HEPATOPROTECTIVE EFFECT OF GOAT MILK AGAINST ANTI-TUBERCULAR DRUG INDUCED HEPATOTOXICITY IN RATS

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This is to submit that this written submission in my thesis entitled "Hepatoprotective effect of goat milk against anti-tubercular drug induced hepatotoxicity in rats" represents original ideas in my own words and where others ideas or words have been included, I have adequately cited and referenced the original sources. I also declare that I have stuck to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that any violation of the above will be cause for disciplinary action by the School and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when required.

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DEDICATED TO…..

MY PARENTS

ABSTRACT

Aim: To study the hepatoprotective effect of goat milk against anti-tubercular drug induced hepatotoxicity in rats.

Materials and methods: Adult albino rats (Wister strain) of either sex weighing 200-300 gram were used (n=30). Experimental hepatotoxicity was induced by oral administration of Isoniazid (H) + Rifampicin (R) + Pyrazinamide (Z) suspension (27 +54+135mg/kg) for 30 days. Rats were divided into five groups (n=6). Group I: Vehicle control (gum acacia, 2%), Group II: H+R+Z suspension (27+54+135mg/kg), Group III: H+R+Z suspension (27+54+135mg/kg) + Goat milk (20ml/kg), Group IV: H+R+Z suspension (27+54+135mg/kg) + Goat milk (40ml/kg), Group V: H+R+Z suspension (27+54+135mg/kg) + Silymarin (50mg/kg). The study period was of 30 days, in which animals were treated with H+R+Z suspension and the test group was administered goat milk. After 30 days, the animals were sacrificed for the investigation of various morphological, histopathological and biochemical parameters.

Results: Anti-tubercular drug induced hepatotoxicity was successfully reproduced. At the same time administration of goat milk along with anti-tubercular drugs significantly prevented the rise in levels of serum alanine aminotransferase, serum aspartate aminotransferase and tissue malondialdehyde. Administration of goat milk reduced inflammation, degeneration and necrotic changes in hepatocytes. Similarly goat milk significantly prevented fall in serum total protein and superoxide dismutase as compared to group II, which received anti-tubercular drug suspension alone.

Conclusion: These results showed that goat milk is effective as hepatoprotective agent and prevented the anti-tubercular drug induced hepatotoxicity. However, the effects produced by both the groups receiving goat milk were not statistically different.

Keywords: Hepatotoxicity, goat milk, anti-tubercular drug induced hepatotoxicity, silymarin.

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LIST OF ABBREVIATION

Abbreviations	Full Forms
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AST	Aspartate transaminase
ATP	Adenosine triphosphate
CMA	Cow's milk allergy
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
GGT	Gamma-glutamyl transpeptidases
GI	Gastrointestinal
H	Isoniazid
IL	Interleukin
INF	Interferon
R	Rifampicin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SGOT	Serum glutamate pyruvate transaminase
SGPT	Serum glutamate oxaloacetate transaminase
SOD	Superoxide dismutase
TB	Tuberculosis
TNF	Tumor necrosis factor
Z	Pyrazinamide

1. INTRODUCTION

1. INTRODUCTION

Liver plays a pivotal role in the transformation and clearance of chemicals, drugs, and xenobiotics, so it is the main target for the toxicity of these agents. The damage to the liver is known as hepatotoxicity and these damage causing agents are called hepatotoxins. (Swaroop et al., 2012) Hepatotoxins get converted into chemically reactive metabolites in liver, which have ability to interconnect with cellular macromolecules such as lipids, proteins, nucleic acid and then lead to lipid peroxidation, protein dysfunction and DNA damage. This damage of cellular function can lead to cell death and likely liver failure. These hepatotoxins also cause the depletion of antioxidants profile of hepatic tissues and induce lipid peroxidation. (Singh et al., 2012) Hepatotoxins promote the further liver damage by accumulation of bile acid inside the liver and this is caused due to injury to hepatocyte. The damaging hepatocyte results in the activation of innate immune system kupffer cells, natural killer cells and natural killer T cells and results in producing pro-inflammatory mediators which produces liver injury. (Singh et al., 2012) Certain medicinal agents when taken in overdoses and sometimes even within therapeutic ranges may cause hepatotoxicity. Infact, drugs are one of the main reasons for hepatotoxicity. Hepatotoxicity is a serious adverse effect of currently used first line anti-tubercular drugs like isoniazid, rifampicin and pyrazinamide. These drugs individually have been associated with hepatotoxicity and the risk is enhanced when these drugs are used in combination. (Swaroop et al., 2012) Anti-tubercular drug induced hepatotoxicity is one of the most challenging clinical problems and the main cause of treatment interruption during tuberculosis treatment course that causes hospitalization and life threatening event. Anti-tubercular drug induced hepatotoxicity has a wide spectrum of presentations, ranging from asymptomatic mild rise in liver biochemical tests to acute hepatitis and acute liver failure. It is often suggested to be mediated through oxidative stress, leading to generation of lipid peroxidation and alteration in antioxidants. (Chandane et al., 2013) Lipid peroxidation occurs as a chain reaction initiated by excess production of free radicals. In response to deleterious effects of free radical induced lipid peroxidation, cells activate antioxidant defense mechanisms in which superoxide dismutase and reduced glutathione act synergistically to detoxify the effects of lipid peroxidation. Therefore, it is suggested that the agents which reduce the lipid peroxide content in tissue and increase the intracellular antioxidant defenses may have protective effects on the liver in people taking anti-tubercular treatment.

Currently very few reliable liver-protective drugs are available in the allopathic armamentarium. They results are unsatisfactory and they add to the pill burden. Management of drug induced hepatotoxicity is still a challenge to the modern medicine. So, there is a strict need to screen herbal products or nutraceuticals which can be taken by the patients as food during treatment of tuberculosis.

Goat milk is a natural product obtained from *Capra aegagrushirus* (a species of goat, Family Bovidae). It is rich in various physiologically functional components, including proteins, vitamins (such as vitamins E and C), flavonoids, and carotenoids with antioxidant properties. Goat milk is considered to possess high antioxidant activity that resists oxidative stability and highly protects consumers from exposure to oxidative stress. (Rahmat et al., 2001) A study has reported that goat milk consumption potentiates liver divalent metal transporter 1 (DMT1) expression thereby enhancing Fe metabolism and storage indicating its potential in anaemia. Furthermore, few studies have also demonstrated its anti-inflammatory and anti-oxidant properties which indicate that goat milk may possess hepatoprotective activity. (Daddaouna et al., 2006) The hepatoprotective activity of goat milk in anti-tubercular drug induced hepatotoxicity has not been investigated yet. So the rationale of the present study was to explore the effect of goat milk on antitubercular drug induced hepatotoxicity in rats.

2. REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Liver

The liver is among the most complex and important organ in the human body. It is both the largest internal organ and largest gland in the human body. It lies below the diaphragm in the abdominal pelvic region of the abdomen. It is an organ of supreme importance and plays a crucial role not only in metabolism and disposition of exogenous toxins but also in biochemical regulation of fats, carbohydrates, amino acids, proteins, blood coagulation and immune modulation. (Singh et al., 2012)

2.1.1 Structure of liver

The liver is a reddish brown organ with four lobes of unequal size and shape. It is connected to two large blood vessels, one called hepatic artery and one called the portal vein. It constitutes about 2.5% of an adult's body weight. During rest, it receives 25% of the cardiac output via the hepatic portal vein and hepatic artery. The hepatic portal vein carries the absorbed nutrients from the GI tract to the liver, which takes up, stores and distributes nutrients and vitamins. It produces bile, an alkaline compound which aids in digestion via the emulsification of lipids. Two major types of cells populate the liver lobes: parenchymal and nonparenchymal cells. 80%of the liver volume is occupied by parenchymal cells commonly referred to as hepatocytes. Non-parenchymal cells constitute 40% of the total number of liver cells but only 6.5% of its volume. Sinusoidal endothelial cells, kupffer cells and hepatic stellate cells are some of the non-parenchymal cells that line the hepatic sinusoid. (Singh et al., 2012)

2.1.2 Functions of liver

The liver has well over 500 functions and is known as the laboratory of the human body. The liver is tied to all bodily processes because it is responsible for filtration of all incoming foods and fluids. The body relies upon the liver to remove toxins so that nutrients supplied to the body are pure and capable of providing nourishment. Many scientists believe the liver is connected to or at least aware of every disease or dysfunction that is happening inside the body. (Swaroop et al., 2012)

2.1.2.1 Carbohydrate metabolism

Liver maintains the normal blood glucose level. It can converts glucose to glycogen (glycogenesis) when blood sugar level is high and breakdown of glycogen to glucose

(glycogenolysis) when blood sugar level is low. Also liver can convert amino acid and lactic acid to glucose (gluconeogenesis) when blood sugar level is low.

2.1.2.2 Lipid metabolism

Liver stores some triglycerides breakdown fatty acids into acetyl coenzyme-A, this process is called as oxidation and converts excess acetyl coenzyme A into ketone bodies (ketogenesis). It synthesis lipoproteins. Hepatic cells synthesize cholesterol and use cholesterol to make bile salts

2.1.2.3 Protein metabolism

The liver removes the amino group from amino acids so that they can be used for ATP production. It converts the resulting toxic ammonia into the much less toxic urea for excretion of urine. Hepatic cells synthesize plasma protein such as alpha and beta globulin, albumin, prothrombin and fibrinogen.

2.1.2.4 Haematological function

The liver produces coagulation factors 1, 2, 5, 7, 9, 10 and 11 as well as protein C, protein S and antithrombin. In the first trimester foetus, the liver is the main site of red blood cell production. By the 32^{nd} week of gestation, the bone marrow has almost completely taken over that task.

2.1.2.5 Secretion and exertion of bile

Bile is partially an excretory product and partially a digestive secretion. Each day the hepatic cells secrete 800-1000ml of bile, a yellow or olive green liquid. It has pH of 7.6-8.6. Bile mainly consists of water, bile salts, cholesterol and a phospholipid called lecithin, bile pigments and several ions. The principle bile pigment is bilirubin. When worn out red blood cells broken down, iron, globin's and bilirubin are released.

2.1.2.6 Breakdown

The breakdown of insulin and other hormones. The liver glucoronidates bilirubin, facilitating its excretion into bile. The liver breaks down or modifies toxic substances and most medicinal products in a process called drug metabolism. This sometimes results in toxication, when the metabolite is more toxic than its precursor. Preferably, the toxins are conjugated to avail excretion in bile or urine. The liver converts ammonia to urea (urea cycle)

2.1.2.7 Other functions

The liver stores a multitude of substances, including glucose, vitamin A, and vitamin D, vitamin B12, iron and copper. The liver is responsible for immunological effects. The liver produces albumin, the major osmolar component of blood serum. The liver synthesizes angiotensinogen, a hormone that is responsible for raising the blood pressure when activated by rennin, an enzyme that is released when kidney senses low blood pressure. The liver also produces insulin like growth factor, a polypeptide protein hormone that plays an important role in childhood growth and continues to have anabolic effects in adults. The liver is the main site of thrombopoietin production. Thrombopoietin is a glycoprotein hormone that regulates the production of platelets by the bone marrow. (Swaroop et al., 2012)

2.2 Hepatotoxicity

Hepatotoxicity implies chemical-driven liver damage. Certain medicinal agents, when taken in overdoses and sometimes even when introduced within therapeutic ranges, may injure the organ. Other chemical agents, such as those used in laboratories and industries, natural chemicals and herbal remedies can also induce hepatotoxicity. Chemicals that cause liver injury are called hepatotoxins. (Saim et al., 2013) These agents are converting in chemically reactive metabolites in liver, which have ability to interconnect with cellular macromolecules such as proteins, lipids and nucleic acids, leading to protein dysfunction, lipid per oxidation, DNA damage and oxidative stress. This damage of cellular function can dismiss in cell death and likely liver failure. More than 900 drugs have been implicated in causing liver injury and it is most common reason for a drug to be withdrawn from the market. Chemicals often cause subclinical injury to liver which manifests only as abnormal liver enzyme tests. Liver plays a pivotal role in regulating various physiological processes. It is also involved in several vital functions, such as metabolism, performance and regulating homeostasis of the body. It involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction. It aids metabolism of carbohydrate, protein and fat, detoxification, secretion of bile and storage of vitamins. The role played by this organ in the removal of substances from the portal circulation makes it susceptible to first and persistent attack by offending foreign compounds, culminating in liver dysfunction.

2.2.1 Hepatotoxicants

Hepatotoxicants are of two major groups, direct and indirect hepatotoxicants. The direct hepatotoxicants are those which damage the hepatocytes directly resulting interference in cell

metabolism. On the other hand, the indirect hepatotoxins are which causes hepatic injury as a result of selective interference with metabolic pathways.

Toxins affecting the liver are of three classes, namely biological, chemical and physical toxins.

Biological toxins: Aflatoxins, Senecio alkaloids and Amantia mushrooms

Physical toxins: Hyperthermia, Burns and Irradiation

Chemical toxins: Carbon tetrachloride, Tetrachloroethane, Chloropenitone, Benzene derivatives, Tannic acid, Phosphorus, Iron and Arsenic

2.2.2 Drug induced hepatotoxicity

Drug induced liver injury is responsible for 5% of all hospital admissions and 50% of all acute liver failures. More than 75% of cases of idiosyncratic drug reactions result in liver transplantation or death. (Kshirsagar et al., 2008) Figure 2.1 showing the types of drug induced hepatotoxicity.

