A

Project work on

"HEPATOPROTECTIVE ACTIVITY OF LEAVES OF *RHODODENDRON ARBOREUM* IN CCl_4 INDUCED HEPATOTOXICITY IN WISTAR RATS"

By

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Rajiv Gandhi University of Health Sciences, Karnataka, Bangalore

In partial fulfillment of the requirements for the degree of

MASTER OF PHARMACY
in
PHARMACOLOGY

Under the guidance of

Mr. T. PRAKASH, M. Pharm.

Asst. Prof.

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September-2007

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LIST OF ABBREVIATIONS USED

ALP	:	Alkaline phosphatase
CCl_4	:	Carbon tetrachloride
CPCSEA	:	Committee for the Purpose of Control and Supervision on Experiments on Animals
et al.	:	(L) et al. i.e. = Excetra and all
H_2O_2	:	Hydrogen peroxide
NADPH	:	Nicotinamide adenine dinucleotide
NaOH	:	Sodium hydroxide
O_2	:	Superoxide radical
OECD	:	Organisation for Economic Co-operation and Development
OH	:	Hydroxyl radical
rpm	:	Rotation per minute
s.c.	:	Subcutaneous
SEM	:	Standard error of mean
SGOT	:	Serum Glutamate Oxaloacetate Transaminase
SGPT	:	Serum Glutamate Pyruvate Transaminase
TCA	:	Trichloroacetic acid

ABSTRACT

Objective: To investigate the hepatoprotective activity of ethanolic extract of leaves of *Rhododendron arboreum* against Carbon tetrachloride-induced hepatotoxicity in Wistar rats.

Methods: Liver damage was induced in Wistar rats by administering CCl_4 on 2nd and 3rd day. The ethanolic extract of leaves of *Rhododendron arboreum* was given for five days. Silymarin (100 mg/kg) was given as the reference drug. Hepatoprotective effect was studied by assaying the activities of serum marker enzymes like serum glutamate pyruvate transaminase, serum glutamate oxaloactetate transaminase and serum alkaline phosphatase, serum direct bilirubin, serum total bilirubin, serum triglycerides, serum cholesterol and estimation of ascorbic acid in urine. Histopathological study of the liver in experimental animals was also undertaken.

Result: The activities of all the marker enzymes registered a significant elevation in Carbon tetrachloride treated rats, which were significantly recorved towards an almost normal level in animals co-administered with ethanolic extract of leaves of *Rhododendron arboreum* at a dose of 100 mg/kg. Ethanolic extract of leaves of *Rhododendron arboreum* prevented decrease in the excretion of ascorbic acid in CCl_4 induced hepatotoxicity in rats. Histopathological analysis confirmed the biochemical investigations.

Conclusion: The present study demonstrates that the ethanolic extract of leaves of *Rhododendron arboreum* possess hepatoprotective property.

Keywords: Hepatoprotective; Marker enzymes; Carbon tetrachloride; *Rhododendron arboreum*; Ascorbic acid.

