabolism to be associated with the Diclofenac-induced hepatotoxicity. A recent study shows association between an upstream (C-161T), UGT2B7, polymorphism and Diclofenac-induced liver injury **[116]**.

Some studies conducted in humans have shown that Diclofenac modified proteins are formed in the liver that are associated with the hepatotoxicity, and that they elicit a selective antibody response **[117]**, hence, acyl glucuronides formation may be responsible for the antibody response in Diclofenac induced hepatotoxicity. Further, the frequency of variant alleles for the IL10, and IL4 have also been found to be five times higher in patients with liver injury, providing an evidences for the immune mechanism in pathogenesis of liver disease.

Low IL10 could increase the presentation of Diclofenac related neoantigens by the monocytes and lead to subsequent activation of T-cells and the immune mediated liver injury; while high levels of IL-4 promote T_H2 mediated immune response and induce B cell differentiation **[118]**.

The animal studies have shown that ferrous iron release from rat liver microsomes have contributed to the Naproxen induced microsomal lipid peroxidation;

while Diclofenac has been demonstrated to be more cytotoxic to the drug metabolizing cells than to the non-metabolizing cell line (HepG2; FaO), thus indicating that both, impairment of ATP synthesis by the mitochondria and the drug metabolism is reduced by the addition of CYP 450 inhibitors (Prothiaden and Ketoconazole) to the culture medium. Therefore, indicating that inhibition of the ATP synthesis with Mitochondrial Permeability Transition resulting in the generation of reactive oxygen species; mitochondrial swelling and oxidation of NADP and protein Thiols; all have been shown to be important mechanisms involved in the Diclofenac induced Liver injury **[104]**.

2.10.4. Therapeutic uses of Diclofenac sodium:

Diclofenac sodium is one of the most extensively used Non-steroidal Anti-Inflammatory agents used in the treatment of rheumatoid arthritis, osteoarthritis, bursistis, ankylosing spondylitis, toothache, dysmehorrhoea, post-traumatic and post operative inflammatory conditions to relieve the pain and wound edema.

Diclofenac is Non-Steroidal Anti-Inflammatory Drug (NSAID), indicated in the relief of all grades of pain and inflammation associated with a wide range of conditions, including arthritic conditions, acute musculo-skeletal disorders and other painful conditions resulting from trauma **[73, 107]**.

2.10.5 Laboratory findings in Diclofenac sodium induced liver injury:

In case of hepatotoxicity the serum alanine aminotransferase [ALT] increases abnormally. The increased serum ALT is a consequence of release by the dead or the dying hepatocytes and thus is considered as a sensitive semi quantitative measure of liver injury. As a general rule, rise in the serum levels when found to be three times greater than the upper limit of normal (ULN) are identified as sensitive specific signal

for liver toxicity. However, the three times rise in the serum ALT than the ULN in the absence of an increase in the serum bilirubin levels, reflects very mild injury.

a) Hence, the biochemical markers such as Alanine Aminotransferase [ALT] or Serum Glutamic-Pyruvic Transaminase (SGPT); Aspartate Aminotransferase [AST] or Serum Glutamic-Oxaloacetic Aminotransferases (SGOT); Alkaline Phosphatase [ALP] and Bilirubin are relevant markers of hepatotoxicity; the increased bilirubin levels indicate overall liver function [76, 107].

The levels of serum liver enzymes, such as, transaminases, Alkaline Phosphatase, Gamma-Glutamyl Transpeptidase (GGTP) or Gamma-Glutamyl Transferase (GGT); help in detecting the injury to the hepatocytes.

i. Alanine Aminotransferase [ALT] or Serum Glutamic-Pyruvic Transaminase (SGPT):

It is considered as a standard biomarker for studying the hepatotoxicity induced by any drug or chemical substance. It plays an important role in gluconeogenesis and amino acid metabolism. It catalyzes the reductive transfer of an amino group from alanine to α-ketoglutarate, in order to yield glutamate and pyruvate. The normal serum levels are in the range of 5-50 U/L, which are elevated during the hepatotoxic state. Hence, estimation of this enzyme is a more specific test to detect the hepatic damage or hepatocellular necrosis [26].

ii. Aspartate Aminotransferase [AST] or Serum Glutamic-Oxaloacetic Aminotransferases (SGOT):

Serum glutamic oxaloacetate transaminase [SGOT] catalyzes the reductive transfer of an amino group from aspartate to α-ketoglutarate to yield oxaloacetate and glutamate. The normal levels of which range from 7-40 U/L are elevated in

case of hepatocellular injury, yet it is considered as a less specific marker to indicate hepatotoxicity because, since the enzyme is also found in other organs like heart, muscle, brain and kidneys apart from the liver; damage to any of these organs may also elevate the SGOT levels. However, the ratio between serum AST and serum ALT can be made use of to differentiate the liver damage from that of the other organ damage [26].

iii. Alkaline Phosphatase [ALP]:

Alkaline phosphatase is eliminated in the bile that hydrolyzes monophosphates at an alkaline pH and is particularly present in the biliary ducts of the liver. The normal levels of the enzyme ranges from 20-120U/L, the level of which may be elevated when bile excretion is inhibited, as seen in the cases of hepatotoxicity, due to either congestion or obstruction of the biliary tract. Hence, it is considered as a biomarker for the hepato-biliary effects [30, 77, 78].

iv. Gamma-Glutamyl Transpeptidase (GGTP) or Gamma-Glutamyl Transferase (GGT):

γ-Glutamyl Transferase [GGT] or Transpeptidase [GGTP] is found in liver, the normal levels of which range from 0-51 U/L. The enzyme catalyzes the transfer of γ-glutamyl groups to amino acids and short peptides, and is more useful to indicate hepatic injury as compared to ALP. However, the comparison of the two enzymes, GGT and ALP, helps in determining the hepatotoxicity. The normal GGT level with an elevated ALP level is suggestive of bone disease as GGT is not found in bone; while an elevated level of both the enzymes i.e. GGT and ALP are suggestive of the liver or bile duct disease. Hence, GGT is considered as a

specific biomarker of hepatobiliary injury, in human beings. It was also reported as a specific indicator of bile duct lesions in the rat liver.

v. Total Bilirubin Levels (TBL):

Bilirubin is derived by the degradation of hemoglobin from the red blood cells (RBCs) and is excreted from the liver. The normal levels of it range from 0.2 to 1.2 mg/dL. In case of hepatotoxicity or liver damage, the excretion of bilirubin doesnot occurs in the normal manner, thus causing an increase in the bilirubin levels in the blood and extracellular fluid. The increased serum bilirubin is mainly due to decreased hepatic clearance, thus an increase in bilirubin level with little or no increase in ALT indicates cholestasis, as in case of hepatic injury, however, total bilirubin can be a better indicator of liver disease and severity as compared to ALT [26].

2.10.6. Histopathological examination:

To understand about the liver injury causing hepatotoxicity, histopathological findings and their correlation with the biochemical markers is important. The histopathology reveals the histopathological features, the degree of cholestasis, duct injury, duct paucity, microvesicular changes, inflammation and the necrosis. The pattern of classification is critical to define the pathological differential diagnosis.

The histological findings may range from minor fatty change, hepatocytes anisonucleus, mild portal based inflammation and focal necrosis to more severe hepatocellular necrosis, fibrosis and cirrhosis. Further, the hepatic injury based on the histopathological findings may be divided in two forms, such as, pure (bland) cholestasis and cholestatic hepatitis as commonly seen with anabolic steroids [30].

2.10.6.i Histopathological findings in Diclofenac-induced hepatotoxicity:

Diclofenac sodium on histopathological examination shows typically an association with the findings of acute hepatitis with **necrosis** which is **more prominent centrally**. The necrosis is focal with inflammation. However, chronic hepatitis like prominent portal inflammation, fibrosis may also be found on prolonged use. In some cases, mixed-hepatocellular cholestatic injury, with varied degree of inflammation has also been observed **[119]**.

2.11 DL-Methionine or Racemethionine:

DL-Methionine is 2-amino-4-methylsulfanylbutanoic acid, also known as Methionine; Racemethionine; Acimetion; Banthionine; which is also one of nine essential amino acids in humans [120].

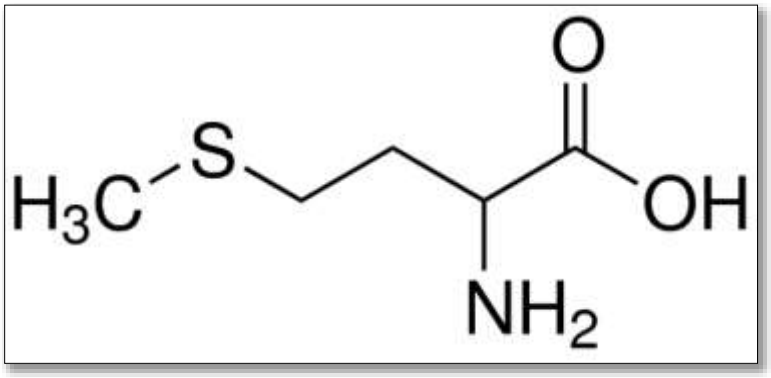


Figure 5: Chemical Structure of DL-Methionine.