2.2.3 Factors influencing drug induced hepatotoxicity

- Ethnicity and race
- Age
- Gender
- Nutritional status
- Underlying liver disease
- Renal function
- Pregnancy
- Duration and dosage of drug
- Enzyme induction
- Drug to drug interaction

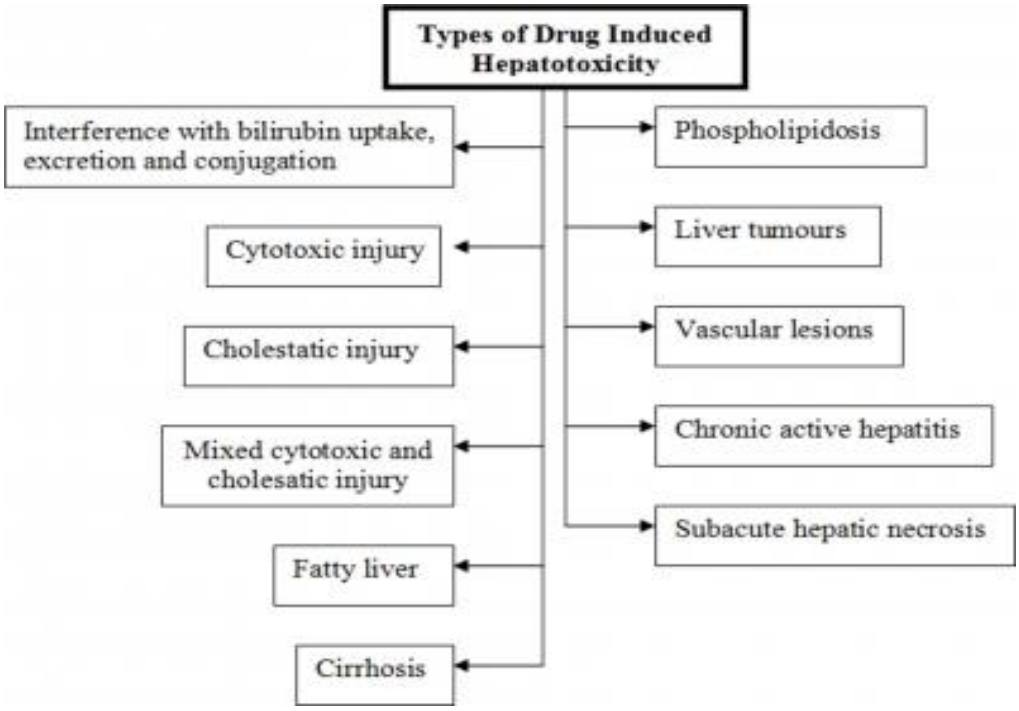


Figure 2.1: Types of drug induced hepatotoxicity

2.2.4 Different models for hepatotoxicity

2.2.4.1 Carbon tetrachloride (CCl_4) induced hepatotoxicity

CCl_4 was previously used as fire extinguisher, grain fumigant and for degreasing the metals. It is used as hepatotoxic agent for inducing both acute and chronic liver failure. The injury caused by this can be attributed to a number of mechanisms. The formation and release of free radicals and lipid peroxides is one of the mechansims. (Ansari et al., 2014)

2.2.4.2 Paracetamol induced hepatotoxicity

It can cause liver damage in higher doses. The liver injury by paracetamol is mediated by its toxic reactive metabolites, N-acetyl p-benzoquineimine. The metabolite is detoxified by binding to glutathione. The unconverted metabolite then binds to nucleophilic macromolecules of the hepatocytes. This causes hepatocytes necrosis. (Ansari et al., 2014)

2.2.4.3 Galactosamine-induced hepatotoxicity

Galactosamine is supposed to produce hepatic injury by depleting the uridine content of the liver. This depletion leads to decreased formation of uracil nucleotide dependent synthesis of macromolecules such as RNA, protein synthesis and ultimately alter hepatocellular functions. Hepatic injury is induced by intraperitoneal single dose injection of galactosamine.(Ansari et al., 2014)

2.2.4.4 Lead induced hepatotoxicity

The mechanism of lead induced hepatotoxicity is occurring by lipid peroxidation and generation of reactive oxygen species (Gurer et al., 2000) Lead toxicity lead to free radical damage by two separate pathway: 1) generation of ROS, including hydroperoxides and hydrogen peroxide 2) the direct depletion of antioxidant reserves.

2.2.4.5 Erythromycin induced hepatotoxicity

Erythromycin estolate is a potent macrolide antibiotic, generates free radicals and has been reported to induce hepatotoxicity. Erythromycin when given as erythromycin stearate or erythromycin esolate to albino rats produces hepatotoxicity in them (Ahmad et al., 2012)

2.2.5 Mechanism of hepatotoxicity

The hepatotoxic agents activated some enzyme activity in the cytochrome p-450 systems such as CYP2E1 also leads to oxidative stress. Injury to hepatocyte and bile duct cells lead to accumulation of bile acid inside liver. This body as such has important immune function in maintaining body veracity. Damaging hepatocyte results in the activation of innate immune system like kupffer cells, natural killer cells and natural killer T cells and results in producing proinflammatory mediators such as tumor necrosis factor-α (TNF), interferon-γ (INF) and interleukin-β (IL) produced liver injury. Many agents damage mitochondria, an intracellular organelle that produces energy. In mitochondria hepatocellular death is a direct result of drugs acting on these organelles. An indirect result ensuing from mitochondrial participation in programs of cell death. These programs lead to necrosis or apoptosis; they are mediated through signaling mechanisms arising at cell membrane or in subcellular compartments. Its dysfunction releases oxidants, which in turn injure hepatic cells. Modern medicines offer limited success in providing effective cure and there is a severe need to develop new drug capable of healing toxic liver damages. In traditional system of medicine, plant were claimed to be effective and used successfully to alleviate multiple liver disorders. Ayurveda has a clinical specialty called rasayana, which prevents disease and counteracts the aging process by means of optimization of

homeostasis. It has been reported that rasayanas are rejuvenators, nutritional supplemenst and possess strong antioxidant activity. Hepatic injury leads to disturbances in transport function of hepatocytes resulting in leakage of plasma membrane thereby causing increased enzyme level in serum. All the hepatotoxic used in the present study induced ROS production in the body. This leads to depletion of antioxidants status of hepatic tissue and induced lipid per oxidation degradation of bio membrane in the control group. Administration of antioxidants, which can scavenge the free radicals, could reduce the hepatic injury. There are many herbal medicine plant having antioxidant properties which show hepatoprotective activity**.**

2.3 Anti-tubercular drug induced hepatotoxicity

Anti-tubercular drugs can cause varied degree of hepatotoxicity from a transitory asymptomatic rise in transaminases to acute liver failure and the frequency of hepatotoxicity in different countries varies widely from 2-39%. The occurrence of drug induced hepatotoxicity is unpredictable but it is observed that certain patients are at a relatively higher risk than other populations. (Kishore et al., 2007)

Hepatotoxic potential of first line anti-tubercular Drug:

Hepatotoxic potential	Drugs
High	INH, Rifampicin, Pyrazinamide
Less	Ethambutol, Streptomycin

2.3.1 Isoniazid

Isoniazid hepatotoxicity is a common complication of anti-tuberculosis therapy that ranges in severity from asymptomatic elevation of serum transaminases to hepatic failure requiring liver transplantation. This is not caused by high plasma isoniazid levels but appears to represent an idiosyncratic response. Isoniazid is metabolized to monoacetyl hydrazine, which is further metabolized to a toxic product by cytochrome P450 leading to hepatotoxicity. Human genetics studies have shown that cytochrome P4502E2 (CYP2E1) is involved in anti-tubercular drug hepatotoxicity.

2.3.2 Rifampicin

Patients on concurrent Rifampicin therapy have an increased incidence of hepatitis. This has been postulated due to rifampicin-induced production of the toxic metabolities form acetyl hydrazine. Rifampicin also increases the metabolism of isoniazid to isonicotinic acid and

hydrazine, both of which are hepatotoxic. The plasma half-life of acetyl hydrazine is shortened by rifampicin and it is quickly converted to its active metabolites by increasing the oxidative elimination rate of acetyl hydrazine, which is related to the higher incidence of liver necrosis by isoniazid and rifampicin in combination. Rifampicin also interacts with antiretroviral drugs and affects the plasma levels of these drugs as well as risk of hepatotoxicity.

2.3.3 Pyrazinamide

Pyrazinamide is converted to pyrazinoic acid and further oxidised to 5-hydro-oxypyrazinoic acid by xanthine oxidase. The serum half-life of pyrazinamide is not related to the length of treatment, indicating that pyrazinamide does not induce the enzymes responsible for its metabolism. The mechanism of pyrazinamide-induced toxicity is unknown; it is unknown what enzymes are involved in pyrazinamide-toxicity and whether toxicity is caused by pyrazinamide inhibited the activity of several CYP450 isoenzymes, but a study in human liver microsomes showed that pyrazinamide has no inhibitory effect on CYP450 isoenzymes.

2.3.4 Ethambutol

There are fewer reports of hepatotoxicity with ethambutol in the treatment of Tuberculosis. Abnormal liver function tests have been reported in some patients taking ethambutol however, these patients was also taking other anti-TB drugs known to cause liver dysfunction.

2.4 Epidemiology of hepatotoxicity related to anti-tubercular drugs

Tuberculosis (TB) remains a major global health problem despite the availability of highly efficacious treatment for decades. World Health Organization (WHO) declared TB a global public health emergency in 1993, at a time when an estimated 7-8 million new cases and 1.3-1.6 million deaths occurred each year. In 2010, there was an estimated 8.8 million new cases reported and 1.4 million deaths occurred. TB is major public health issue with an estimated prevalence of 256 per 100,000 population and 26 per 100,000 populations dying of TB. Although about 85% of TB cases are successfully treated, treatment-related adverse events including hepatotoxicity, skin reactions, gastrointestinal and neurological disorders account for significant morbidity leading to reduced effectiveness of therapy. (Ramappa et al., 2013)

Anti-TB drugs are one of the commonest group underlying idiosyncratic hepatotoxicity worldwide. The incidence of anti-TB drug induced hepatotoxicity varies widely dependent upon the characteristics of the particular cohort, drug regimens involved, threshold used to define

hepatotoxicity, monitoring and reporting practices. Overall, hepatotoxicity attributes to anti-TB drugs has been reported in 5%-28% of people treated with anti-TB drugs.

Age has been associated with an increased risk of drug induced hepatotoxicity. In one study including 519 patients on standard anti-TB medications, age over 60 years was associated with a 3.5 fold risk of drug induced hepatotoxicity. In a case-control study, the incidence of hepatotoxicity was 17% in patients below 35 years of age and 33% in age above 35 years. Studies show that women are more susceptible to drug induced hepatotoxicity from anti-TB therapy with reported 4-fold risk.

2.5 Hepatoprotection

Hepatoprotection is the ability to prevent damage to the liver. This damage is known as hepatotoxicity. Hepatoprotectives are the class of therapeutic agents that includes both synthetic as well as natural products. Medicinal herbs are significant source of hepatoprotective drugs.

2.5.1 Silymarin

Silymarin is a unique flavonoid complex containing silybin, silydanin and silychrisin that is derived from the milk thistle plant. (Pradhan et al., 2006) These unique phytochemicals from the milk thistle have been the subject of decades of research into their beneficial properties. Silymarin is used to:

- Regenerate liver cells damaged by alcohol or drug
- Decongest the liver
- Increase the survival rate of patients with cirrhosis
- Complement the treatment of viral hepatitis
- Protect against industrial poisons, such as carbon tetrachloride
- Protect the liver against pharmaceuticals that stress the liver, such as acetaminophen and tetracycline
- Antidote and prevent poisoning from the death cup mushroom, Amanita phalloides.

2.5.2 Mechanism of action of Silymarin

- As an antioxidant, silymarin scavenges for free radicals that can damage cells exposed to toxins. Silymarin has been said to be atleast ten times more potent in antioxidant activity than vitamin E

- It increases glutathione in the liver by more than 35% in healthy subjects and by more than 50% in rats. Glutathione is responsible for detoxifying a wide range of hormones, drugs and chemicals. High levels of glutathione in the liver increases its capacity for detoxification**.**
- Silymarin also increases the level of the important enzyme superoxide dismutase in cell cultures.
- It stimulates protein synthesis in the liver, which results in increase in the production of ner liver cells to replace the damaged ones.
- Silymarin inhibits the synthesis of leukotrienes.

2.5.3 Some important hepatoprotective medicinal plants: a bird's eye view (Dey et al., 2013)

Botanical name	**Family**	**Parts used**	**Reference**
Andrographic paniculata	Acanthaceae	Whole plant	Trivedi NP, 2001
Hemidesmus indicus	Asclepiadaceae	Root	Murali A, 2013
Taraxacum officinale	Asteracceae	Root	Malki AL, 2013
Berberis lycium	Berberidaceae	Leaf	AD Kshirsagar, 2011
Bryonia alba	Cucurbitaceae	Root	Manvi, 2011
Euphorbia nerifolia	Euphorbiaceae	Fruit	Datta S, 2015
Fumaria officinalis	Fumariaceae	Whole plant	AD Kshirsagar, 2011
Garcinia indica	Guttiferae	Fruit	Deore AB, 2011
Swertia chirata	Gentianaceae	Whole plant	Sharma N, 2013

Mentha longifolia	Labiatae	Leaf	AD Kshirsagar, 2011
Trigonella Foenumgraceum	Leguminosae	Seed	Subhashini N, 2011
Ficus carica	Moraceae	Fruit	AD Kshirsagar, 2011
Aphanamixis polystachya	Meliaceae	Bark	Apu AH, 2012
Myristica fragrans	Myristiceae	Seed	Jumaily EF, 2012
Tinospora cordifolia	menispermaceae	Stem	Kumar V, 2013
Nelumbo nucifera	Nymphaceae	Flower	Sheikh SA, 2014

Table 2.1: **List of some important hepatoprotective medicinal plants**

2.5.4 Hepatoprotectives in preclinical studies

(i) A study has been carried out to check the pharmacological potential of silymarin in combination with hepatoprotective plants against experimental hepatotoxicity in rats. Hepatotoxicity was induced in rats by administration of Paracetamol or Carbon tetrachloride, it significantly increase in serum Alanine transaminase (ALT), Aspartate transaminase (AST), Alkaline phosphatase (ALP) and bilirubin levels as compared to normal untreated control rats. Pretreatment with Silymarin(50mg/kg) and combination of Silymarin(25mg/kg) with each Plant extract: *Picrorrhiza kurroa, Tephrosia purpurea, Phyllanthus amarus and Aspasragus racemosus* significantly attenuated the increase in these serum markers, in comparison to respective Paracetamol or Calcium teterachloride control rats. The effect of Silymarin with each plant extract in lowering serum ALT level was significant in comparison to silymarin 25mg/kg.(Singh G et al., 2012)

(ii) A previous study has reported the effect of honey on hepatotoxicity induced by antitubercular drugs in albino rats. Many *in vitro* and *in vivo* studies revealed that honey possess antioxidant property. (Chandane et al., 2013) This study was carried out to study the prophylactic and curative value of honey with its antioxidant activity in antitubercular drugs induced

hepatotoxicity in albino rats. Results have shown that treatment with honey significantly reversed levels of serum proteins as compared to antitubercular drugs treated groups.