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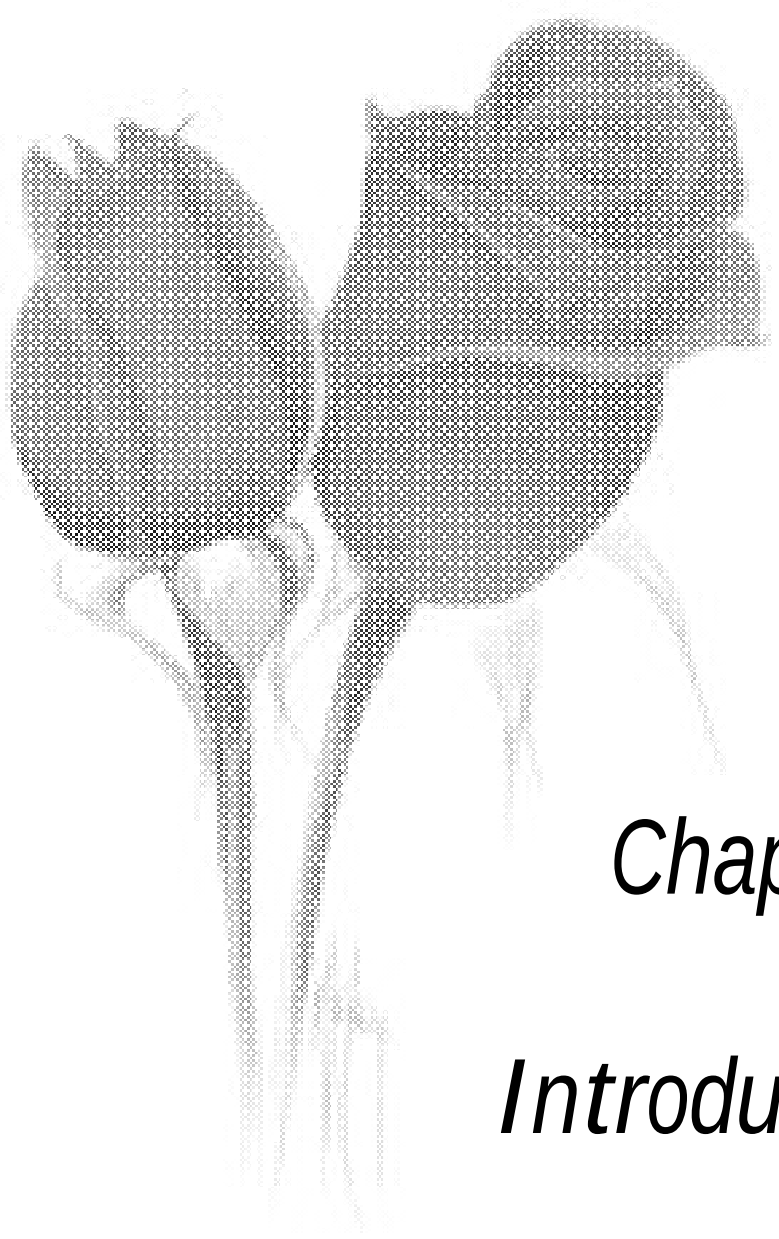


Chapter-1

Introduction

INTRODUCTION

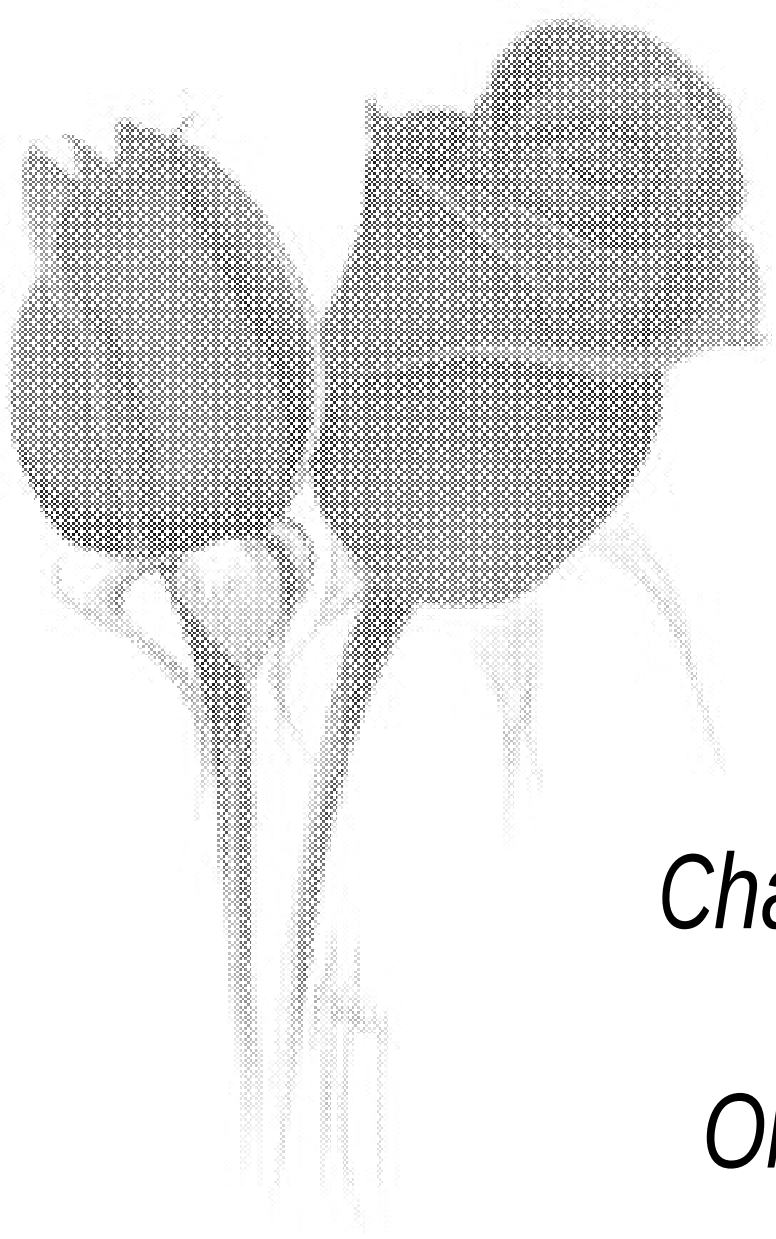
Liver is considered the key organ in the metabolism, detoxification, and secretory functions in the body, and its disorders are numerous with no effective remedies, however, the search for new medicines is still ongoing. Many folk remedies from plant origin have been long used for the treatment of liver diseases[1].