Methionine is an organic substance, which is considered as sulphur containing essential amino acid that is important for many of the body functions. Methionine is required for growth and tissue repair and is also involved in many detoxifying processes; sulphur provided by Methionine protects cells from pollutants and slows cell aging. It is an important sulphur donor. It is used in protein synthesis, including the formation of S-adenosyl-L-Methionine (SAMe), L-homocysteine, L- cysteine, taurine, and sulphate. It is also considered as a component of enkephalins and various endorphins which are pain-relieving peptides, coenzyme A, heparin, biotin, and tripeptide glutathione which are important antioxidant and detoxifying agents. Also essential trace element selenium needs Methionine for its absorption, transportation and bioavailability. It also acts as a lipotropic agent and prevents excess fat build-up in the liver. Low levels of Methionine are known to cause temporary folic acid deficiency by trapping the folate in the liver [120, 121].

2.11.1. Mechanism of action of DL-Methionine:

The hepatoprotective activity of L-Methionine is not clear. In high doses of Paracetamol, the hepatic glutathione levels decreases causing increased oxidative stress and hepatic injury. L-methionine, a precursor of L-cysteine, which is considered to have antioxidant activity, is found to be a precursor to glutathione as well [120, 121]. Antioxidant activity of L-Methionine and its metabolites are therefore, attributed for their possible hepatoprotective activity.

S-Adenosyl L-Methionine (SAMe), biologically active metabolite of Methionine is found to be depleted in chronic liver condition. Hence, supplementation of the same has been proposed to have a greater role in reducing the toxicity in liver diseases. Since, it is a principle methyl donor, used in transmethylation reaction. Glycine-n-methyltransferase is most abundant in the liver, pancreas, and prostrate. It has been proposed that, supplementation of SAMe could both ameliorate liver injury and reduce the development of Hepatocellular carcinoma (HCC) in chronic liver disease. Therefore, Methionine is particularly important in opposing the toxicity of free radicals generated by various toxins including that of alcohol [122, 123].

To counter the oxidative stress and cellular damage, several antioxidant enzymes have been developed including superoxide dismutase (SOD), Glutathione peroxidase and catalase, that detoxify the reactive oxygen species and the cells contained endogenous antioxidants that scavenge the free radicals and reduces the cellular damage of which glutathione plays a major role. Since, SAMe is a precursor for cysteine and glutathione, it has effectively been useful in hepatotoxicity [122, 123].

2.11.2 Adverse reactions of DL-Methionine:

Methionine is generally well tolerated upto the dose 250 mg per day. However, in higher doses, it may cause nausea, vomiting and headache. It increases calcium and has shown to precipitate encephalopathy in patients with cirrhosis [120, 121].

2.11.3 Therapeutic uses of DL-Methionine:

Methionine is commonly used nutritional, essential amino acid, is preferred in the treatment of Paracetamol poisoning, to prevent the hepatotoxicity, as an alternative to acetylcysteine. It is also used to lower the urinary pH and as an adjunct in the treatment of liver diseases. It has also been used in the assessment of hypercysteinaemia [120, 121].

It antagonizes the radiation effect, SAMe, alone or in combination with Vitamin B12, B6 and folate supplementation is used in the treatment of Non-alcoholic steatohepatitis (NASH). Also as an adjunct to pegylated Interferon – alpha/Ribavarin; SAMe is beneficial in the treatment of chronic Hepatitis C viral infection (HCV), in it has also been useful in reducing the incidence of Hepatocellular Carcinoma (HCC), in chronic liver diseases such as Hepatitis B viral infection (HBV) [122, 124].

2.11.4 Hepatoprotective action of DL-Methionine:

Methionine is also found to be a hepatoprotective substance, although its mechanism of action to produce hepatoprotection is not clear. It has been shown that the metabolism of high dose of Paracetamol the **liver reduce the level of hepatic glutathione and oxidative stress.** Moreover, DL-Methionine is a precursor of L-Cysteine, which by itself may **have antioxidant activity** and additionally L-Cysteine and its metabolites are accountable to their hepatoprotective action. There are also evidences suggesting that Methionine by itself has the **free radical scavenging** activity due to the sulphur moiety and the chelating ability [120, 121].

2.12 N-Acetylcysteine:

N-Acetyl-L-cysteine is the N-acetyl derivative of Cysteine, is also known as N-Acetyl-L-cysteine; Acetylcysteine; N-Acetylcysteine; Mercapturic acid; Acetadote, etc.

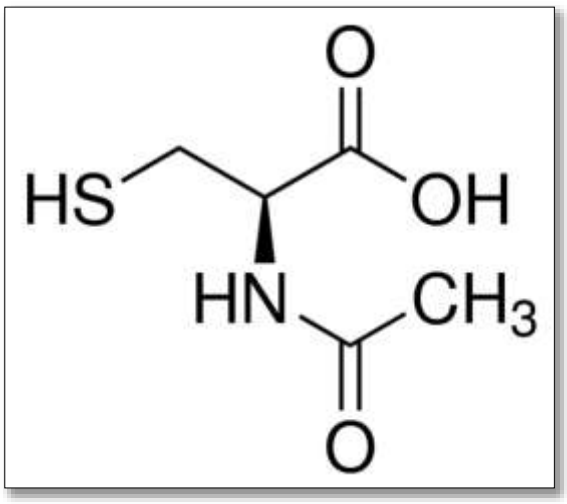


Fig: 6. Shows chemical structure of N-Acetylcysteine.

Acetylcysteine, also known as N-acetylcysteine (NAC), is a modified amino acid, i.e. Cysteine. Acetylcysteine is the nonproprietary name for the N-acetyl derivative of the naturally occurring amino acid, L-cysteine (N-acetyl-L-cysteine).

N-Acetylcysteine (NAC) was first used in the treatment of Paracetamol toxicity. Later, it got established as an effective and safe treatment for Paracetamol toxicity, which was also analyzed for its effectiveness in the treatment of non-paracetamol liver failure for which N-Acetylcysteine was found to be hepatoprotective [84, 125].

It had also shown that N-acetylcysteine was associated with mortality benefit, when it was studied for its effect on non-Paracetamol acute failure; in patients without facility for liver transplantation. It reduced the hospitality stay and improved the survival. Apart from its hepatoprotective effect in Paracetamol and non-Paracetamol liver failure, N-Acetylcysteine has been beneficial in the treatment of drug induced

liver injury due to several other substances such as Amanita phalloides mushroom poisoning [126].

Acetylcysteine or N-Acetylcysteine is used as a specific drug in the treatment of Paracetamol overdose. It serves as a prodrug to L-Cysteine, which is a precursor to the antioxidant Glutathione. Hence, it replenishes the glutathione stores [127].

2.12.1 Mechanism of action of N-Acetylcysteine:

The liver injury that occurs due to Paracetamol is a form of non-idiosyncratic drug-induced liver injury. While the mechanism involved in idiosyncratic hepatotoxicity, may be due to either direct cell injury, immune-mediated damage or the mitochondrial injury due to oxidative stress.

N-acetylcysteine has been beneficial in both the types of liver injuries, is understood that the metabolism of Paracetamol produces excessive hepatotoxic metabolite N-acetyl-p-Benzoquinoneimine (NAPQI), also known as Acetimidoquinone. It is normally produced only in small amounts, and then almost immediately detoxified in the liver. However, under some conditions in which NAPQI is not effectively detoxified (usually in case of Paracetamol overdose), it causes severe damage to the liver. This NAPQI is highly reactive arylating metabolite which is detoxified by conjugating Glutathione. When a large dose of Paracetamol is taken, glucuronidation capacity is saturated, and more of the minor metabolite is being formed, with the depletion of glutathione levels. The metabolite binds covalently to the proteins in the liver cells and the renal tubules causing necrosis. Hence, the toxicity shows a threshold effect only when the glutathione is depleted to a critical level [131].

N-Acetylcysteine replenishes the GSH stores of the liver and prevents binding of the toxic metabolite to other cellular constituents and thus acts as hepatoprotective

agent [128]. Furthermore, it is also beneficial in the idiosyncratic liver injury, which has been mainly attributed to its antioxidant effect [126, 129]. In general, N-Acetylcysteine also benefits by improving the systemic hemodynamic and tissue oxygen delivery which are considered as other favourable actions to benefit the injured liver [67, 63].