(iii) Studies also revealed the protective role of camel Milk against paracetamol induced hepatotoxicity in male rats. Eighteen white rats were used, these animals were divided into three groups each group contain six animals: First group I is the control group treatment with normal saline for seven days, second group II treated with paracetamol at the first day and fourth day of the experimental 7 days, third group III treated with paracetamol at the first day and the fourth day of the experimental 7 days, and then treated orally with camel's milk for seven days. The results indicate that the paracetamol caused a significant increase in the level of the serum glutamate pyruvate transaminase (SGOT), serum glutamate oxaloacetate transaminase (SGPT), alkaline phosphate (ALP), cholesterol and triglyceride levels. Also, there was a significant decreased in albumin, while there was a non-significant decrease in total protein and globulin levels compared with the control group. Protective activity of camel's milk against toxicity of paracetamol observed in decreasing SGOT, SGPT, ALP, cholesterol and triglyceride levels and increased in the total protein and albumin levels.(Fartosi et al., 2011)

(iv) A previous study also revealed the effect of aqueous extract of *Azadirachta Indica* (Neem) leaves on hepatotoxicity induced by antitubercular drugs in rats.(Kale et al., 2003)

2.6 Goat milk

Goat milk is a natural product obtained from *Capra aegagrus hirus*(a species of goat). The goat is the member of the family Bovidae. Milk is a naturally valuable source of vitamins and minerals such as vitamin A, vitamin B6, vitamin B12, thiamine, riboflavin, niacin, calcium, phosphorus, magnesium, zinc and potassium. Goat milk naturally contains these nutrients. Other milks such as soya, rice and oat milks do not and so are often fortified with vitamins and minerals. Goat milk is easily available and it is easy to consume and digest and can be taken as food during drug treatment of tuberculosis. (Harden et al., 2011)

2.6.1 Comparison of nutritional value between goat and cow milk

Compositions(Per100 grams)	Goat Milk	Cow Milk
Protein (g)	3.1	3.2
Fat % (g)	3.5	3.9
Calories/100ml	60	66
Vitamin A (1U/gram fat)	39	21
VitaminB1(thiamine UG/100/ml)	68	45
Riboflavin (Ug/100ml)	210	159
Vitamin C (mg ascorbic acid/ 100 ml)	2	2
Vitamin D (1U /gram fat)m	0.7	0.7
Calcium %	0.19	0.18
Iron %	0.07	0.06
Phosphorus %	0.27	0.23
Cholestreol (mg/100ml)	10	14
Sugars (lactose)	4.4	4.8
Saturated fatty acid	2.3	2.4
Monosaturated fatty acid	0.8	1.1
Polyunsaturated fatty acid	0.1	0.1

Table.2.2: **Comparison of nutritional value between goat and cow milk**

2.6.2 Benefits of goat milk

2.6.2.1 Goat milk is less allergenic: In US, the most common food allergy for children under three is cow's milk. Mild side effects include vomiting, diarrhea and skin rashes and severe

effects can be serious as anaphylactic shock. The allergic reaction can be blamed on a protein allergen known as alpha S1 casein found in high levels in cow's milk. The levels of alpha S1 casein in goat's milk are about 89% less than cow's milk providing a far less allergenic food. In fact a recent study of infants allergic to cow's milk found that nearly 93% could drink goat's milk with virtually no side effects.

2.6.2.2 Goat milk is easier to digest: Goat milk has smaller fat globule as well as higher levels of medium chain fatty acids. This means that during digestion, each fat globule and individual fatty acid will have a larger surface to volume ration resulting in a quicker and easier digestion process. Also, when the proteins found in milk denature (clump up) in stomach, they form a much softer bolus (curd) than cow's milk. This allows the body to digest the protein more smoothly and completely than when digesting cow's milk**.**

2.6.2.3 Goat's milk rarely causes lactose intolerance: All milk contains certain levels of lactose which is also known as 'milk sugar'. A relatively large portion of the population suffers from a deficiency (not an absence) of an enzyme known as lactase which is used to digest lactose. This deficiency results in a condition known as lactose intolerance which is fairly common ailment. Lactose intolerance and cow's milk allergy (CMA) are 2 distinct conditions. CMA is due to protein allergens, while lactose intolerance is due to carbohydrate sensitivity. Goat's milk contains less lactose than cow's milk and therefore is easier to digest for those suffering from lactose intolerance.

2.6.2.4 Goat's milk matches up to the human body better than cow's milk: This matter is both an issue of biochemistry as well as thermodynamics. Regarding the biochemistry of the issue, we know that goat's milk has a greater amount of essential fatty acids such as linoleic acid and arachidonic acid than cow's milk as well as significantly greater amounts of vitamin B6, vitamin A and niacin. Goat's milk is also a far superior source of vitally important nutrient potassium. This extensive amount of potassium causes goat's milk to react an alkaline way within the body whereas cow's milk is lacking in potassium and ends up reacting in an acidic way. Thermodynamically speaking goat's milk is better for human consumption. A baby usually starts life at around 7-9 pounds, a baby goat (kid) usually starts life at around 7-9 pounds and a baby cow (calf) usually starts life at around 100 pounds. Now speaking from a purely thermodynamic position, these 2 animals have very significant and different nutritional needs for

both maintenance and growth requirements. Cow's milk is designed to take a 100 pound calf and transform into 1200 pound cow. Goat's milk were both designed and created for thermodynamically a 7-9 pounds baby/kid into an average adult/goat of anywhere between 100-120 pounds. This significant discrepancy along with many others is manifesting on a nutritional level as obesity rates in US.

2.6.3 Therapeutic significance of goat milk

2.6.3.1 Anti-inflammatory activity

An animal study (Daddaouna et al., 2006) found that goat's milk contains powerful anti-inflammatory molecules called oligosaccharides. In short, these useful were shown to:

- Prevent pro-inflammatory bacteria from collecting on stomach and intestinal lining
- Reduce bacterial translocation which can trigger the severe and potentially deadly inflammatory response known as sepsis
- Promote the growth of healthy friendly bacteria (prebiotic). Friendly bacteria are crucial for healthy digestion, immune function, weight loss/obesity-prevention, insulin sensitivity, diabetes prevention and more.

2.6.3.2 Antioxidant effect

Goat milk is rich in various physiologically functional components, including proteins, vitamins, flavonoids and carotenoids with antioxidant properties. Therefore, goat milk is considered to possess high antioxidant activity that resists oxidative stability and highly protects consumers from exposure to oxidative stress, which is an important characteristic of numerous acute and chronic diseases. Antioxidants may positively affect human health by protecting the body against damage caused by reactive oxygen species (ROS), which attack membrane lipids, protein and DNA. These compounds are also involved in several important protective functions in many diseases such as cardiovascular diseases, cancer, diabetes mellitus and Alzheimer's. Antioxidants also protect organisms against free radicals, but a sufficient concentration of antioxidants is necessary to balance the disruption caused by these radicals. (Alyaqoubi et al., 2014)

2.6.3.3 Anaemia and malabsorption disorders

Researchers have carried out a comparative study on the properties of goat's milk compared to those of cow's milk. They found reason to believe that goat's milk could help prevent diseases

such as anaemia and bone demineralization. Goat's milk was found to help with the digestive and metabolic utilization of minerals such as iron, calcium, phosphorus and magnesium. (Castro JD et al., 2015)

2.6.3.4 Cardiovascular disorders

Goat milk is rich in **angiotensin converting enzyme inhibitory peptides.** ACE inhibitory peptides are mainly derived from milk proteins and are especially interesting because of their beneficial effect in the treatment of hypertension. The peptides are released from milk proteins by hydrolysis with digestive enzymes or fermentation with proteolytic starter culture. ACE inhibitory peptides may be extracted both from goat whey and casein proteins. k-casino macropeptide (k-CMP) is a main component of whey and is obtained as a byproduct in cheese making. Goat k-CMP and its decomposition products from trypsin treatment have been found to inhibit human platelet aggregation. That is a crucial incidence in cardiovascular diseases called thrombosis. (Manso et al., 2002)

2.6.3.5 Antimicrobial activity

Milk proteins have also proved to be presursors for antimicrobial peptides. The most well-known peptides are those derived from lactoferrin. Lectoferrin is an iron-binding glycoprotein, those main function is iron transport. It is involved in many physiological functions, including regulation of iron absorption and immune responses. It also exhibit antioxidant activity and has both anticarcinogenic and anti-inflammatory properties. However, its antimicrobial properties are its most widely studied function. (Garcia et al., 2011)

2.6.3.6 Inflammatory bowel disorder

Previous studies have shown that components of goat milk like conjugated linoleic acid (CLA) (Rodrigues et al., 2014) and oligosaccharide (Daddaouna et al 2006) has been used to treat various disorders. A study has shown that CLA reduces body fat and protects against cardiovascular diseases and goat milk oligosaccharides have anti-inflammatory effects in rats with experimental colitis.

2.6.3.7 Hepatic function and hepatocarcinoma

A recent study has reported that the habitual consumption of goat milk has positive effects on the plasma lipid profile, biliary composition and hepatic antioxidant defence. (Alyaqoubi et al., 2014) A study has reported that goat milk consumption potentiates liver divalent metal

transporter 1 (DMT1) expression thereby enhancing Fe metabolism and storage indicating its potential in anaemia. (Castro JD et al. 2014) A previous study has also reported the effect of goat's milk along with soybean's milk on tumor-marker enzyme activities during hepatocarcinogenesis in rats. Results have shown that activities of enzymes Gamma-glutamyl Transpeptidases (GGT) and Alkaline Phosphatase (ALP) decreased in hepatatoacrcinogenic rats after administration of goat's milk and soybean's milk. (Rahmat et al., 2001)

3. RESEARCH VISUALIZED & WORK PLAN

3. RESEARCH VISUALIZED AND WORK PLAN

3.1 Rationale of the study

Milk is a physiological fluid possessing high nutritional value as it is naturally rich in energy, proteins, vitamins and minerals. In comparison to cow's milk, goat milk is considered as superior and an excellent natural food alternative due to its unique macro- and micronutrient composition. It is valuable source of vitamins and minerals such as vitamin A, vitamin B 6, vitamin B 12, thiamine, riboflavin, niacin, calcium, phosphorus, magnesium, zinc and potassium. It is easily available and is easy to digest. And it is also less allergenic due to less presence of protein allergen known as alpha S1 casein. Traditionally and popularly goat milk has been considered to possess a variety of nutritional and medical benefits like tuberculosis, autism, arthritis, asthma, eczema, migraines, stomach ulcers, liver complaints, chronic catarrh, cystic fibrosis, gallbladder diseases, childhood epilepsy and stress-related symptoms such as insomnia and constipation. (Alyaqoubi et al., 2014) However the lack of scientific evidence for these benefits appeals for the investigation on goat milk.

Studies in animal models have indicated that goat milk has high antioxidant activity. (Alyaqoubi et al., 2014) It has demonstrated positive effects on enzymatic antioxidant defense and possesses anti-inflammatory activity. (Daddaouna et al., 2006) It has been found effective in malabsorption disorders and inflammatory bowel diseases. It has been reported that goat milk supplemented with folic acid protects cell biomolecules from oxidative stress-mediated damage after anaemia recovery in comparison with cow milk. A study has reported that goat milk consumption potentiates liver divalent metal transporter 1 (DMT1) expression thereby enhancing Fe metabolism and storage indicating its potential in anaemia. (Castro JD et al., 2015) Furthermore, a previous study has also reported the effect of goat's milk along with soybean's milk on tumor-marker enzyme activities during hepatocarcinogenesis in rats. Results have shown that activities of enzymes Gamma-glutamylTranspeptidases (GGT) and Alkaline Phosphatase (ALP) decreased in hepatatoacrcinogenic rats after administration of goat's milk and soybean's milk. (Rahmat et al., 2001)

A recent study has reported that the habitual consumption of goat milk has positive effects on the plasma lipid profile, biliary composition and hepatic antioxidant defence. (Alyaqoubi et al., 2014) As goat milk is rich in antioxidants, which may positively affect human health by protecting the body against damage caused by reactive oxygen species (ROS), it may possess

hepatoprotective activity. The hepatoprotective activity of goat milk in anti-tubercular drug induced hepatotoxicity has not been investigated yet. So the rationale of the present study was to explore the effect of goat milk on anti-tubercular drug induced hepatotoxicity in rats.

3.2 Aim and objectives

AIM: To study the hpatoprotective effect of goat milk against anti-tubercular drug induced hepatotoxicity in rats.