Liver regulates various important metabolic functions. Hepatic damage is associated with distortion of these metabolic functions. Liver disease is still a world wide health problem. Unfortunately, conventional or synthetic drugs used in the treatment of liver diseases are inadequate and sometimes can have serious side effects. This is one of the reasons for many people in the world over including those in developed countries turning complimentary and alternative medicine. Many traditional remedies employ herbal drugs for the treatment of liver ailments[2, 3].

Rhododendron arboreum is an important medicinal plant which is used for treatment of various ailments in Ayurvedic system of medicine. Flavonoids, isolated from the leaves of *Rhododendron arboreum* were found to have potent antioxidant property[4]. and the plant *Rhododendron arboreum* have been reported for anti-inflammatory[5], antidiarrhoeal activities[6].

In the absence of reliable liver protective drugs in modern medicine, there are numbers of medicinal preparations in the ayurvedic system of Indian medicine recommended for the treatment of liver disorders. Their usage is in vogue since centuries and are quite often claimed to offer significant relief. However, no scientific information is available regarding the hepatoprotective effect of *Rhododendron arboreum*. Since, antioxidants are known to reduce the development of chemically induced liver damage,

the effect of ethanolic extract of leaves of ***Rhododendron arboreum*** has been evaluated for hepatoprotective activity.



Chapter-2

Objectives

OBJECTIVES

The main objective of the present study was undertaken for the evulate hepatoprotective activity on the wistar rats by adapting following steps.

Step I : Preparation of different extracts of leaves of *Rhododendron arboreum*.

Step II : Preliminary phytochemical screening of different extracts like petroleum ether, chloroform, and ethanol of leaves of *Rhododendron arboreum*.

Step III : To evaluate the toxicity study (LD_{50}) of the ethanolic extract of leaves of *Rhododendron arboreum.*

Step IV : To study the hepatoprotective activity, following parameters were selected.

i) Estimation of biochemical parameters

a) Serum Glutamate Pyruvate Transaminase (SGPT)

b) Serum Glutamate Oxaloactetate Transaminase (SGOT)