2.12.2 Adverse reactions of N-Acetylcysteine:

N-Acetylcysteine is mostly associated with anaphylactoid reactions such as rashes and pruritus, which may be accompanied by flushing, nausea, bronchoconstriction, angioedema and hypertension which occur more when administered intravenously. Other common adverse effect includes arthralgia, blurred vision, acidosis, convulsions, and cardiac or respiratory arrest [130].

2.12.3 Therapeutic uses of N-Acetylcysteine:

N-Acetylcysteine has been primarily hepatoprotective drug, benefiting in protecting from the liver injury induced by either drug or in case of idiosyncratic liver injury.

It is also known to exert mucolytic action through its free sulfhydryl group, which opens the disulfide bonds and lower the viscosity of the mucus, thus proving to be a good mucolytic agent. It is used in the treatment of aspergilloma, as well as burns in children and nephropathy [130].

Other uses of N-Acetylcysteine:

Idiopathic pulmonary fibrosis, dry eye, meconium ileus in neonates and distal obstruction syndrome in children with cystic fibrosis; and with its antiapoptic effect, in prevention of cancer cell growth, which is still under investigation [130].

2.12.4 Hepatoprotective action of N-Acetylcysteine

N-Acetylcysteine is the principle antidotal treatment for the hepatotoxicity, either drug-induced or idiosyncratic. It is a sulfhydryl compound which probably acts by replenishing the hepatic stores of Glutathione (GSH). GSH is required to inactivate an intermediate metabolite of Paracetamol, which is thought to be hepatotoxic. In Paracetamol overdose, excessive amount of this metabolite are formed because the primary metabolic (glucuronides and sulphate conjugation) pathways become saturated. Hence, Acetylcysteine may act by reducing the metabolite to the parent compound and/or by providing the sulfhydryl group for conjugation of the metabolite. Experimental evidence also suggests that a sulfhydryl-containing compound such as Acetylcysteine may directly inactivate the metabolite **[130]**.

N-Acetylcysteine is effective when given orally or intravenously. It is recommended if less than 36 hours have lapsed, since the ingestion of the Paracetamol, although the treatment with N-Acetylcysteine is more effective when given less than 10 hours after Paracetamol ingestion. An oral loading dose of 140 mg/kg is given, followed by 70 mg/kg every 4 hourly for 17 doses. However, the treatment is terminated if the assay of plasma Paracetamol **indicates** low risk of hepatotoxicity **[131]**.

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.

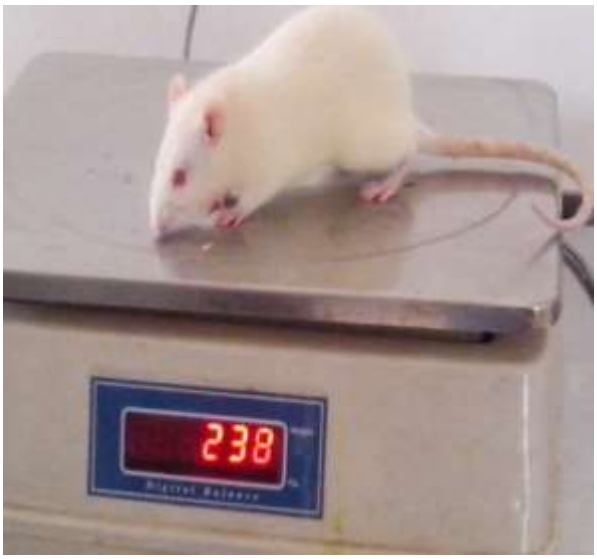
Albino rats of either sex weighing between 100 - 400g were used. All the animals used were housed separately in poly-propylene rat-cages and were allowed to acclimatize under controlled environmental conditions of temperature 24° ± 2°C and 55% ± 5%, relative humidity, in a 12-hour light-/dark cycle throughout 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.

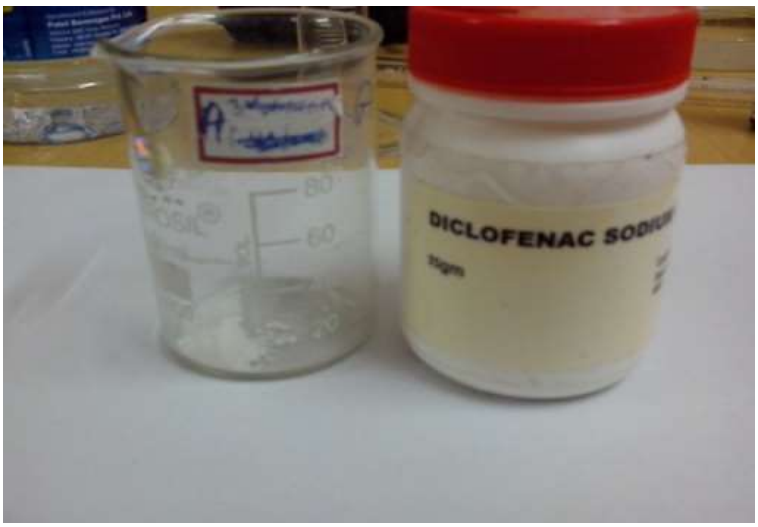
PHOTOGRAPHS:

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.

Group I	**Control - Distilled Water 10 ml/kg p.o.**
Group II	**Diclofenac sodium 72 mg/kg p.o.[119]**
Group III	**Diclofenac sodium 96 mg/kg p.o.[119]**
Group IV	**Diclofenac sodium 240 mg/kg p.o.[119]**
Group V	**DL-Methionine per se 700 mg/kg p.o.[132]**
Group VI	**DL-Methionine per se 1400 mg/kg p.o. [132]**
Group VII	**N-Acetylcysteine per se 450 mg/kg p.o.[133]**
Group VIII	**Diclofenac sodium 96 mg/kg p.o. + DL-Methionine 700 mg/kg p.o.**
Group IX	**Diclofenac sodium 240 mg/kg + DL-Methionine 700 mg/kg p.o.**
Group X	**Diclofenac sodium 96 mg/kg + N-Acetylcysteine 450 mg/kg p.o.**
Group XI	**Diclofenac Sodium 240 mg/kg + N-Acetylcysteine 450 mg/kg p.o.**
Group XII	**Diclofenac Sodium 96 mg/kg + DL-Methionine 1400 mg/kg p.o.**
Group XIII	**Diclofenac Sodium 240 mg/kg + DL-Methionine 1400 mg/kg p.o.**

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 [119] 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 haemato-biochemical 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°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 [134].

3.9 Demonstration of hepatoprotective effect of N-Acetylcysteine

After overnight fasting, the albino rats belonging to group VII was administered with N-Acetylcysteine [133] 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 Transpeptidase (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, γ-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:

L-Aspartate + 2-oxoglutarate $\xrightarrow{\text{SGOT/AST}}$ Oxaloacetate + L-Glutamate

Oxaloacetate + NADH $\xrightarrow{\text{MD}}$ Malate + NAD^+

Sample pyruvate + NADH $\xrightarrow{\text{LDH}}$ 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 $\xrightarrow{\text{ALT/GPT}}$ Pyruvate + L-Glutamate

Pyruvate + NADH $\xrightarrow{\text{LDH}}$ L-Lactate + NAD^+

Sample pyruvate + NADH $\xrightarrow{\text{LDH}}$ L-Lactate + NAD

1. The amino group is enzymatically transfered 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 I 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]**.

$$AMP + 4\text{-}NPP + H_2O \xrightarrow[Mg\ 2^{++}/Alkaline\ pH]{ALP} 4\text{-nitrophenol} + phosphate$$

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.

3.12.1 d 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.

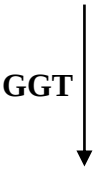
3.12.1 e Determination of serum Gamma glutamyltransferase (Serum GGT):

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 γ-glutamyl-3-carboxy-4-nitroanilide to glycylglycine forming glutamyl glycylglycine and 5-amino-2-nitrobenzoate.

L-γ-glutamyl-3-carboxy-4-nitroanilide + glycylglycine



L-γ-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 (**$p \leq 0.05$**).

CHAPTER :1

INTRODUCTION

The safety and efficacy of the drugs used in the treatment of various clinical conditions in any individual remains complex and multifactorial and difficult to analyse or identify the suspected drug that causes the Adverse Drug Reaction (ADR).

1.1 Role of liver in drug-induced hepatotoxicity:

Liver being a principle organ for playing several vital roles in the body, is involved in several biochemical pathways, metabolism of nutritional factors, metabolising the administered drugs or any substance that is ingested, which could be either herbal or even natural chemicals. Thus, making it important to observe, for the drug-induced hepatotoxicity, at all phases of drug development that includes the pre-clinical toxicity studies, the different phases of clinical trial including the post-marketing surveillance.