OBJECTIVES:

1. To induce hepatotoxicity in rats by anti-tubercular drugs.
2. To evaluate the effect of goat milk on gross morphology of liver in experimentally induced hepatotoxicity.
3. To evaluate the effect of goat milk on histopathology of liver in experimentally induced hepatotoxicity.
4. To evaluate the effect of goat milk on the levels of serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST).
5. To estimate lipid peroxidation by assessing the malondialdehyde levels.
6. To evaluate the effect of goat milk on serum superoxide dismutase activity (SOD).

4. EXPERIMENTAL WORK

4. EXPERIMENTAL WORK

4.1 Materials and methods

The current study was conducted in Department of Pharmacology, Lovely School of Applied Medical Sciences, Lovely Professional University, Phagwara.

4.2 Experimental animals

The study was approved by Institutional Animal Ethics Committee (IAEC) (LPU/LSPS/IAEC/CPCSE/MEETING NO. 4/PROTOCOL NO. 3). Adult Albino rats (Wister strain) of either sex weighing 200-300 gram were obtained from the institute's central animal house. The animals were accommodated in standard laboratory conditions at 25±2˚C and 12 hours light and dark cycle. Animals were given free admittance to rat chow diet and water *ad libitum.* Before the conduction of experiments, the animals were habituated to laboratory conditions for ten days.

4.3 Drugs and chemicals

The drugs required for the current study i.e. isoniazid, rifampicin and pyrazinamide were provided as gift samples by Kwality Pharmaceuticals Pvt.Ltd. Amritsar, India. Goat milk was purchased from Sonu Verka Milk Agency, Ludhiana, India.

4.4 Experimental groups

Total 30 animals were incorporated in the study. After 10 days adaptation period, the animals were divided into five groups. Each group was consisting of six animals. The groups were treated as follows:

Groups	Treatment	Animal used	Dose (mg/kg)	Route of administration
I.	Vehicle control (2% gum acacia)	6	10ml/kg	p.o

II.	Isoniazid(H)+ Rifampicin(R)+ Pyrazinamide(Z) suspension	6	(27+54+135)	p.o
III.	(H+R+Z) suspension+ goat milk	6	(27+54+135)+ 20ml/kg	p.o
IV.	(H+R+Z) suspension+ goat milk	6	(27+54+135)+ 40ml/kg	p.o
V.	(H+R+Z) suspension+ Standard (Silymarin)	6	(27+54+135) + 50mg/kg	p.o

Table 4.1: Protocol of the experimental study

4.5 Induction of hepatotoxicity

Experimental anti-tubercular drug induced hepatotoxicity was produced by administration of isoniazid, rifampicin and pyrazinamide (H+R+Z) suspension daily orally for 30 days. The animals were sacrificed after 30 days.

4.6 Experimental Design

Group I: Vehicle control i.e. 2% gum acacia orally daily for 30 days.

Group II: (H+R+Z) suspension orally daily for 30 days.

Group III: (H+R+Z) suspension + goat milk 20ml/kg orally daily for 30 days.

Group IV: (H+R+Z) suspension + goat milk 40ml/kg orally daily for 30 days.

Group V: (H+R+Z) suspension + Standard (Silymarin) orally daily for 30 days.

Blood samples of animals from groups I, II, III, IV and V were taken on 30th day for liver function tests and antioxidant tests by cardiac puncture under ether anaesthesia and livers were removed for histopathological and biochemical parameters examination.

4.7 Gross morphological evaluation

Livers were removed from the rats and were washed with phosphate buffer. Afterthat they were weighed. The liver indices were calculated as the percentage body weight. (Farkaad et al. 2013) Then gross morphological evaluation was done for hepatic lesions based on the qualitative procedure developed by Mitchell et al. 1973. They were graded as:

0+ no lesions

1+ minimal damage

2+ mild to moderate damage

3+ severe damage

4.8 Histopathological examination

The liver tissues were dissected out and fixed in 10% formalin. The paraffin sections were prepared and stained with haematoxylin and eosin and examined microscopically for histopathological changes.

4.9 Assessment of liver damage

Biochemical estimations- Serum alanine aminotransferase (ALT), Serum aspartate aminotransferase (AST) and Seum total protein were estimated by Reitman, Frankel and Biuret method. Serum malondialdehyde (MDA) and Serum Superoxide dismutase activity (SOD) were estimated by Okhawa et al and Kakkar et al method.

4.9.1 Serum alanine aminotransferase (ALT)

ALT is present in high concentration in the liver and to a lesser extent in kidney, heart, skeletal muscle, pancreas, spleen and lungs. Increased levels are generally a result of primary liver diseases such as cirrhosis, carcinoma, viral or toxic hepatitis and obstructive jaundice. Decreased levels may be observed in renal dialysis and those with vitamin B_6 deficiency.

Principle

Alanine + 2-oxoglutarate → (ALT) → pyruvate + L-glutamate

Pyruvate + NADH → (LDH) → L-lactate + NAD

Pyridoxine 5'-phosphate is a coenzyme for the ALT reaction; its addition to the reaction mixture ensures that all the apoenzyme is catalytically active and measured. Pre-incubation is required to remove any endogenous oxo-acids from the reaction mixture, the reaction then being started by the addition of 2-oxoglutarate.

Procedure:

Reagent	**Blank**	**Standard**	**Control**	**Test**
Substrate reagent	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Deionised water	0.1 ml	-	-	-

Sample	-	-	-	0.1 ml
Standard	-	0.1 ml	-	-

Mix and incubate at 370C for 30 min.

SGPT	0.5 ml	0.5ml	0.5ml	0.5ml
Sample	-	-	0.1ml	-

Mix and incubate for 20 min. at room temp.

Add 3ml of alkali reagent to each test tube. Read the absorbance against distilled water at 505 nm.

4.9.2 Serum aspartate aminotransferase (AST)

AST occurs in all tissues and is present in large amounts in liver, renal, cardiac and skeletal muscles tissue. Increased levels are associated with liver diseases or damage, myocardial infarction, muscular dystrophy and cholecystitis. Decreased levels are observed in renal dialysis and in those with vitamin B_6 deficiency.

Principle

L-Aspartate + 2-Oxoglutarate $\xrightarrow{\text{AST}}$ Oxaloacetate + L-glutamate

Oxaloacetate + NADH $\xrightarrow{\text{MDH}}$ Malate + NAD

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

Procedure

Reagent	**Blank**	**Standard**	**Control**	**Test**
Substrate reagent	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Deionised water	0.1 ml	-	-	-
Sample	-	-	-	0.1 ml
Standard	-	0.1 ml	-	-

Mix and incubate at 370C for 60 min.

SGOT colour	0.5 ml	0.5ml	0.5ml	0.5ml

reagent				
sample	-	-	0.1ml	-

Mix and incubate for 20 min. at room temp.

Add 3ml of alkali reagent to each test tube. Read the absorbance against distilled water at 505 nm.

4.9.3 Serum total protein

Total protein is useful for monitoring gross changes in protein levels caused by various disease states. It is usually performed in conjugation with other tests such as serum albumin, liver function tests or protein electrophoresis. An albumin / globulin ratio is often calculated to obtain additional information.

Principle

The peptide bonds of protein react with copper II ions in alkaline solution to form blue-violet complex, (biuret reaction). Each copper ion complexes with 5 or 6 peptide bonds. Tartarate is added as a stabilizer whilst iodide is used to prevent auto-reduction of the alkaline copper complex. The colour formed is proportional to the protein concentration and is measured at 546nm (520 – 50nm).

Procedure

Pipette into tubes marked	Blank	Standard	Test
Reagent	1000µl	1000µl	1000µl
Distilled water	20µl	-	-
Standard	-	20µl	-
Test	-	-	20µl

Incubate for 10 min. at 37°C. Read absorbance of the Standard and each test at 546nm against reagent blank.

4.9.4 Malondialdehyde assay (MDA)

Lipid peroxidation represents tissue injury due to inflammation. Excessive lipid peroxidation leads to increase in malondialdehyde level. Therefore, measuring malondialdehyde levels gives an indirect estimate lipid peroxidation. Assay was done by ferrous sulphate method to assess the

lipid peroxidation in the liver tissue (Okhawa et al., 1978) Liver tissue of amounting to 700 mg was minced with a mixture of 0.5 ml of sodium dodecyl sulphate, 4 ml H_2SO_4 and 1ml of thiobarbituric acid in a test tube. The mixture was placed in a boiling water bath at 90^oC for one hour. After cooling to room temperature the tubes were centrifuged at 2000 rpm for 10 minutes, the optical density of the supernatant fluid was analyzed spectrophotometrically at 535nm. The extinction coefficient of 1.56×10^5 was used to calculate the amount of MDA formed.

4.9.5 Superoxide dismutase (SOD) activity

SOD activity was assayed by the Kakkar *et al* method. This method is based on the principle of the inhibitory effect of SOD on reduction of nitroblue tetrazolium (NBT) dye by super oxide anions, which are generated by the photo oxidation of hydroxylamine hydrochloride ($NH_2OH.HCl$). The values were expressed as units/mg protein/min and one unit of SOD activity was defined as the amount of enzyme required for 50% inhibition of NBT reduction/min/mg protein. The liver tissue was homogenized in chilled phosphate buffer (0.1 M, pH 7.4) to prepare a 10% homogenate (w/v). The supernatant was centrifuged at 10,500 *g* for 30 min at 4°C to obtain the post-mitochondrial supernatant (PMS). The assaying reagents system consisted of solution A: 0.1 mM EDTA dissolved in 50 mM sodium carbonate solution (pH 10.0), solution B: 96 µM of NBT in solution A (2 mg NBT/25.0 ml), solution C: 0.6% Triton X-100 in solution A and solution D: 20 mM Hydroxylamine hydrochloride (14 mg/10ml) in double distilled water (pH adjusted to 6.0 with 0.1 N NaOH).

Procedure

To the test and reference cuvettes, 1.3 ml of solution A, 0.5 ml of solution B and 0.1 ml of solution C were added. The contents were incubated at 37°C for 10 minutes. Reaction was initiated by the addition of 0.1 ml of solution D to the reaction mixture in test cuvette and the rate of NBT reduction in the absence of the enzyme source was recorded. Following this, small aliquots of liver tissue homogenate were added to the test as well as reference cuvettes. Finally, percentage inhibition in the rate of NBT reduction was noted at 560 nm.

4.10 Statistical analysis

The values were expressed as mean ± SEM. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Tukey multiple comparison tests. Differences were considered significant at $p<0.05$.

5. RESULTS & DISCUSSION

5. RESULTS AND DISCUSSION

5.1 Body and liver weight changes

The mean body and liver weight noted in all groups is shown in Table 5.1. Two rats from group I and one from group II died at the beginning of the experiment for unknown reasons. Dead rats were excluded from statistical analysis, as they did not live long enough for full development and maturation of the lesions or evaluation of other parameters. There was a significant reduction in the body weight and increase in the liver weight in H+R+Z suspension treated rats when compared to corresponding control rats. Treatment with goat milk and silymarin caused a marked increase in the body weight and decreased the liver weight.

Table 5.1Body and liver weight changes in control and experimental groups

S. No.	Experimental groups	Initial body weight (g)	Final body weight (g)	Percent change (%)	Liver weight (g)	Relative liver weight (g)
1	Group I	326±3.33	352±9.69	7.97	9.76±0.51	2.90
2	Group II	293±14.7	260±17.7	-11.26***	11.65±0.52	4.49***
3	Group III	200±3.33	225±8.46	12.5###	10.95±0.32	3.10
4	Group IV	241±8.33	266±8.71	10.37###	9.95±0.35	3.28
5	Group V	310±8.47	330±7.71	6.45###	9.84±0.69	2.86###

The values were expressed as Mean ±SEM. (*****, #, † = p<0.05; ******, ##, †† = p<0.01; *******, ###, ††† = p<0.001) ***** = when compared with vehicle control group, # = when compared with anti-TB drug group, † = when compared with silymarin group.The data was analyzed using One-Way ANOVA followed by *Tukey HSD Test*.

5.2 Gross morphological assessment

The severity of liver necrosis was assessed qualitatively following inspection of the liver gross morphology (Table 5.2, Fig. 5.2) Gross morphological scores indicated that goat milk significantly decreased the degeneration and tissue necrosis in liver as compared to group II.