c) Serum direct Bilirubin

d) Serum total Bilirubin

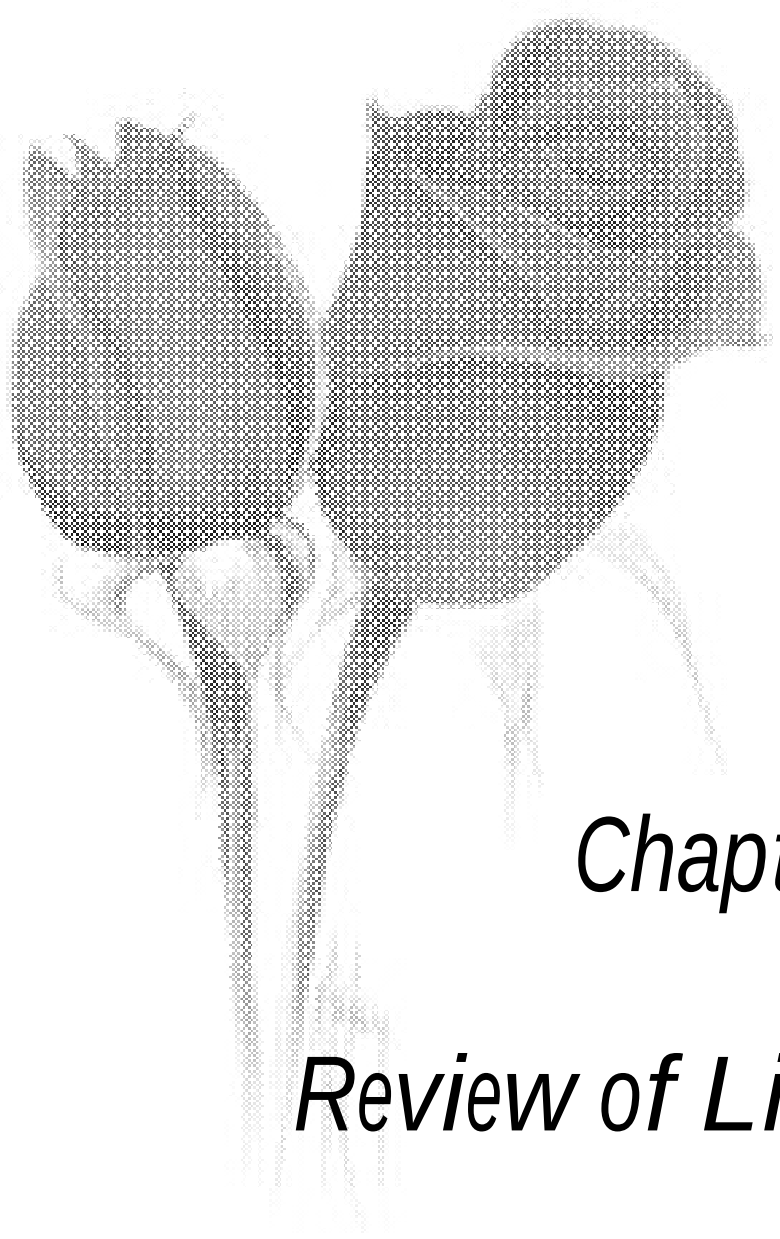
e) Serum Alkaline Phosphatase (ALP)

f) Serum triglycerides

g) Serum cholesterol

ii) Histopathological studies of rat liver

iii) Estimation of ascorbic acid in CCl_4- intoxicated rat urine



Chapter-3

Review of Literature

Figure-1

Rhododendron arboreum

Family : Ericaceae

REVIEW OF LITERATURE

Plant name : ***Rhododendron arboreum***

Family : Ericaceae

The plant is known by various names in different languages as under.

Almora	-	Brons
Canarese	-	Bili, Pu
English	-	Rose tree[7]
Hindi	-	Buruns[8]
Kumaon	-	Bras, Brus
Malayalam	-	Kuttupuvarasu
Nepali	-	Bhorans, Ghonas, Guras, Tagghu.
Punjabi	-	Ardawal, Aru, Broa
Sinhalese	-	Maratmal
Tamil	-	Alingi, Billi

Distribution:

Temperate Himalaya, from Kashmir to Bhutan 5000-11000 ft. Khasia Hills, 4000-6000 ft. Burma, Nilgiris, Pulneys, Travancore, above 5000 ft.

Description:

A small ever green tree up to 1.5 m girth and 7.5 m high. Bark pinkish brown, some what rough, exfoliating in thin flakes. Young shoots clothed with white scales. Leaves 7.5-15 by 3-5.5 cm, crowded towards the ends of the branches, lanceolate or

oblong, narrowed at both ends, glabrous and glossy green above; pale beneath from a film of small white scale, the midrib and nerves prominent beneath. Petiole stout, 1.3-2.5 cm long, clothed with white scales when young. Flowers 2.5-5 cm long, deep red or pale pink, crowded in large rounded corymbs. Ovary mealy or rusty-woolly. Capsule 2.5 cm by 7.5 mm, cylindric, curved, mealy, longitudinally ribbed [9].