The Drug Induced Liver Injury (DILI) is defined as the injury caused by exposure to a drug or non-infectious toxic agent and is associated with different levels of organ dysfunction [1]. Despite the advancement in research at molecular level, understanding and characterizing the mechanisms involved in causing the Drug induced Liver Injury, it is still difficult to diagnose and identify the suspected drug.

1.2 Types of drug induced liver injury:

The drug induced liver injury are mainly of two types**:(1)** D**ose-dependent**, which is also called as predictable, direct toxicity, reproducible and occurs after the consumption of the drug that exceeds a known toxic threshold level. In such cases, the **liver injury** that occurs is **proportional** to the **administered dose** [2], example

Paracetamol; **(2)** while the **Dose-independent** Drug induced Liver Injury is also called as unpredictable and idiosyncratic that occurs even at the therapeutic doses, and the **liver injury** caused is **not always proportional** to the **administered dose**, further, the time of damage, onset can also vary example Diclofenac, Sulindac, Trovafloxacin [3, 4].

1.3 An overview of drug induced liver injury:

Paracelsus stated that, "all substances (drugs) are poisons; there is none which is not a poison. The right dose differentiates a poison and a remedy." Any drug, therefore, despite of its trivial therapeutic action has a potential to harm. With the limitations on toxicity studies and clinical trials, in the process of a new drug development, the adverse drug effects that occur may not be in total are detected, before introduced into the market for the patient's use. Therefore, it becomes imperative to detect the infrequent yet significant adverse drug reactions that occur when the drug has entered the market. This can be achieved by the post-marketing surveillance.

The liver injury caused by the drug may vary with the extent of the damage, ranging from mild fatty liver to necrosis. Though uncommon and rare, it is contributing to the morbidity and mortality in the general population and remains as a potential complication for most of the prescribed drugs [5, 6]. Despite of the relative frequency, little information is available on the long-term outcome of drug induced liver injury. The reasons could include missed diagnosis, difficulty in establishing definite diagnosis, particularly in cases where the hepatotoxicity is reversible following the drug withdrawal with limited long term follow up (Dantrolene-induced chronic hepatitis or Flucloxacillin-induced cholestasis) [7, 8].

1.4 Incidence of drug induced liver injury:

Although, the incidence of drug induced liver injury is found to be low, the probability of it should always be considered in any case of the liver injury. According to the literature study, the incidence was between 1 in 10,000 and 1 in 100,000 which was found to be increased from the evidences of the recent study. The information from the recent registries show an annual incidence of 19.1 cases per 10,0000 inhabitants in Iceland, 13.9 cases per 100,000 inhabitants in France, with hospitalization of 5% and mortality 6% [4].

A prospective study conducted in US have shown that, 13% of the total cases were diagnosed as idiosyncratic hepatotoxicity; while 39% with acetaminophen-induced hepatotoxicity, however, it was interesting to know with the recent prevalence rates in south-east Asian registries, which revealed that 70% of the drug induced liver injury cases occurred due to Herbal And Dietary Supplements (HDS), which is surprisingly found to be increased in its prevalence; even through the Western registers, attributing to 16% of the total drug induced liver injury to be due to Herbal And Dietary Supplements [9]. The drug induced liver injury has been found as an important cause of hospital admissions, which are increased over the decades and is 45% in Spain [10].

In India, the drug induced liver injury contributes to 1.4% of the gastrointestinal admissions and 2.5% of hepatobiliary admissions, with gradual increase in the numbers over a period of years, of which 0.7% were found to be Idiosyncratic Drug induced Liver Injury (IDILI) [11, 12]. Although, there occurs geographical difference in the common drugs causing Drug induced Liver Injury, **worldwide antimicrobials** are considered the most common particularly in Europe, Amoxicillin and flucloxacillin are found to be the common drugs in the Europe, while in **India, Antituberculosis** drugs

are contributing more to the drug induced liver injury[11, 13]. As compared to the Western world, where Paracetamol or Acetaminophen was found to be the leading cause of Acute Liver Failure (ALF), followed by the antimicrobials. In India, both in adults and children, the antituberculosis drugs have been the leading cause of for drug induced liver injury, followed by the Non-Steroidal Anti Inflammatory Drugs (NSAIDs) 10% [14]. The incidence of liver injury caused by the Non-Steroidal Anti Inflammatory Drugs is ranging from 1 – 9 cases per 100,000 persons exposed, indicating an increased risk of these preparations which remains as a common drug used in the treatment of the most painful conditions. Diclofenac sodium, widely used among the Non-Steroidal Anti Inflammatory Drugs, across the world is known for its hepatotoxicity, where more than 60 cases were reported by Bank and co-workers in 1995 [15], indicating that small number of hospitalisation 0.023% is the strongest evidence for it to bear hepatotoxic effect.

1.5 Mechanisms of drug induced liver injury:

The exact mechanisms of the drug induced liver injury remains unclear and depends on the hepatotoxicity that could be either predictable (Paracetamol) or unpredictable (Diclofenac, Sulindac, and Flucloxacillin). The mechanism involved, in causing hepatic injury-induced hypersensitivity and metabolic aberration, in case of **predictable hepatotoxicity**, massive hepatocellular **necrosis**, when the Paracetamol is consumed in large doses. It is known to release a toxic **metabolite N-acetyl-p-benzoquinone imine** (NAPQI), which depletes the hepatoprotective glutathione, which in turns results in mitochondrial dysfunction, oxidative stress, that culminates into cellular damage, causing necrosis and death [16], while in case of **idiosyncratic**; the **inflammatory stress** hypothesis is considered, which results to conjugate with the drug

metabolite, that has a potential to precipitate Drug induced Liver Injury, with an evidence of important role of the innate and adaptive immune system through; involved in the pathogenesis of Drug induced Liver Injury [17].

1.6 Risk factors of drug induced liver injury:

With a wide range of drugs, including Antimicrobials, NSAIDs, Antiepileptic, Antipsychiatric drugs etc., causing the drug induced liver injury, several factors are known to influence the drug induced liver injury, and are hence considered as the **risk factors** these includes; the age, gender, alcohol, concomitant use of drugs, nutrition, HIV, genetic factors, the dose and the body mass of the individual.

1.7 Evaluation of drug induced liver injury:

Apart from the clinical evaluation, the diagnosis includes the causality assessment to identify the suspected drug; evaluation of the biochemical parameters which indicate the liver functioning status, and further; the histopathological studies to reveal and confirm the clinical diagnosis. Liver imaging can also remain the infiltrative hepatic diseases and fatty live diseases. The histopathological information could be drug-specific and would indicate the severity and latency of the biochemical pattern.

Although, 90% of recoveries have been registered on discontinuation of the drug, some may progress with the outcome as chronic liver disease [18]. The prognosis has been poor in women, elderly, individuals with pre-existing liver disease; those habituated to alcohol and individuals with genetic defect. Hence, it is always important to monitor the liver enzymes which are indicative of the hepatotoxicity.

1.8 Treatment of drug induced liver injury:

The treatment for Drug induced Liver Injury mainly consists of discontinuation of the involved drug, followed by treatment with specific drugs. The specific drugs for the treatment of Drug induced Liver Injury are very scarce. However, N-Acetylcysteine (NAC) remains as a specific antidote for Paracetamol or Acetaminophen-induced toxicity, where it is known to benefit by replenishing the Glutathione stores. Similarly, as symptomatic treatment, drugs like Corticosteroids, Antihistamines, Cholestyramine, L-Carnitine, Folic acid, Methionine and Ursodeoxycholic acid have been used in the treatment of Drug induced Liver Injury [19, 20,].

1.9 Aim and Objectives of the Study:

1.9.1 AIM:

The present research was conducted to explore the hepatoprotective action of DL-Methionine and N-Acetylcysteine on the albino rats on dose-related hepatotoxicity of the hepatotoxic drug Diclofenac sodium.

1.9.2 OBJECTIVES:

1) To evaluate dose-dependent hepatic injury by orally administered Diclofenac sodium.

2) To evaluate the hepatic changes due to the dose dependent hepatic injury caused by Diclofenac sodium.

3) To demonstrate the hepatoprotective effect of DL-Methionine against the hepatotoxic drug Diclofenac sodium by oral route of administration in small animals.

4) To demonstrate the hepatoprotective effect of N-Acetylcysteine against the hepatotoxic drug Diclofenac sodium by oral route of administration in small animals.

5) To compare the hepatoprotective effect of DL-Methionine with N-Acetylcysteine.

6) To demonstrate the hepatoprotective effect of N-Acetylcysteine on hepatotoxic drug other than Paracetamol.