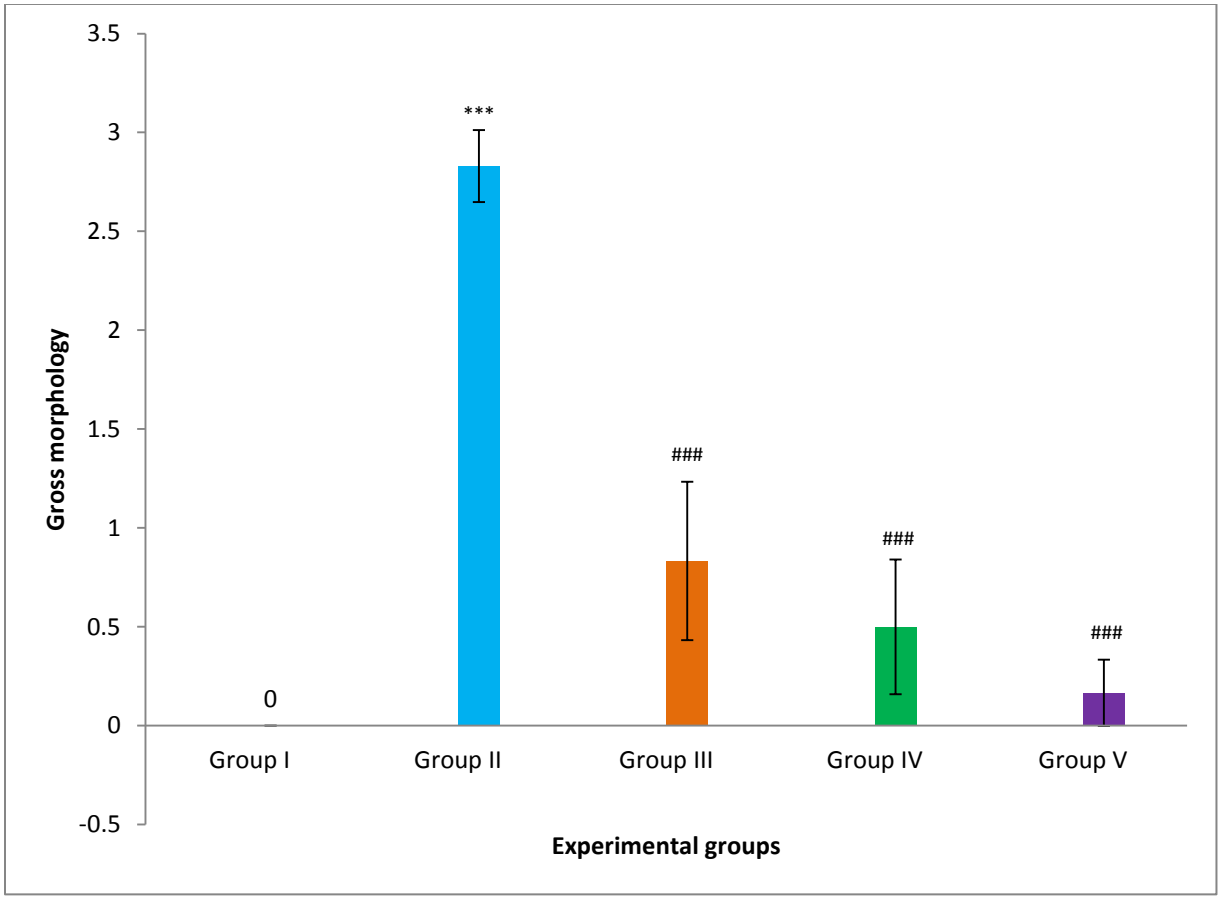
Table 5.2 Liver gross morphological score of different experimental groups

S. No.	Experimental groups	Score 0	Score 1+	Score 2+	Score 3+	Total
1	Group I	4 (100%)	-	-	-	4
2	Group II	-	-	1 (20)	4 (80%)	5
3	Group III	3 (30%)	1 (20%)	2 (30%)	-	6
4	Group IV	4 (60%)	1 (20%)	1 (20%)	-	6
5	Group V	5 (80%)	1 (20%)		-	6

Table 5.3 Mean gross morphological assessment of different experimental groups

S. No.	Experimental groups	Mean ±SEM
1	Group I	0±0
2	Group II	2.83±0.182***
3	Group III	0.833±0.401###
4	Group IV	0.5±0.341###
5	Group V	0.166±0.167###

The values were expressed as Mean ±SEM. (*, #, † = p<0.05; **, ##, †† = p<0.01; ***, ###, ††† = p<0.001) * = when compared with vehicle control group, # = when compared with anti-TB drug group, † = when compared with silymarin group.The data was analyzed using One-Way ANOVA followed by *Tukey HSD Test*.



The values were expressed as Mean ±SEM. (*, #, † = p<0.05; **, ##, †† = p<0.01; ***, ###, ††† = p<0.001) * = when compared with vehicle control group, # = when compared with anti-TB drug group, † = when compared with

silymarin group.The data was analyzed using One-Way ANOVA followed by *Tukey HSDTest*

Figure 5.1 Liver gross morphological assessments of different experimental groups

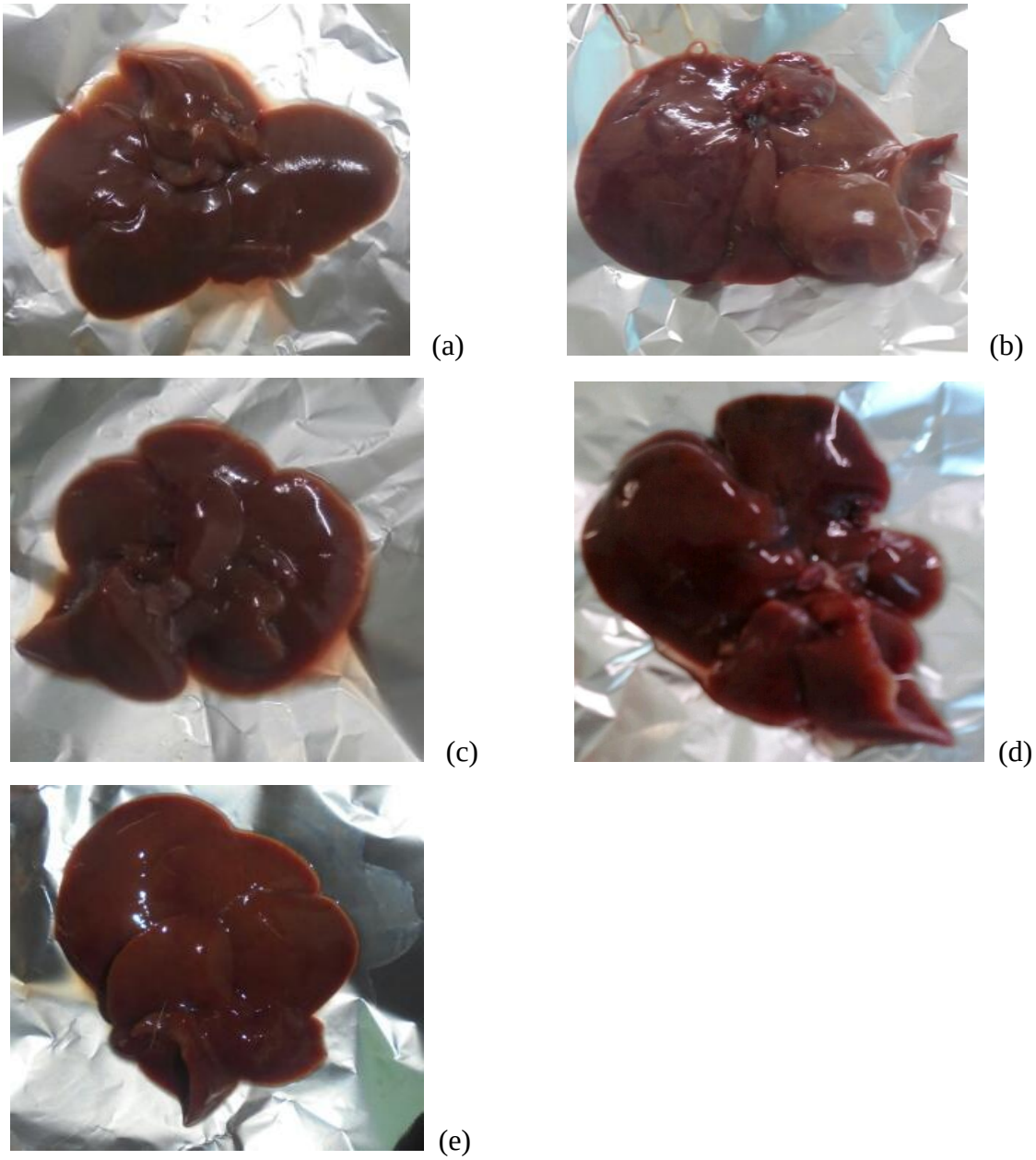


Fig 5.2 Comparative gross morphology of the intact livers all the experimental groups

(a) Vehicle control group- showing smooth surface of liver

(b) Anti-TB drug suspension- showing shrinkage of liver with some spots in liver

(c) Anti-TB drug suspension + Goat milk (20ml/kg)- showing smooth surface of liver

(d) Anti-TB drug suspension + Goat milk (40ml/kg)- showing smooth surface of liver

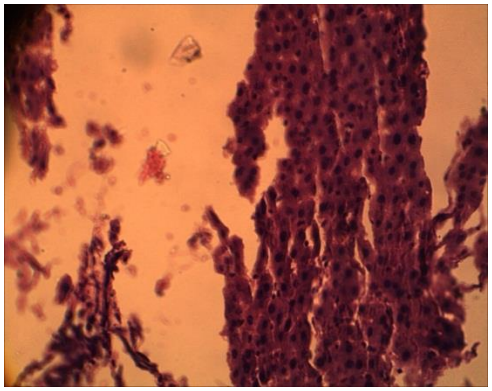
(e) Anti-Tb drug suspension + standard (Silymarin)- showing smooth surface of liver

5.3 Histopathological analysis

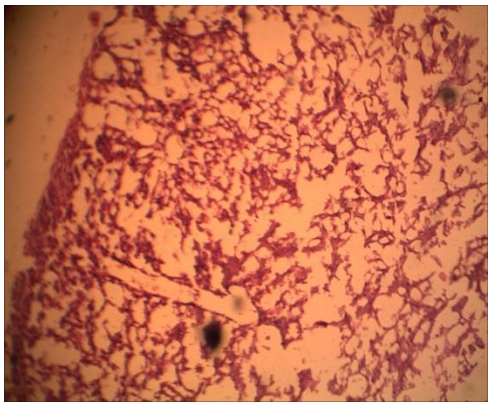
In the histopathological studies, the liver sections of rat treated with vehicle showed normal hepatic architecture (Fig. 5.2a). Administration of antitubercular drugs for 30 days to group II produced inflammation, degeneration and necrosis on histological examination of rat livers (Fig. 5.2b). Co-administration of goat milk (20ml/kg) along with antitubercular drugs decreased histological changes like inflammation, degeneration and necrosis (Fig. 5.2c) Co-administration of goat milk (40ml/kg) along with antitubercular drugs almost recovered from necrotic and degenerative changes. (Fig. 5.2d) and is comparable to silymarin.

Table 5.4 Summary of histopathological observations in different experimental groups.

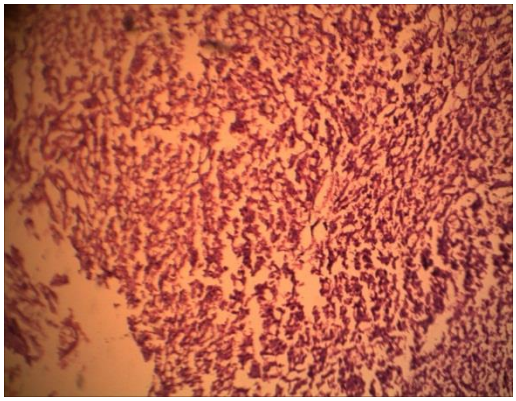
S. No.	Experimental groups	Histopathological observations
1	Group I	Normal histology of liver tissue with no damage
2	Group II	Hepatocellular damage indicated by degeneration, necrosis and inflammation of hepatocytes
3	Group III	Shows decreased degeneration, necrosis and inflammation
4	Group IV	Almost recovery from degenerative and necrotic changes. Inflammation is also scanty
5	Group V	Shows reduced degeneration, necrosis and inflammation.



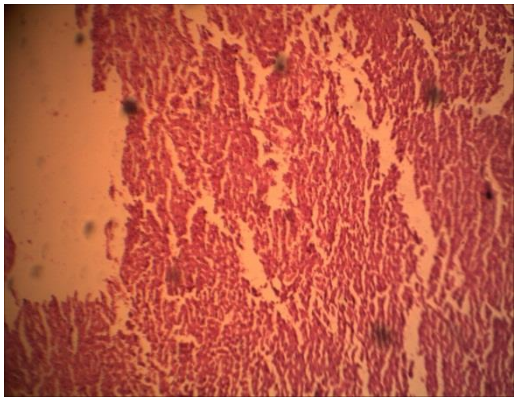
a. Vehicle control



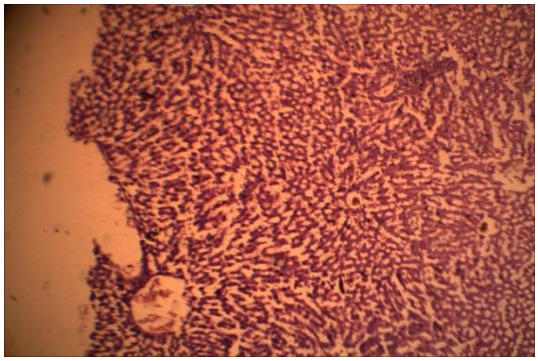
b. Anti-TB drugs



c. Anti-TB drugs+goat milk (20ml/kg)



d. Anti-TB drugs+goat milk (40ml/kg)



e. Anti-TB drugs + standard (Silymarin)

Figure 5.3 Photomicrograph of the liver tissue:

a) Vehicle control: showing normal histology of liver tissue

b) Anti-TB group: showing the moderate degree of inflammation in hepatocytes in the form of degeneration, necrosis and inflammation

c) Anti-TB group + goat milk (20ml/kg): shows decreased degeneration, necrosis and inflammation

d) Anti-TB group + goat milk (40ml/kg) : recovery from degeneration, necrosis and inflammation

e) Anti-TB group + standard (silymarin): shows reduced degeneration, necrosis and inflammation

5.4 Biochemical evaluations

5.4.1 Estimation of Serum ALT, AST and Total protein level in liver tissue

Anti-tubercular drugs causes a considerable degree of hepatotoxicity and the tissue injury in the rat liver. (Chandane et al., 2013) So, there is increase in serum ALT and AST and fall in serum Total protein. In the present study, the group that received only Anti-TB drug suspension i.e. Group II showed significant fall in serum protein level and rise in ALT and AST as compared to vehicle control i.e. Group I. Treatment with goat milk along anti-TB drugs i.e. Group III significantly reversed level of serum protein, serum ALT and AST as compared to anti-TB drug treatment group II. (Table 5.5)