Chemical constitutes present in plant

The family Ericaceae produces phenolic acids, phenolic glycosides, aucubin glycosides, diterpenoids, triterpenoids[10]. The leaves of ***Rhododendron arboreum*** were reported to contain Quercetin 3-O- beta -D-glucopyranosyl [1->6]-O- alpha -L-rhamnopyranoside, pectolinarigenin 7-O-rutinoside, 7,2'-dimethoxy-4',5-methylenedioxyflavanone[11]. The flowers of ***Rhododendron arboreum*** were reported to contain quercetin, rutin, coumaric acid[12].

Traditional uses

The young leaves are astringent and poultice. They are made into a paste and then applied to the forehead in the treatment of headaches. The juice of the bark is used in the treatment of coughs, diarrhoea and dysentery. A decoction of the flowers is used to check a tendency to vomit, especially if there is also a loss of appetite. The juice of the flowers is used in the treatment of menstrual disorders. The petals are eaten to assist the removal of any animal bones that have become stuck in the throat.

Edible Uses

The tender leaves are used as a cooked vegetable. Caution is advised, because leaves are poisonous. Flowers - raw or cooked A sweet-sour taste, they are said to make a

good sub-acid jelly. The flowers are sometimes simply pickled by adding salt and chili. Caution is advised, large quantities can cause intoxication[13].

Scientific publications of the plant *Rhododendron arboreum*

1) Anti-inflammatory activity of flowers of *Rhododendron arboreum* in rat's hind paw oedema induced by various phlogistic agents[5].
2) Status of medicinal plants in the disturbed and undistured sacred forests of Meghalaya, northeast India: population structure and regeneration efficacy of some important species[14].
3) Developing cells and tissue engineering for colors and dyes[12].
4) Flavonoidic constituents of *Rhododendron arboreum* leaves[11].
5) Free radical scavenging activities of Himalayan rhododendrons[4].

LIVER

The liver is the largest gland in the body weighing about 1.4 kg in an adult. It is situated under the diaphragm in the upper abdominal cavity and is held in place by several ligaments. It is a reddish-brown colour and comprises of four anatomical lobes. When viewed from the front, the dominant left and right lobes can be seen which are separated by the falciform ligament.

Situated in a depression on the posterior surface of the liver is the gall bladder, a pear-shaped sac which stores bile synthesized by the liver. The liver performs many vital metabolic functions. It has the ability to store and metabolize useful substances such as nutrients, but it breaks down or detoxifying harmful substances to render them inert and less harmful.

Internal Structure

The liver lobes are made up of microscopic units called lobules which are roughly hexagonal in shape. These lobules comprise of rows of liver cells (hepatocytes) which radiate out from a central point. The hepatic cells are in close contact with blood-filled sinusoids and also lie adjacent to canaliculi into which bile is secreted.

Situated around the perimeter of the lobule are branches of the hepatic artery, hepatic portal vein and bile duct. These clusters together at the "corners" of the lobule forming what is called the portal triad. At the mid-point of the lobule is the central vein. Blood flows out of the sinusoids into the central vein and is transported out of the liver.

Blood flow

Venous blood from the entire gastrointestinal tract (containing nutrients from the intestines) is brought to the liver by the hepatic portal vein. Branches of this vein pass in

between the lobules and terminate in the sinusoids. Oxygenated blood is supplied in the hepatic artery. The blood leaves the liver via a central vein in each lobule, which drains in the hepatic vein.

Hepatic vein - one of several short veins originating within the lobes of the liver as small branches, which unite to form the hepatic veins. These lead directly to the inferior vena cava, draining blood from the liver.

Inferior vena cava - formed by the union of the right and left common iliac veins, collects blood from parts of the body below the diaphragm and conveys it to the right atrium of the heart.

Hepatic artery - a blood vessel which supplies the liver with oxygenated blood. It supplies 20% of the liver's blood.

Hepatic portal vein - a blood vessel which drains venous blood into the liver from the entire gastrointestinal tract. It supplies the remaining 80% of the liver's blood.

Lobule Activity

The hepatic portal vein and hepatic artery deliver oxygen and nutrients into the blood sinusoids. This close relationship between the hepatocytes and surrounding blood enables many metabolic processes to take place.