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Ervilla Dass

CHAPTER : 6

CONCLUSION

1. The positive control drug Diclofenac sodium was to be hepatotoxic in the dose of 96 and 240 mg/kg as evident with the changes that were observed by the biochemical parameters and histopathological studies.

2. Per se DL-Methionine and N-Acetylcysteine, although showed alteration in the biochemical parameters, they were not found to be significant so as to be considered as hepatotoxic agent.

3. It was observed that both DL-Methionine and N-Acetylcysteine had hepatoprotective effect against the single oral dose diclofenac sodium 96 and 240 mg/kg.

4. However, there was no much of a difference of hepatoprotective effect of both DL-Methionine (700 and 1400 mg/kg) and N-Acetylcysteine (450 mg/kg).

5. Thus, with our study, we conclude that although no much of a statistically significant difference is found between DL-Methionine and N-Acetylcysteine on its hepatoprotective activity, both have found to be hepatoprotective, in the doses used against the hepatotoxicity caused by diclofenac sodium (96 and 240 mg/kg) thus opening with a new area to evaluate more on the mechanism involved in DL-Methionine hepatoprotection which may also be confirmed by evaluating its hepatoprotective effect against known hepatotoxic drugs.

CHAPTER: 7

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ORIGINAL RESEARCH PAPER

Pharmacology

DICLOFENAC-INDUCED LIVER TOXICITY IN ALBINO RATS: DOSE-DEPENDENT STUDY.

KEY WORDS: Diclofenac, drug-induced hepatotoxicity, Serum markers, liver injury, NSAIDs

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ABSTRACT

Liver is the major organ which helps in detoxifying the drugs; during the process the long standing use of some may cause hepatotoxicity. The NSAIDs are major class, known to cause hepatotoxicity ex; Diclofenac, Sulindac and Aspirin. Hence, Diclofenac sodium was evaluated for its hepatotoxic effect. Albino rats were administered with Diclofenac sodium (72, 96 and 240 mg/kg) respectively as a single oral dose & 24-hours of post-treatment, serum levels of the liver enzymes were evaluated to demonstrate its hepatotoxic effect. Further, the liver was subjected for histopathological study. On statistical analysis Diclofenac had shown significant rise in the levels of serum SGOT & serum SGPT (< 0.05), when compared with the control, which was evident for the hepatotoxic effect of the Diclofenac sodium. It is concluded that, Diclofenac in above mentioned doses has hepatotoxic effect in rats.

INTRODUCTION

Diclofenac sodium was introduced in late 70's as a potent anti-inflammatory and analgesic preparation, which on long term use has shown hepatotoxic effects, which presented in the form of hepatic injury ranging from mild to fatal liver injury [1,2].

Liver being a principle organ for maintenance and regulation of the internal milieu is involved for the structural alterations of the administered drugs. It is the target organ, which gets exposed to the drugs in higher concentration, than other organs of the body, when they are orally administered[3]. Hence, it is the most vulnerable organ to be injured by the chemicals and the drugs, which leads to hepatic dysfunction. Generally, any drug in excess could be a burden on the liver causing toxic effects, but, sometimes, even the drugs introduced in therapeutic ranges may also injure the liver. Some of the commonly hepatotoxic drugs, include, antitubercular drugs such as Isoniazid, Rifampicin, Pyrazinamide, which contribute to hepatotoxicity, with the toxicity ranging between 2% to 28% (Girling 1978, D. Hong 1986)[4,5]. More than 900 drugs are known to be hepatotoxic[6,7,8] for example, Non-Steroidal Anti-inflammatory Drugs (NSAIDs) such as Acetaminophen, Nimesulide, Diclofenac, Ibuprofen, which are very commonly used in the treatment of rheumatological conditions and apart from which they are the most commonly used analgesics and antipyretics[9]. They also make the most important group of drugs used over-the-counter as OTC preparations and contribute to the adverse drug reactions (ADRs) [10]. Concurrent administration of Acetaminophen along with other hepatotoxic drugs is usually seen in many clinical situations[11]. Drug-induced liver injury (DILI) according to the recent estimates show the incidence of 14-19 cases per 100,000[4,5].

Hence, the study was taken up to evaluate the most commonly used preparations in today's practice, i.e. Diclofenac sodium, for its hepatotoxic effect.

MATERIALS AND METHODS

The study was conducted after obtaining the approval from the Institutional Animal Ethics Committee (IAEC), of S.B.K.S.M.I. & R.C., Sumandeep Vidyapeeth Deemed to be University, Piparia, Vadodara, considering the rules and regulations of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Albino rats of either sex weighing between 100 - 400g were used. Each animal was used only once. The animals were housed separately in poly-propylene rat-cages under controlled environmental conditions temperature 24° ± 2°C and 55% ± 5%, relative humidity, in a 12-hour light-/dark cycle throughout the experiment, which were kept fasting for 24 hours, before administering the drug.

The drugs and chemicals used were Diclofenac sodium from Aatur Instru Chem, Vadodara. The chemicals included 10% Formalin, Xylene, Hemotoxylin and Eosin stains.

To evaluate the levels of liver enzymes, serum Glutamic-Pyruvic Transaminase (SGPT), Serum Glutamic-Oxaloacetic Aminotransferases (SGOT), Serum Alkaline Phosphatase, Serum bilirubin – Direct and Indirect Bilirubin, Total Bilirubin ; Serum Gamma-Glutamyl Transpeptidase (GGTP) the diagnostic kit reagents (Erba Diagnostics, Manheim) was used.

All the drug solutions were freshly prepared before use and were administered orally with the volume of 10 ml/kg. The animals were divided in four groups, with each group containing 6 rats. The dose of Diclofenac sodium was selected based on the LD_{50} dose in rats, when administered orally[12].

The 24-hour fasted rats were administered orally with Distilled Water for the control group while the remaining three groups were administered with Diclofenac sodium in the doses of 72, 96 and 240 mg/kg respectively and were re-housed in the individual polypropylene cages.

Following 24 hours of post-treatment, under light ether anaesthesia, upto 3 ml of blood sample was collected from retro-orbital plexus by capillary method technique in the test tube, which was centrifuged at 3000 rpm for 10 minutes to separate serum that was subjected to analyse the Liver Function Tests (LFTs) so as to evaluate its hepatotoxic effect.

Liver from each animal was immediately dissected out and cleaned with normal saline and was preserved into the specimen collection jars that contained 10% formalin. The liver samples were quickly fixed in 10% formalin and embedded in paraffin. Sections of about 4-6 µm were stained with haematoxylin for 5 minutes at room temperature, 15 minutes later was counterstained with eosin for 2 minutes, washed with xylene and blocked by eosin for histopathological studies and were observed under photomicroscope.

RESULTS

Statistical analysis:

All the observed data were subjected for statistical analysis and the results were expressed as Mean± SEM. All calculations were

performed using statistical software SPSS version 21.0 computer-based. Values were considered to be significant when P values were less than or equal to 0.05 ($p \leq 0.05$).

Observations and results:

Albino rats (n=6) that were administered Distilled Water (10 ml/kg) was considered as control (Group- I). Diclofenac sodium administered as a single oral dose in each group (n=6) Group II (Diclofenac sodium 72 mg/kg), Group III (Diclofenac sodium 96 mg/kg) and Group IV (Diclofenac sodium 240 mg/kg), respectively, when compared to the control group showed statistical significant rise ($p < 0.0001$) in the serum SGPT and serum SGOT levels as indicated in the **table number 1** below, and as depicted in the figure 1 and figure 2, respectively.

Table - 1: Shows changes in the levels of liver enzymes, following administration of Diclofenac sodium (72 mg/kg, 96 mg/kg and 240 mg/kg).

Sr. no.	Liver Function Tests	Group (n=6)	Mean± SEM
1.	SGPT (IU/L)	Control DW 10 ml/kg	32.83 ± 2.91
		Diclofenac 72 mg/kg	85.67 ± 7.33***
		Diclofenac 96 mg/kg	147.67 ± 13.72***
		Diclofenac 240 mg/kg	236.50 ± 24.01***
2.	SGOT (IU/L)	Control DW 10 ml/kg	126.00 ± 15.07
		Diclofenac 72 mg/kg	528.33 ± 86.50***
		Diclofenac 96 mg/kg	1220.83 ± 130.50***
		Diclofenac 240 mg/kg	1490.00 ± 168.88***
3.	Total Serum Bilirubin (µmol/L)	Control DW 10 ml/kg	0.70 ± 0.08
		Diclofenac 72 mg/kg	01.13 ± 0.12
		Diclofenac 96 mg/kg	1.07 ± 0.12
		Diclofenac 240 mg/kg	1.25 ± 0.11
4.	Serum ALP (IU/L)	Control DW 10 ml/kg	106.17 ± 23.15
		Diclofenac 72 mg/kg	120.17 ± 23.551
		Diclofenac 96 mg/kg	153.83 ± 32.01
		Diclofenac 240 mg/kg	229.00 ± 32.06
5.	Serum GGTP (IU/L)	Control DW 10 ml/kg	2.33 ± 0.56
		Diclofenac 72 mg/kg	04.95 ± 1.45
		Diclofenac 96 mg/kg	3.03 ± 1.40
		Diclofenac 240 mg/kg	1.60 ± 0.28

Note:

* p value < 0.05 = significant, values are presented as Mean± SEM

Serum Glutamic-Pyruvic Transaminase (SGPT), Serum Glutamic-Oxaloacetic Aminotransferases (SGOT), Total serum bilirubin, Alkaline Phosphatase (ALP) and Gamma Glutamyl Transpeptidase (GGTP) or γ-Glutamyl Transferase (GGT), DW = Distilled Water.