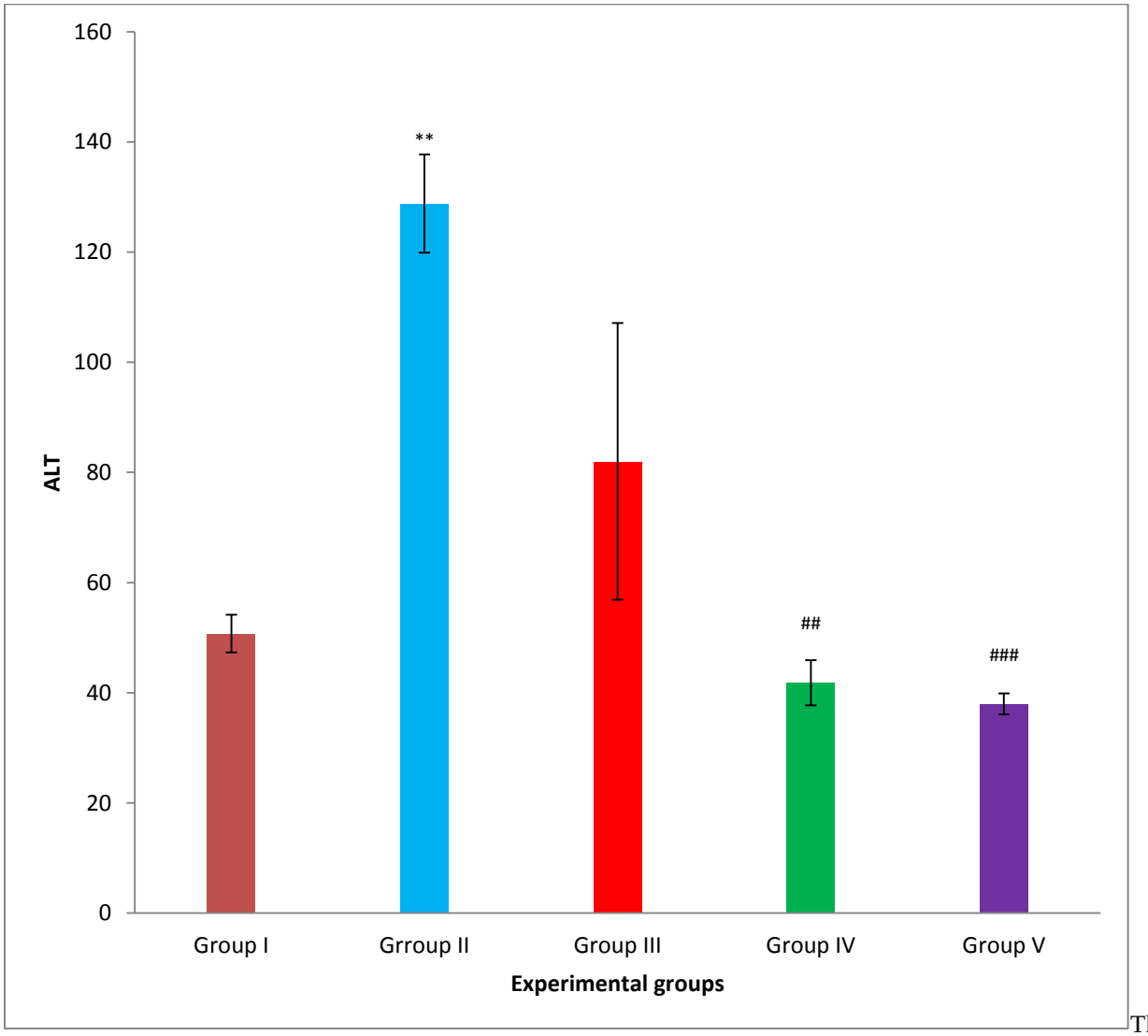
Table 5.5 Serum ALT, AST, Total protein level in liver tissue in different experimental groups

S. No.	Experimental groups	Sr. ALT (units/ml)	Sr. AST (units/ml)	Sr. Total Protein (g/dl)
1	Group I	50.75±3.4	181.75±7.88	7.2±0.129
2	Group II	128.8±8.9**	296.4±56.4*	5.06±0.07***
3	Group III	82±25.1	174.7±31.5##	6.32±0.209###
4	Group IV	41.8±4.1##	128.8±8.54###	6.72±0.153###

5	Group V	38±1.9$^{###}$	121.2 ± 8.61$^{###}$	6.12±0.195$^{###}$

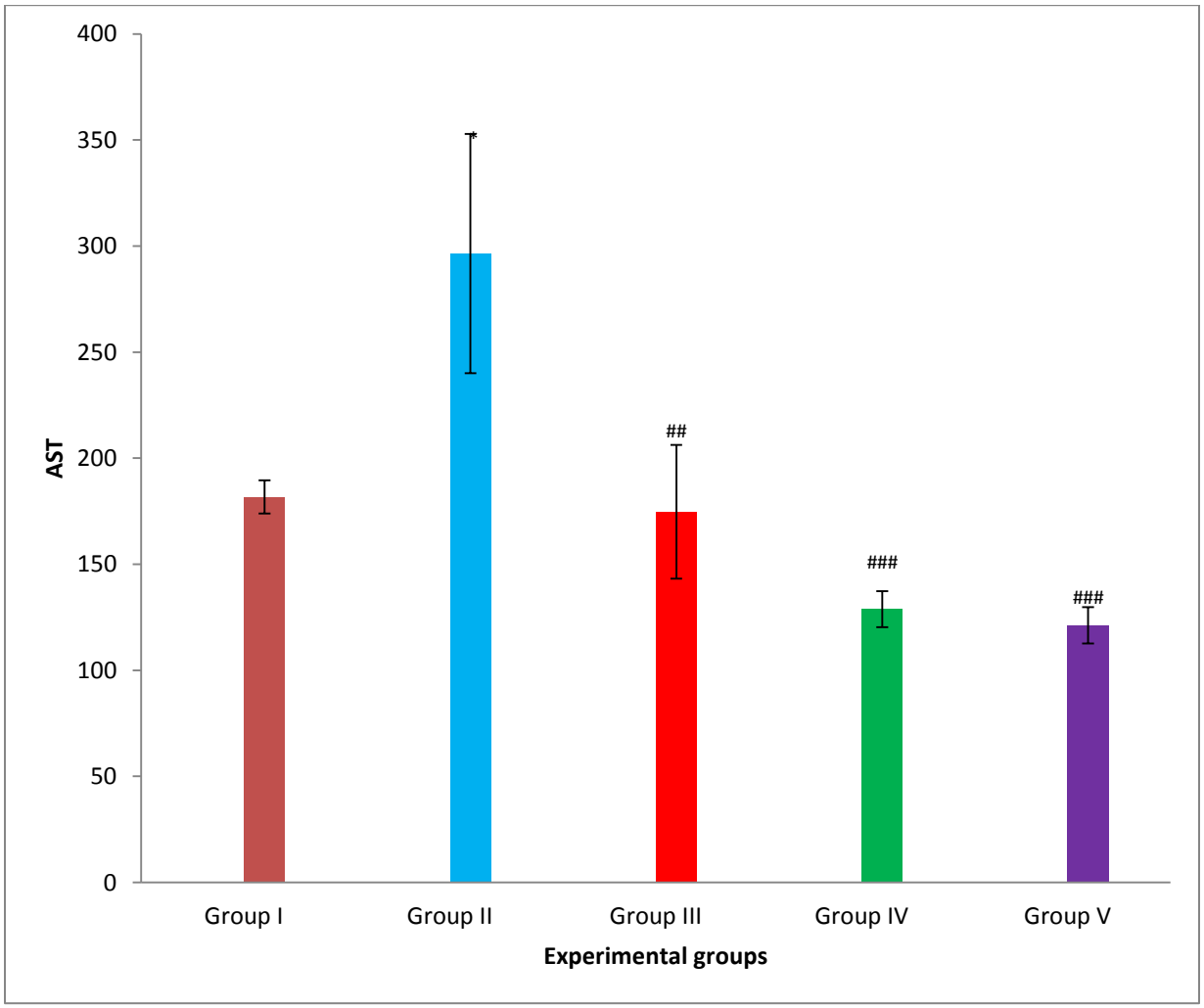
The values were expressed as Mean ±SEM. (*****, #, † = p<0.05; ******, ##, †† = p<0.01; *******, ###, ††† = p<0.001) ***** = when compared with vehicle control group, # = when compared with anti-TB drug group, † = when compared with silymarin group.The data was analyzed using One-Way ANOVA followed by *Tukey HSD Test.*

Figure 5.4 Effect of different pharmacological interventions on (a) Serum ALT (b) Serum AST and (c) Total Protein level in rats



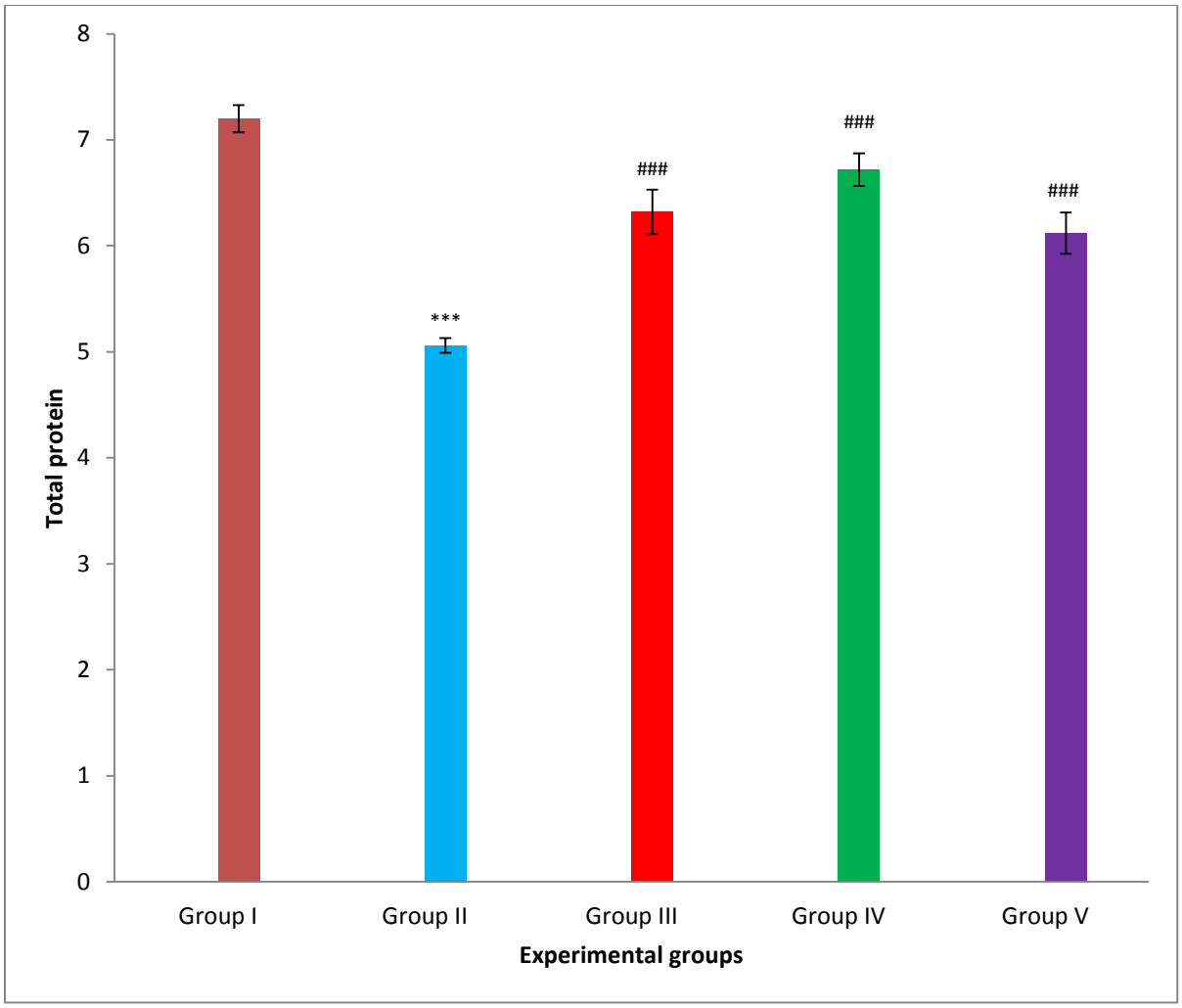
The values were expressed as Mean ±SEM. (*****, #, † = p<0.05; ******, ##, †† = p<0.01; *******, ###, ††† = p<0.001) ***** = when compared with vehicle control group, # = when compared with anti-TB drug group, † = when compared with silymarin group.The data was analyzed using One-Way ANOVA followed by *Tukey HSD Test*

(a) Effect of different pharmacological interventions on serum ALT level in rats



The values were expressed as Mean ±SEM. (*****, #, † = p<0.05; ******, ##, †† = p<0.01; *******, ###, ††† = p<0.001) ***** = when compared with vehicle control group, # = when compared with anti-TB drug group, † = when compared with silymarin group.The data was analyzed using One-Way ANOVA followed by *Tukey HSDTest*

(b) Effect of different pharmacological interventions on serum AST level in rats



The values were expressed as Mean ±SEM. (*****, #, † = p<0.05; ******, ##, †† = p<0.01; *******, ###, ††† = p<0.001) ***** = when compared with vehicle control group, # = when compared with anti-TB drug group, † = when compared with silymarin group.The data was analyzed using One-Way ANOVA followed by *Tukey HSDTest*

(c) Effect of different pharmacological interventions on Total protein level in rats

54.2 Estimation of MDA level in liver tissue

The level of MDA was determined from the standard calibration curve of TEP as shown fig 5.5