Blood flows out of the sinusoids into the central vein, removing detoxified substances and metabolic end products. The central vein ultimately reunites with the hepatic vein transporting these substances out of the liver.

Bile that is produced by the hepatocytes drains into tiny canals called bile canaliculi (singular *canaliculus*). These drain into bile ducts located around the lobule perimeter.

Hepatocytes

Hepatocytes are the predominant cell type in the liver. An estimated 80% of the liver mass is made of these cells. The hepatocytes are round in shape containing a nucleus and an abundance of cellular organelles associated with metabolic and secretory functions.

Organelles include endoplasmic reticulum (smooth and rough) and Golgi apparatus for secretary functions. Also there are high numbers of mitochondria to provide energy to support the many metabolic functions on the liver.

Some of the hepatocytes lie adjacent to endothelial cells which form the walls of the sinusoids. These two cell types are separated by small space called the space of Disse[15].

Liver Function

The liver receives 30% of the resting cardiac output and acts as a giant chemical processing plant in the body. These chemical reactions, called metabolism, are central in the regulation of body homeostasis.

The liver cells, called hepatocytes, contain thousands of enzymes essential to perform vital metabolic functions. They are supermodels in the world of cellular metabolism.

The liver metabolises both beneficial and harmful substances. It stores nutrients and other useful substances, as well as detoxifying or breaking down harmful compounds. These can be then excreted from the body in bile via the liver; in urine via the kidney or by other means.

Nutrient Metabolism

The liver is involved in the metabolism of nutrients. It receives digestive products in the form of glucose, amino acids, fatty acids and glycerol.

The metabolism of carbohydrate, fat and protein takes place in the liver, although specific functions are carried out by fat depots and skeletal muscle. Metabolic end products are often stored in the liver and utilized at a later stage if required.

How the hepatocytes deal with the nutrients depend on whether each nutrient is in abundance or whether levels are low in the body and they are therefore in demand. The hepatocytes alter their metabolic pathways accordingly.

Carbohydrate

Glucose is a vital energy source for cells and levels in the blood stream must remain constant. The liver helps maintain blood glucose levels in response to the pancreatic hormones insulin and glucagons.

After a meal, glucose enters the liver and levels of blood glucose rise. This excess glucose is dealt with by glycogenesis in which the liver converts glucose into glycogen for storage. The glucose that is not stored is used to produce energy by a process called glycolysis. This occurs in every cell in the body.

In between meals or during starvation, blood glucose levels fall. The hepatocytes detect this change, and restore glucose levels by either glycogenolysis which converts glycogen back to glucose or gluconeogenesis in which non-sugars such as amino-acids are converted to glucose.

Fat

The liver is involved in fat metabolism and synthesises lipoproteins, cholesterol and phospholipids essential for many body functions. Lipids also provide a valuable alternative energy source to glucose and so the metabolic fate of fats and lipids will depend on the levels of intake in the diet and energy expenditure.

If fat is in excess, the liver prepares for storage. Lipogenesis is the metabolic process in which fats, composed of fatty acids and glycerol, are converted for storage in subcutaneous tissue and other storage depots.

If energy and glucose levels are low, stored fat is converted back into glycerol and fatty acids by a process called lipolysis. This occur in adipose cells, but the fatty acids and glycerol are transported to the liver for use as an alternative energy supply.

Protein

Amino acids are transported to the liver during digestion and most of the body's protein is synthesised here.

If protein is in excess, amino acids can be converted into fat and stored in fat depots or if required, made into glucose for energy by gluconeogenesis. However, before amino acids can be utilized in these ways, the first step is to remove the nitrogen-containing amino group NH_2. This very important metabolic process is called deamination.

In the hepatocytes, NH_2 (the amino group) quickly changes into ammonia NH_3, which is highly toxic to the body. The liver acts fast to convert ammonia into urea that then can be excreted in the urine and eliminated from the body.

Storage

The liver plays an important role as a storage facility. The hepatocytes take up many types of vitamins and minerals from the blood and store them. These include vitamins A, B_{12}, D, E, K and minerals like iron and copper.

Glycogen which is formed from excess glucose is also stored by the liver, although muscle tissue can also store glycogen too.