However, there was no statistically significant rise in the serum levels of total serum bilirubin, alkaline phosphatase and γ-Glutamyl Transferase, as shown in figure 3, 4 and 5 as below.

Figure 1: Changes in the Serum Glutamic-Pyruvic Transaminase (SGPT) levels

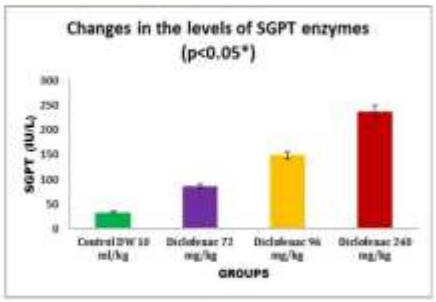


Figure 2: Changes in the Serum Glutamic-Oxaloacetic Aminotransferases (SGOT) levels.

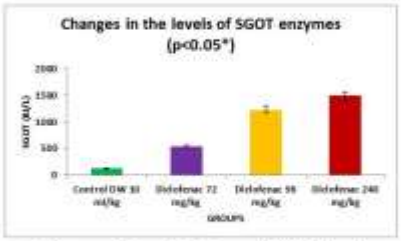


Figure 3: Changes in the Total Serum Bilirubin levels.

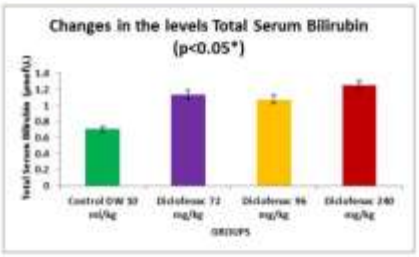


Figure 4: Changes in the serum Alkaline Phosphatase (ALP) levels.

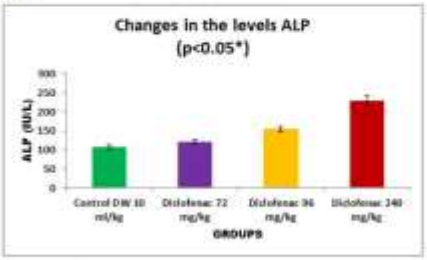
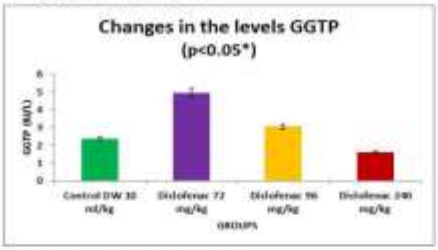


Figure 5: Changes in the Serum Gamma Glutamyl Transpeptidase (GGTP) levels.



Histopathological Observations:

i. Gross appearance of the liver sample:

The gross appearance of liver of albino rats administered with Distilled Water, did not show any abnormal changes in texture, shape, size or colour. It was reddish brown and showed typical lobular architecture; whereas, those treated with Diclofenac sodium were pale yellow to pale brown colour.

ii. Microscopic examination of liver:

The evidence of changes in the liver cells from the liver sections of Diclofenac sodium at 72 mg/kg, 96 mg/kg & 240 mg/kg, p.o. showed dose-dependent changes in the liver section.

The histopathological changes observed in the liver tissue sections shows mainly hepatocellular changes along with the changes in the portal area, which also revealed microvesicular vacuolation, that is diffuse hepatic vacuolation (degeneration) seen which was dose-dependent and marked congestion. Whereas, irreversible changes in the tissue such as severe hepatocellular degeneration or centrilobular focal necrosis was not seen.

Figure number 6: Liver sections from control rats showing central vein low power

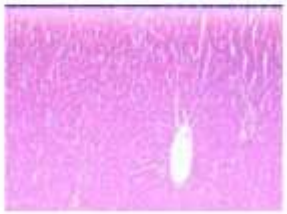


Figure number 7a: – Liver section from diclofenac sodium 72 mg/kg

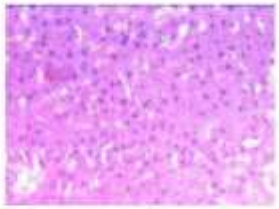


Figure number 7b – Liver section from diclofenac sodium 96 mg/kg

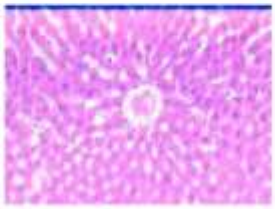
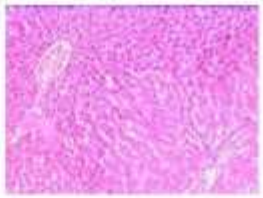


Figure number 7c - Liver section from diclofenac sodium 240 mg/kg



DISCUSSION

The NSAIDs are considered as the major groups to cause hepatotoxicity, since they are used as both prescriptive and OTC preparations [9]. Of the currently used NSAIDs, the most common drugs associated with liver disease include; Didofenac, Sulindac and Aspirin [9].

Diclofenac sodium being widely used Non steroidal anti-inflammatory and analgesic compound, we have observed for the hepatotoxic effect of single dose of Didofenac sodium in dose-dependent manner, in the albino rats.

Diclofenac had shown statistically significant rise ($p < 0.0001$) in the levels of serum SGOT and serum SGPT, when compared with the control group, which was evident for the hepatotoxic effect of the diclofenac sodium in all the three doses 72 mg/kg, 96 mg/kg & 240 mg/kg as a single oral dose. This observation of ours concurs with the observations made by D. Schapira et al [12].

Along with the rise in the serum levels, the histopathological studies have demonstrated the toxic effects of diclofenac sodium in the form of sinusoidal dilatation, cytoplasmic vacoulation and mild portal congestion.

CONCLUSION

With observations made by the authors, Diclofenac in the doses of 72 mg/kg, 96 mg/kg & 240 mg/kg as a single oral dose has shown to be hepatotoxic in albino rats.

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DECLARATIONS

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Original Research Article

Hepatoprotective effect of DL-methionine on diclofenac-induced hepatotoxicity in albino rats: an experimental study

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ABSTRACT

Background: Liver is the main detoxifying organ, which is affected by most of the drugs and xenobiotic agents that could result in liver damage. The present study was designed to evaluate the hepatoprotective effect of DL-Methionine against experimentally induced liver injury in albino rats.
Methods: Hepatotoxicity was induced by administering high doses of positive control drug Diclofenac sodium in albino rats, which was confirmed by estimating Liver Function Tests. Hepatoprotective effect was determined by administering DL- Methionine concomitantly with positive control drug. Albino rats were administered with DL-Methionine (700 mg/kg and 1400 mg/kg) respectively as a single oral dose, concomitantly with positive control drug Diclofenac sodium (96 mg/kg and 240 mg/kg) respectively. After 24-hours of post-treatment, serum levels of the liver enzymes were evaluated to demonstrate the hepatoprotective effect of DL-Methionine on drug-induced hepatotoxicity, and all the liver samples were examined for the histopathological study.
Results: Significant increase in serum transaminase enzymes were observed by the positive control drug Diclofenac sodium. There was significant reduction in the serum transaminases on concomitant administration of DL-Methionine with Diclofenac sodium. Liver injury induced by positive control drug, and its protection with DL-Methionine was revealed by histopathological study. The combination of Diclofenac sodium and DL-Methionine showed no significant histopathological difference when compared to the normal liver section.
Conclusions: The results reveal that, DL-Methionine significantly prevented the rise in transaminases levels produced by hepatotoxic doses of the positive control drug.