Table 5.6 Absorbance of TEP

S.no	Concentration (µmol/ml)	Absorbance (nm)
1	0	0
2	1	0.006
3	2	0.017
4	3	0.027
5	4	0.04
6	5	0.048

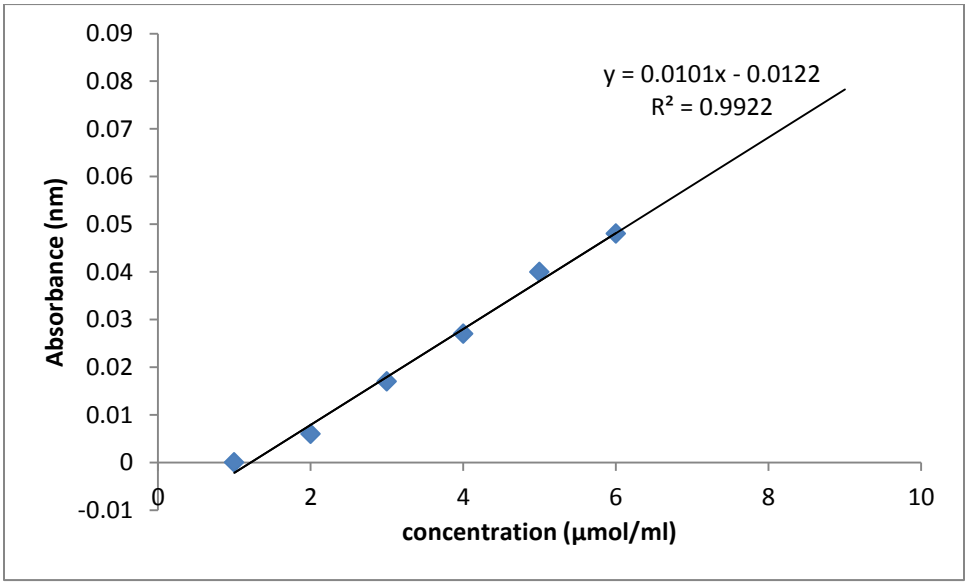


Figure 5.5 Standard plot of TEP

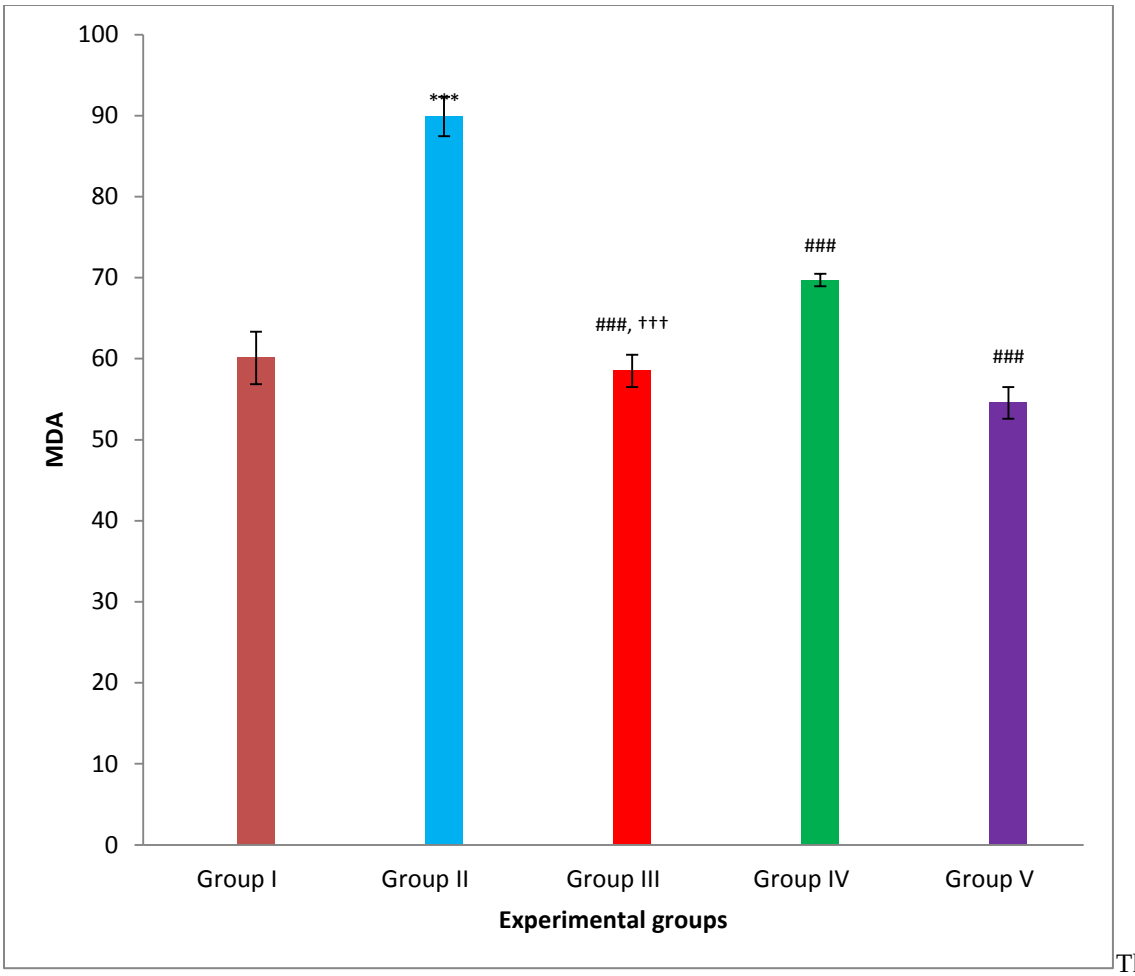
TBARS are formed as the byproduct of lipid peroxidation and it is also the index of oxidative stress. Assay of TBARS measures the level of MDA in the tissue which is related to the extent of damage caused in the tissue (Marquez et al., 2010)

The level of MDA in Isoniazid (H) + Rifampicin (R) + Pyrazinamide (Z) suspension group (89.88±2.42) was significantly higher than the vehicle control group (60.1±0.78). The administration of goat milk (20ml/kg) and goat milk (40ml/kg) was moderately effective in reversing the rise in MDA level as compared to anti-TB drug suspension group.

Table 5.7 MDA level in liver tissue in different experimental groups

S.no.	**Experimental groups**	**MDA µmol/ml of tissue**
1	Group I	60.1 ±3.24
2	Group II	89.88 ±2.42***
3	Group III	58.5 ±2### †††
4	Group IV	69.7 ±0.78###
5	Group V	54.55±1.96###

The values were expressed as Mean ±SEM. (*****, #, † = p<0.05; ******, ##, †† = p<0.01; *******, ###, ††† = p<0.001) ***** = when compared with vehicle control group, # = when compared with anti-TB drug group, † = when compared with silymarin group.The data was analyzed using One-Way ANOVA followed by *Tukey HSD Test*.



The values were expressed as Mean ±SEM. (*****, #, † = p<0.05; ******, ##, †† = p<0.01; *******, ###, ††† = p<0.001) ***** = when compared with vehicle control group, # = when compared with anti-TB drug group, † = when compared with silymarin group.The data was analyzed using One-Way ANOVA followed by *Tukey HSDTest*

Figure 5.6 Effect of different pharmacological interventions on MDA level of in rats

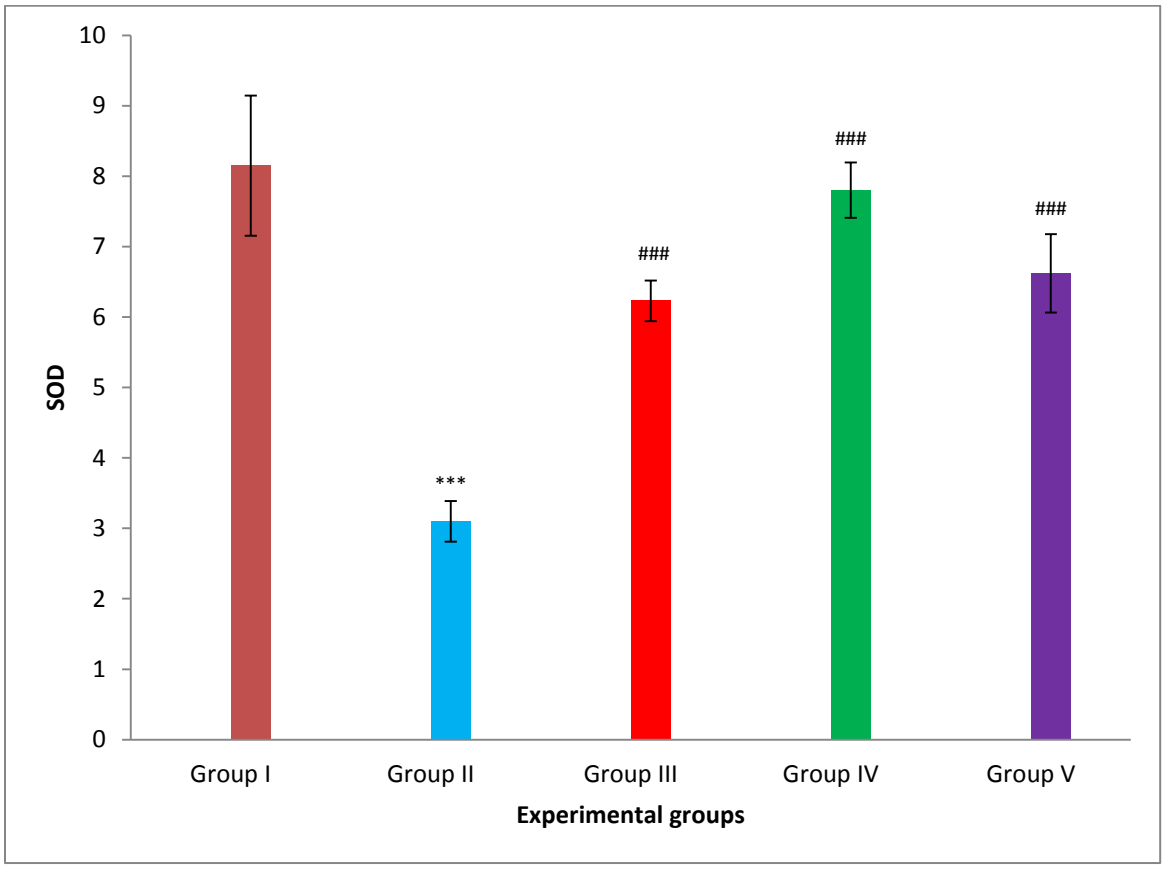
5.4.3 Estimation of SOD level in liver tissue

In the present study (Table 5.7) the antioxidant enzyme Superoxide dismutase level in anti-tubercular drug treated group II showed a significant decrease in SOD as compared to control group. The antioxidant enzyme was significantly increased in group III and IV as compared to anti-tubercular drug group and were approximately equivalent to the group I and V.

Table 5.8 SOD level in liver tissue in different experimental groups

S.no.	Experimental groups	SOD (units/ml)
1	Group I	8.15±0.995
2	Group II	3.1 ±0.288***
3	Group III	6.23 ±0.29###
4	Group IV	7.8±0.392###
5	Group V	6.62 ±0.556###

The values were expressed as Mean ±SEM. (*, #, † = p<0.05; **, ##, †† = p<0.01; ***, ###, ††† = p<0.001) * = when compared with vehicle control group, # = when compared with anti-TB drug group, † = when compared with silymarin group.The data was analyzed using One-Way ANOVA followed by *Tukey HSD Test.*



The values were expressed as Mean ±SEM. (*, #, † = p<0.05; **, ##, †† = p<0.01; ***, ###, ††† = p<0.001) * = when compared with vehicle control group, # = when compared with anti-TB drug group, † = when compared with silymarin group.The data was analyzed using One-Way ANOVA followed by *Tukey HSDTest*

Figure 5.7 Effect of different pharmacological interventions on SOD level in rats

5.5 Discussion

The anti-tubercular drugs induced hepatotoxicity is mediated through oxidative stress and free radical damage to hepatocytes. Due to leakage of enzymes from damaged hepatocytes into vascular compartment serum AST and ALT levels increase in hepatic damage. Liver damage leads to decrease in synthetic capability leading to fall in serum protein levels. Rise of serum MDA due to anti-tubercular drugs treatment may be attributed to the chronic pathology of liver leads to disturbances in circulation and oxygenation which in turn cause lipid peroxidation and subsequently increase serum MDA concentration. Therefore, lipid peroxidation may cause severe damage and play a key role in pathogenesis of several human diseases. Superoxide dismutase is an antioxidant enzyme which can destroy the superoxide anion, $O2^{-}$. The decrease in SOD activity could be due to the increased production of reactive oxygen species as evident from the increased lipid peroxidation levels due to anti-tubercular drugs treatment. Liver biopsy is the most reliable index of liver damage. Liver damage is indicated by degeneration, necrosis and fibrosis while reduction in these parameters and evidence of regeneration are suggestive of hepatoprotection.

In the present study, concurrent administration of goat milk (20ml/kg, 40ml/kg) along with anti-tubercular drugs significantly prevented the rise in level of serum ALT, AST and tissue malondialdehyde. Similarly goat milk significantly prevented fall in serum total protein and superoxide dismutase (SOD) as compared to group receiving anti-tubercular drugs alone. The beneficial results of goat milk in both groups were equivalent to the reference drug silymarin. However, the difference between the two groups receiving goat milk was not statistically significant. Administration of goat milk reduced inflammation, degeneration and necrotic changes. Hence the results show that goat milk prevented the anti-tubercular drugs induced hepatotoxicity and can be considered as an effective hepatoprotective agent.