Keywords: Diclofenac, Drug-induced hepatotoxicity, Liver injury, NSAIDs, Serum markers

INTRODUCTION

Liver as a major organ involved in drug metabolism is susceptible to the injury when exposed to drugs, chemicals and xenobiotics, which is generally indicated by the elevated levels of serum enzymes in the liver.[1] Chemicals that cause liver injury are called hepatotoxins. Hepatotoxicity could be idiosyncratic or non-idiosyncratic.[2] However, the drug-induced liver toxicity has been a major concern with the hepatotoxic drugs such as antitubercular drugs (Isoniazid, Rifampicin, Pyrazinamide), Non-Steroidal Anti-inflammatory Drugs (NSAIDs) such as Ibuprofen, Diclofenac, Sulindac, Aspirin, Paracetamol which are commonly used as, anti-inflammatory and analgesics antipyretic preparations.[3]

The major concern with the group of NSAIDs is they belong to the class of non-prescription and commonly used Over-the-Counter (OTC) preparations.[4] The toxicity induced by these drugs have been the major concern since most of these are used for the long-term treatment, which has been a cause of withdrawal of the preparations

from the market or their termination during the clinical trials.[9]

Hepatocytes death is a characteristic presentation that occurs in case of liver injury, mainly due to the fibrosis and necrosis, which are generally prevented by N-Acetylcysteine, particularly in case of Acetaminophen toxicity.[6-9,3]

Similarly, the essential amino acid, Methionine has also found to be a beneficial hepatoprotective agent which is also been proposed for the treatment of certain disease condition.[3,10-12] Hence, the present study was taken up to demonstrate its hepatoprotective effect on Diclofenac-induced hepatotoxicity.

METHODS

Albino rats of either sex weighing between 100-400gm were used. Each animal was used only once. The animals were housed separately in poly-propylene rat-cages under controlled environmental conditions temperature 24°±2°C and 55%±5%, relative humidity, in a 12-hour light-/dark cycle throughout the experiment, which were kept fasting for 24hours, before administering the drug.

The drugs and chemicals used were Diclofenac sodium and DL-Methionine from Aatur Instru Chem, Vadodara. The chemicals included 10% Formalin, Xylene, Hemotoxylin and Eosin stains. To evaluate the levels of liver enzymes, serum Glutamic-Pyruvic Transaminase (SGPT), Serum Glutamic-Oxaloacetic Aminotransferases (SGOT), Serum Alkaline Phosphatase, Serum bilirubin-Direct and Indirect Bilirubin, Total Bilirubin; Serum Gamma-Glutamyl Transpeptidase (GGTP) the diagnostic kit reagents (Erba Diagnostics, Manheim) was used.

Diclofenac sodium in the doses of 96mg/kg and 240mg/kg are used as positive control drug for their hepatotoxic effects and DL-Methionine 700mg/kg and 1400mg/kg are used as the test drugs to evaluate their hepatoprotective action on toxicity induced by Diclofenac sodium; by evaluating the liver enzymes and histopathological studies of liver.

The animals were grouped into seven groups (n=6). Group I (Control) was treated with Distilled Water 10ml/kg; while Group II, and III were treated with Diclofenac 96mg/kg and 240mg/kg respectively, which were considered as positive control group to demonstrate their hepatotoxic action.

Group IV was treated with DL-Methionine 700mg/kg and Diclofenac 96mg/kg concomitantly, Group V was treated with DL-Methionine 700mg/kg and Diclofenac 240mg/kg concomitantly; Group VI was treated with DL-Methionine 1400mg/kg and Diclofenac 96mg/kg concomitantly and Group VII was treated with DL-Methionine 1400mg/kg and Diclofenac 240mg/kg concomitantly.

The 24-hour fasted albino rats were administered with Diclofenac sodium and DL-Methionine as mentioned above. Later, after 24-hours of post-treatment, 3ml of blood sample was collected from retro-orbital plexus by capillary method technique, under light ether anaesthesia; that was centrifuged at 3000rpm for 10minutes to obtain the serum that was subjected to analyse the levels of liver enzymes. Liver from each animal was immediately dissected out and cleaned with normal saline and was preserved into the specimen collection jars that contained 10% formalin. The liver samples were quickly fixed in 10% formalin and embedded in paraffin. Sections of about 4-6µm were stained with haematoxylin for 5minutes at room temperature; 15minutes later was counterstained with eosin for 2minutes; washed with xylene and blocked by eosin for histopathological studies and were observed under photomicroscope.

Statistical analysis

All the observed data were subjected for statistical analysis and the results were expressed as Mean± SEM. All calculations were performed using statistical software SPSS version 21.0 computer-based. Values were considered to be significant when P values were less than or equal to 0.05 ($P \leq 0.05$).

RESULTS

The positive control drug Diclofenac sodium in the dose of 96mg/kg and 240mg/kg showed significant rise (P value < 0.0001) of serum SGPT and SGOT level when compared to the control group.

On concomitant administration of DL-Methionine 700mg/kg with Diclofenac sodium 96mg/kg and 240mg/kg (Group IV and Group V) respectively; there occurred significant reduction ($p<0.05$) in the serum SGOT and SGPT levels as compared to control and positive control group. Similarly, a significant reduction in the serum SGPT as shown in Table 1 and Figure 1, and serum SGOT levels, as shown in Figure 2, was observed in Groups VI and VII, which were treated concomitantly with DL-Methionine 1400mg/kg and Diclofenac sodium 96mg/kg and 240mg/kg respectively.

However, in both the doses of DL-Methionine (700mg/kg and 1400mg/kg), with Diclofenac sodium 96mg/kg and 240mg/kg, there occurred no statistically significant changes in the other liver enzymes such as, Total Serum Bilirubin, serum Alkaline Phosphatase and serum Gamma-Glutamyl Transpeptidase (GGTP) levels as indicated in Table 1 and Figure 3, 4 and 5.

Histopathological examination

Gross appearance of liver

The gross appearance of liver of albino rats in control group showed reddish to brown colour. On administration

of positive control drug Diclofenac sodium, which were pale yellow, on concomitant administration of DL-Methionine with hepatotoxic drug Diclofenac sodium, showed near normal gross appearance of liver, of mild reddish-brown color.

Table 1: Hepatoprotective action of DL- methionine, on concomitant administration of positive control drug diclofenac sodium.

Group (n = 6)	Biochemical parameters of LFTS Mean± SEM values				
	SGPT (IU/L)	SGOT (IU/L)	Total serum bilirubin (µmol\L)	ALP (IU/L)	GGTP (IU/L)
Control DW 10ml/kg	32.83±2.91	126.00±15.07	0.70±0.08	106.17±23.15	2.33±0.56
Diclofenac 96 mg/kg	147.67±13.72***	1220.83±130.50***	1.07±0.12	153.83±32.01	3.03±1.40
Diclofenac 240mg/kg	236.50±24.01**	1490.00±168.88***	1.25±0.11	229.00±32.06	1.60±0.28
Diclofenac sodium 96mg/kg + DL-methionine 700mg/kg	57.17±5.19**	295.00±22.87**	0.95±0.08	151.17±8.42	2.42±0.30
Diclofenac sodium 240mg/kg + DL-methionine 700mg/kg	69.17±3.57**	395.83±20.95**	1.01±0.09	136.83±27.79	7.38±1.62
Diclofenac sodium 96mg/kg + DL-methionine 1400mg/kg	43.00±4.25***	225.17±9.27**	0.88±0.09	133.83±16.07	2.76±0.88
Diclofenac sodium 240mg/ml + DL-methionine 1400mg/kg	69.50±6.76**	301.83±22.76**	1.11±0.20	134.50±31.48	2.98±0.44

** p value < 0.05 = significant, **p <0.001 = highly significant and ***p value < 0.0001 = very highly significant, values are presented as Mean± SEM, Serum Glutamic-Pyruvic Transaminase (SGPT), Serum Glutamic-Oxaloacetic Aminotransferases (SGOT), Total serum bilirubin, Alkaline Phosphatase (ALP) and Gamma Glutamyl Transpeptidase (GGTP) or γ-Glutamyl Transferase (GGT), DW = Distilled Water*

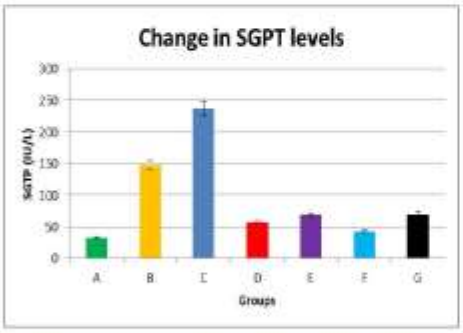


Figure 1: Changes in the Serum Glutamic-Pyruvic Transaminase (SGPT) levels on concomitant administration of DL-methionine and the positive control drug.

Microscopic examination of liver

The histopathological changes observed in the liver tissue in control group revealed normal liver architecture (Figure 6).

Diclofenac treated rats, shows mainly hepatocellular changes in the portal area, mainly microvesicular vacuolation, that is diffuse hepatic vacuolation degeneration, cytoplasmic vacuolation and sinusoidal dilatation.

Portal congestion was markedly seen in the hepatotoxic drug Diclofenac sodium, compared to those concomitantly administered with DL-Methionine. (Figure 7(a), 7(b) and 7(c)).

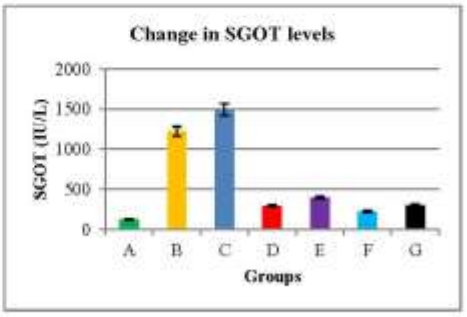


Figure 2: Changes in the Serum Glutamic-Oxaloacetic Aminotransferases (SGOT) levels on concomitant administration of DL-Methionine and the positive control drug.

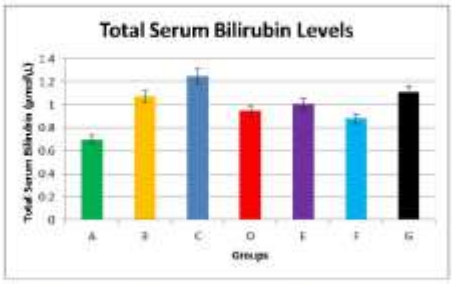


Figure 3: Changes in the Total Serum Bilirubin levels on concomitant administration of DL-methionine and the positive control drug.

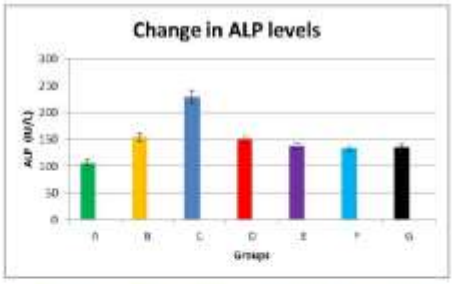
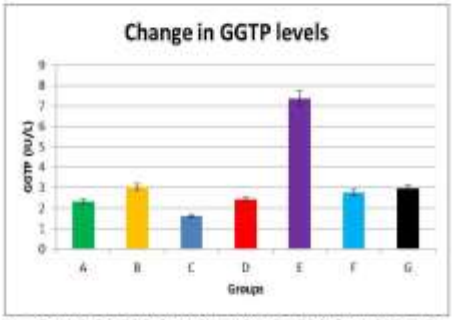


Figure 4: Changes in the serum Alkaline Phosphatase (ALP) levels on concomitant administration of DL-methionine and the positive control drug.



A = Control, B= Diclofenac 96 mg/kg, C= Diclofenac 240 mg/kg, D = Diclofenac sodium 96 mg/kg, + DL-Methionine 700 mg/kg, E = Diclofenac sodium 96 mg/kg + DL Methionine 1400 mg/kg, F = Diclofenac sodium 240 mg/ml + DL Methionine 1400 mg/kg (shown in Figure 1-5).

Figure 5: Changes in the Serum Gamma Glutamyl Transpeptidase (GGTP) levels on concomitant administration of DL-methionine and the positive control drug.

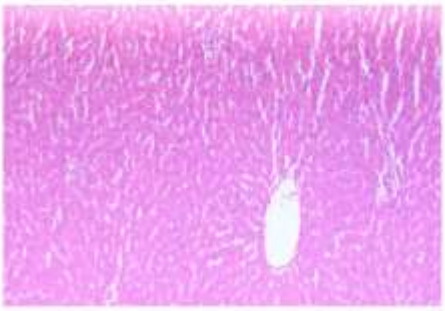


Figure 6: Liver sections from control rats showing central vein.

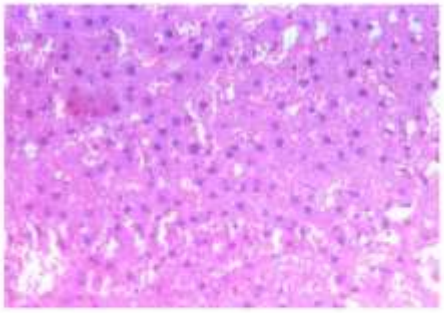


Figure 7(a): Liver section from diclofenac sodium 72mg/kg.

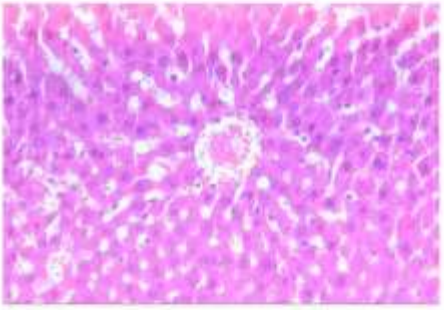


Figure 7(b): Liver section from diclofenac sodium 96mg/kg.

Diffuse hepatic vacoular degeneration was observed prominently in Diclofenac treated rats, which was reduced extensively in the DL-Methionine treated rats (Figure 8(a), 8(b), 8(c) and 8(d)).

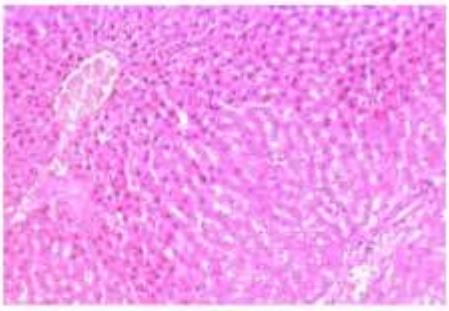


Figure 7(c): Liver section from diclofenac sodium 240mg/kg.

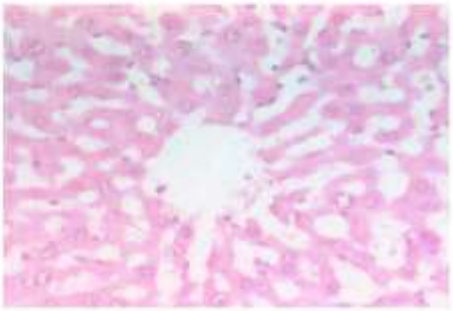


Figure 8(a): Liver section from diclofenac sodium 96mg/kg + DL-methionine 700mg/kg treated rats.

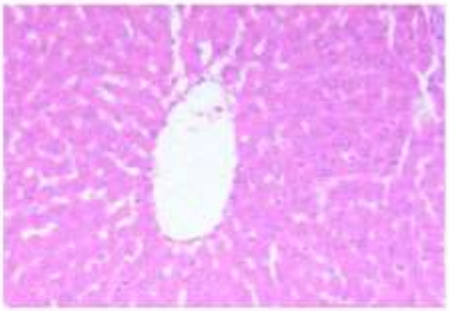


Figure 8(b): Liver section from diclofenac sodium 240mg/kg + DL-methionine 700mg/kg treated rats.

DISCUSSION

It was observed by the authors that, the positive control drug Diclofenac sodium 96mg/kg and 240mg/kg caused hepatotoxicity, which was indicated with the significant rise ($P < 0.05$) of serum enzymes both SGOT and SGPT levels. While on the concomitant administration of the positive control drug with DL-Methionine, it was observed that there occurred a significant reduction ($P < 0.05$) in serum levels of SGOT, SGPT in both the groups which received 96mg/kg and 240mg/kg of the hepatotoxic drug Diclofenac sodium.

This observation concurs with that of observations made by Dass E et al.[3] It also proves DL-Methionine to be a good hepatoprotective agent as shown by Anstt QM et al.[13]

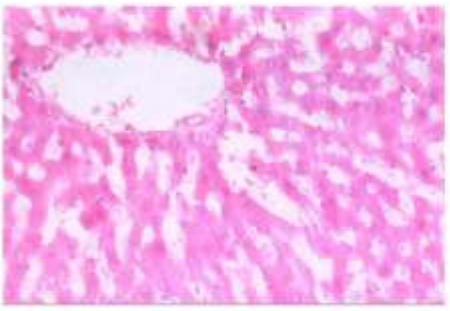


Figure 8(c): Liver section from diclofenac sodium 96mg/kg + DL-methionine 1400mg/kg treated rats.

CONCLUSION

With the observations made in the present study, we conclude that DL-Methionine is a hepatoprotective agent as it has protected the hepatotoxicity induced by Diclofenac sodium, a known NSAID to cause hepatotoxicity. Although, N-Acetylcysteine is an established hepatoprotective agent for paracetamol-induced hepatotoxicity, from the present study, it is evident that Methionine also has been a hepatoprotective agent on Diclofenac sodium which belongs to the class of NSAIDs.

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