6. SUMMARY

&

CONCLUSION

6. SUMMARY AND CONCLUSION

The aim of the present study was to explore the hepatoprotective potential of goat milk in experimentally induced hepatotoxicity in rats. The first step was to establish hepatotoxicity imitating the human liver disease through laboratory animals. Hepatotoxicity was induced by using anti-tubercular drugs i.e. isoniazid, rifampicin and pyrazinamide. The treatment effect of goat milk was studied in comparison with the standard drug silymarin. The study period was of 30 days in which animals were treated with anti-tubercular drugs on 1 to 30 days and treatment was started on same time after the administration of anti-tubercular drugs till the 30^{th} day. On 31^{st} day, the animals were sacrificed for various gross, histopathology and biochemical estimations. Earlier studies have proved the antioxidant and anti-inflammatory activity of goat milk. However, no evidence has been reported regarding its hepatoprotective activity. So present study was designed to investigate the effect of goat milk against anti-tubercular drug induced hepatotoxicity in rats.

The results of the present study are summarized as below:

- Anti-tubercular drug induced hepatotoxicity was successfully reproduced, which was evident from gross morphology and histopathology.
- The orally administered goat milk (20ml/kg and 40ml/kg) was found to be effective in reducing the severity of hepatotoxicity and the effects were similar to the reference drug silymarin. However, the difference between the both groups (III and IV) was not statistically significant.
- The goat milk reduced the histopathological scores. The goat milk was equally effective to standard drug and was found to be an effective treatment
- Goat milk significantly reversed all the biochemical changes i.e. changes associated with serum protein level, serum ALT and serum AST level in the blood of the rats. Total serum protein levels were significantly more in goat milk treated groups and serum ALT and serum AST level significantly less in goat milk treated groups as compared to anti-tubercular drug group. These results showed that goat milk was effective in anti-tubercular drug induced hepatotoxicity. However, the difference between both the milk groups was not statistically significant.

- Goat milk significantly decreased the MDA level in the liver tissue of the rats as compared to anti-tubercular drug treated group. These results showed that the goat milk was effective in treating anti-tubercular drug induced hepatotoxicity.
- SOD levels were significantly increased in goat milk treated groups as compared to anti-tubercular drug treated group. However, the difference between both the milk groups was not statistically significant.

On the basis of present study, it was concluded that goat milk possesses hepatoprotective activity and was found to be effective in anti-tubercular drug induced hepatotoxicity. However, the difference between both the milk groups was not statistically significant. Both the doses were equally effective. Although the higher dose (40ml/kg) was safe and tolerable, but no additional beneficial effects were seen. So goat milk at a dose of 20ml/kg can be investigated for further studies. Hence it can be concluded that goat milk possesses the potential of an effective and promising option for the treatment of hepatotoxicity.

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7. REFERENCES

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8. ANNEXURE

CHEMICALS USED

S. No.	Name of chemical
1.	Ether
2.	EDTA
3.	Formaldehyde
4.	Gum acacia
5.	Hydroxylamine hydrochloride
6.	Nitroblue tetrazolium
7.	Potassium dihydrogen phosphate
8.	Sodium dodecyl sulphate
9.	Sulphuric acid
10.	Sodium dihydrogen phosphate
11.	Sodium hydrogen phosphate
12.	Sodium hydroxide
13.	Sodium carbonate
14.	Thiobarbituric acid
15.	Triton X

EQUIPMENTS USED

S. No.	Equipments	Manufacturer companies
1.	Centrifuge	Remi equipment India
2.	Digital Weighing balance	Shimadzu
3.	Hot air oven	Navyug, India
4.	Homogenizer	Remi equipment India
5.	pH meter	Systronics
6.	UV/VIS spectrophotometer 1800	Shimadzu
7.	Deep Freezer	Blue Star
8.	Water bath	Navyug, India

Annexure I

INDIA'S LARGEST UNIVERSITY

LOVELY PROFESSIONAL UNIVERSITY

Transforming Education Transforming India

Discipline: M. Pharmacy (Pharmacology)

PROJECT/DISSERTATION TOPIC APPROVAL PERFORMA

Name of Student: Sonam Miglani	Registration No.: 11309919
Batch: 2013-2015	Roll No.: .....
Session: 2014-2015	Parent Section.: Y1305

Details of Supervisor:	Name: Dr. Sazal Patyar
Designation: Assistant Professor	Qualification: Ph. D.
UID: 17050	Research Experience: 5 years

PROPOSED TOPICS

1. ✓ Hepatoprotective effect of goat milk against anti-tubercular drug-induced hepatotoxicity in rats.
2. To study the effect of *Crocus sativus* on hepatotoxicity induced by anti-tubercular drugs in rats.
3. To study the effect of a test drug in 1,2-dimethylhydrazine induced colon carcinogenesis in rats.

Sazal Patyal

Signature of Supervisor

*Guide should finally encircle one topic out of three proposed topics and put up for approval before Project Approval Committee (PAC)

*Original copy of this format after PAC approval will be retained by the student and must be attached in the Project/Dissertation synopsis and final report.

*One copy to be submitted to Supervisor.

APPROVAL OF PAC CHAIRPERSON:

Monica Gulati

Signature:

CENTRAL ANIMAL HOUSE FACILITY (CAHF)

Lovely School of Pharmaceutical Sciences, Lovely Professional University

Ludhiana- Jalandhar G.T. Road, Phagwara (Punjab), 144402

Registration Number -954/ac/06/CPCSEA

CERTIFICATE

This is to certify that the project titled *"Hepatoprotective effect of goat milk against anti-tubercular drug induced hepatotoxicity in rats"* has been approved by the IAEC.

Name of Principal Investigator: Dr. Sazal Patyar

IAEC approval number: LPU/LSPS/IAEC/CPCSEA/MEETING NO. 4/2014/2015 PROTOCOL NO.3

Date of Approval: 08/11/2014

Animals approved: 30 Rats

Remarks if any: -

Dr. Monica Gulati	Mrs. Yogyata Vaidya	Mr. Bimlesh Kumar
Biological Scientist, Chairperson IAEC	Scientist, COD Pharmacology	Scientist In-Charge of Animal House

An Associate of
MANN DIAGNOSTIC &
RESEARCH CENTRE PVT. LTD.

Gargi Diagnostics Laboratory
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HEPATOPROTECTIVE EFFECT OF GOAT MILK AGAINST ANTITUBERCULAR DRUG INDUCED HEPATOTOXICITY IN RATS.

Group 1: Shows Normal Liver Architecture.

Group 2: Shows moderate degree of inflammation in hepatocytes in the form of degeneration, necrosis and inflammation.

Group 3: Shows decreased inflammation, degeneration and necrosis.

Group 4: Shows improved picture by reduced degenerative and necrotic changes. Inflammation is also scanty.

Group 5: Shows better picture with reduced degeneration, necrosis and inflammation.

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Analytical Testing Record of Active Pharma Ingredient

(As per Schedule U & G.M.P.)

Certificate of Analysis

ISONIAZID USP

Test Report No: KPA/API/101/ RM15057564

CR. No: RM15057564	Batch Number: 014CI-1352	Standard : USP
Mfg date: 03/2014	Exp date: 02/2019	Quantity: 1 x 15gm
Date of receipt of Sample: 26/08/2014	Testing date: 26/08/2014	Retesting date: 25/08/2015

Sample Drawn by Analyst: Charanjeet Singh

Analytical Data

S.No.	Test	Specification	Observation
01.	Description	Colorless or white crystals or white, crystalline powder. Is odorless and is slowly affected by exposure to air and light.	White colored crystalline powder.
02.	Solubility	Freely soluble in water; sparingly soluble in alcohol; slightly soluble in chloroform; and very slightly soluble in ether.	Freely soluble in water; sparingly soluble in alcohol; slightly soluble in chloroform; and very slightly soluble in ether.
03.	Identification. A) Infrared Absorption B) UV	 Meets the requirements. Meets the requirements.	 Identified Identified
04.	Melting Point	Between 170°C to 173°C	172.4°C
05.	pH	Between 6.0 and 7.5, in a solution (1 in 10).	6.91
06.	Residue on ignition	Not more than 0.2%	0.04%
07	Heavy metals	Not more than 0.002%	Complies
08.	Loss on drying	Not more than 1.0% of its weight.	0.83%
09.	Assay	By titration Between 98%–102% (Dried basis)	 101.2% (Dried basis)

Conclusion: The above product complies with above specifications and is of standard quality as per USP.

Prepared By	Analyzed By	Checked By	Approved By
Jasdeep Singh	Seema	Parul Sharma	Navdeep Singh
Date: 27/08/2014	Date: 27/08/2014	Date 27/08/2014	Date: 27/08/2014

Bankers :
State Bank of India Ranjit Avenue,
Amritsar IFCS : SBIN0004074
SWIFT SBININBB435, Account : 30355858698

C.E.Reg. No. : AAACK6458MXM002
Excise Range : Ranjit Avenue, Amritsar.
Division: The Mall Amritsar
Commissionerate : Ludhiana.

VAT No. : 03391061164 P.S.T/C.S.T. No. 20269913 Dt. 18/8/1980
D.L. No. 1800-OSP, 1804-B I.E. Code No. 1293001210
KWALITY PHARMACEUTICALS PVT. LTD.
6th Mile Stone, Village Nag Kalan, Majitha Road, Amritsar - 143 601 (INDIA)
WHO-GMP Certified Co.
ISO 9001:2008 & ISO 14001:2004

Phone : 91-183-2774321- 25
Mobile : 91-9915743720
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Fax : 91-183-2774477
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ramesh@kwalitypharma.com
Website : www.kwalitypharma.com
Skype ID : ramesh.arora1

Analytical Testing Record of Active Pharma Ingredient

(As per Schedule U & G.M.P.)

Certificate of Analysis

RIFAMPIN USP

Test Report No: KPA/API/101/ RM15057649

CR. No: RM15057649	Batch Number: 1408021	Standard : USP
Mfg Date:08/2014	Exp Date: 07/2018	Quantity: 1x15gm
Date of receipt of Sample: 11/11/2014	Testing date:11/11/2014	Retesting date:10/11/2015

Sample Drawn by Analyst: Charnjeet Singh

Analytical Data

S. No.	Tests	Specifications	Observations
01.	Description	Red-brown, crystalline powder	Red-brown, crystalline powder
02.	Solubility	Very slightly soluble in water; freely soluble in chloroform; soluble in ethyl acetate and in methanol.	Very slightly soluble in water; freely soluble in chloroform; soluble in ethyl acetate and in methanol.
03.	Identification. Infrared Absorption	Spectra of test should be matched with spectra of reference standard.	Identified
04.	pH	Between 4.5 and 6.5, in a suspension (1 in 100).	5.01
05.	Crystallinity	Meets the requirements.	Complies
06.	Related substances	Meets the requirements.	Complies
07.	Loss on drying	Not more than 2.0% of its weight.	1.20%
08.	Assay	By UV Between 95% - 103.0% (Dried Basis)	100.5% (Dried Basis)

Conclusion: The Product complies with above specifications and meets the standard quality as per USP.

Prepared By	Analyzed By	Checked By	Approved By
Jasdeep Singh	Seema	Parul Sharma	Navdeep Singh
Date: 12/11/2014	Date: 12/11/2014	Date: 12/11/2014	Date: 12/11/2014

Bankers :
State Bank of India Ranjit Avenue,
Amritsar IFCS : SBIN0004074
SWIFT SBININBB435, Account : 30355858698

C.E.Reg. No. : AAACK6458MXM002
Excise Range : Ranjit Avenue, Amritsar.
Division: The Mall Amritsar
Commissionerate : Ludhiana.

VAT No. : 03391061164 P.S.T/C.S.T. No. 20269913 Dt. 18/8/1980
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Website : www.kwalitypharma.com
Skype ID : ramesh.arora1

Analytical Testing Record of Active Pharma Ingredient

(As per Schedule U & G.M.P.)

Certificate of Analysis

PYRAZINAMIDE USP

Test Report No: KPA/API/101/ RM15057654

CR. No: RM15057654	Batch Number: PYZ004L14	Standard : USP
Mfg date: 10/2014	Exp. Date : 09/2018	Quantity: 1 x 35gm
Date of receipt of Sample: 30/10/2014	Testing date:30/10/2014	Retesting date:29/10/2015

Sample Drawn by Analyst: Jatinder Kumar

Analytical Data

S. No.	Tests	Specifications	Observations
01.	Description	White to practically white, odorless or practically odorless, crystalline powder	White colored crystalline powder.
02.	Solubility	Sparingly soluble in water; slightly soluble in alcohol, in ether, and in chloroform	Sparingly soluble in water; slightly soluble in alcohol, in ether, and in chloroform
03.	Identification. A) IR Absorption B) UV Absorption C) PPT test	 Meets the requirements. Absorptivities at 268 nm, calculated on the dried basis, do not differ by more than 3.0%. The odor of ammonia should be perceptible.	 Identified Identified Identified
04.	Melting point	Between 188° and 191°.	190.4°
05.	Water	Not more than 0.5%.	0.20%
06.	Residue on Ignition	Not more than 0.1%	Complies.
07.	Heavy metals	Not more than 0.001%	Complies.
08.	Assay	By Titration Between 99.0% - 101.0% (As anhydrous basis)	100.4% (anhydrous basis)

Conclusion: The above product complies with above specifications and is of standard quality as per USP.

Prepared By	Analyzed By	Checked By	Approved By
Jasdeep Singh	Seema	Parul Sharma	Navdeep Singh
Date: 31/10/2014	Date: 31/10/2014	Date: 31/10/2014	Date: 31/10/2014

Bankers :
State Bank of India Ranjit Avenue,
Amritsar IFCS : SBIN0004074
SWIFT SBININBB435, Account : 30355858698

C.E.Reg. No. : AAACK6458MXM002
Excise Range : Ranjit Avenue, Amritsar.
Division: The Mall Amritsar
Commissionerate : Ludhiana.