Bile

The liver synthesises bile which is important for fat digestion and is also a route of excretion from the body. Bile consists of water, bile salts, cholesterol, phospholipids, electrolytes and bile pigments which give it its typical yellowy-green colour.

Bile is stored and concentrated in the gall bladder. The presence of fats in the gut during meals stimulates the gall bladder to empty. Bile enters the duodenum emulsifying fats into smaller globules, which can then be broken down further by lipase enzymes.

Metabolic wastes and drug products may form part of the bile which can then be excreted from the body through the digestive tract in the faeces. Bilirubin, the toxic end product of haemoglobin breakdown, is excreted from the body in this way[16].

Classification of liver diseases

1. Toxic injury to the liver

I Drug induced :

Drugs are an important and common cause of hepatic injury. This is not surprising, as the liver is the predominant site of drug clearance, biotransformation and excretion. Abnormalities cover a wide spectrum from minor nonspecific dearrangements to fulminant hepatic necrosis.

A simplified clinicopathologic classification of important hepatic drug reactions and the agents causing them are presented in Table 1.

Table 1

The agents causing Hepatic damages

Pathologic changes	**Agents**
Acute liver disease	
Zonal necrosis	Carbon tetrachloride, Acetaminophen, Halothane
Massive necrosis	Acetaminophen, Halothane, Methyl dopa
Fatty liver changes	Tetracycline, Salicylates, Methotrexate, Ethanol
Hepatitis	Methyl dopa, Isoniazid, Halothan, Ketoconazole
Granuloma formation	Sulfonamides, Methyl dopa, Quinidine, Allopurinol
Cholestasis	Sex hormones, Chlorpromazine, Nitrofurantoin
Hepatic/portal vein thrombosis	Oral contraceptives
Chronic liver disease	
Fibrosis-cirrhosis	Methotrexate
Focal nodular hyper plasia	Vinyl chloride, Vitamin A, Sex hormones
Adanoma	Sex hormones
Hepatocellular carcinoma	Sex hormones

II Industrial and environmental toxin :

In the case of industrial and environmental toxins, primary disease is caused by the ingestion, injection or inhalation of a toxic substance which adversely affects the

liver. For example, herbicides, such as paraquat, are associated with an increased incidence of liver damage.

III Alcoholic :

Alcohol-induced liver disease, as the name implies, is caused by excessive consumption of alcohol and is a common, but preventable, disease. Examples fatty liver, alcoholic hepatitis, alcoholic cirrhosis.

2. Infectious agents and parasites

I Hepatitis virus :

Hepatitis is most commonly caused by one of three viruses:

the hepatitis A virus

the hepatitis B virus

the hepatitis C virus

II Bacterial and other pathogenic agents:

Helicobacter hepaticus causes chronic hepatitis.

3. Immune disorder

I Autoimmune :

Autoimmune hepatitis is a clinical and laboratory diagnosis that indicates one's immune system is inappropriately attacking liver cells, including bile duct cells. The immune system's lack of control can result in liver destruction, cirrhosis and liver cancer.

II Primary biliary cirrhosis :

Primary biliary cirrhosis is a disease characterized by inflammatory destruction of the small bile ducts within the liver. Primary biliary cirrhosis eventually leads to cirrhosis of the liver.

4. Tumors

I Primary malignant tumors

II Metastatic malignant tumors

III Benign hepatic tumors

IV Tumor-like lesion

5. Inherited

I Wilson's disease :

Wilson's disease is an inherited disorder where there is excessive amounts of copper in the body's tissues. This causes a variety of effects, including liver disease and damage to the nervous system.

II Haemochromatosis :

Haemochromatosis occurs when too much iron builds in the liver. This leads to liver enlargement. The disease may lead to the development of diabetes, skin coloring changes, heart problems, arthritis, testicular atrophy, cirrhosis of liver, liver cancer, hypopituitarism, chronic abdominal pain, severe fatigue and an increased risk of certain bacterial infections.

II Inborn errors :

Congenital hyperbilirubinemia occurs in several forms. The best known congenital jaundice syndromes are Gilbert syndrome, Rotor syndrome and Dubin – Johnson syndrome.

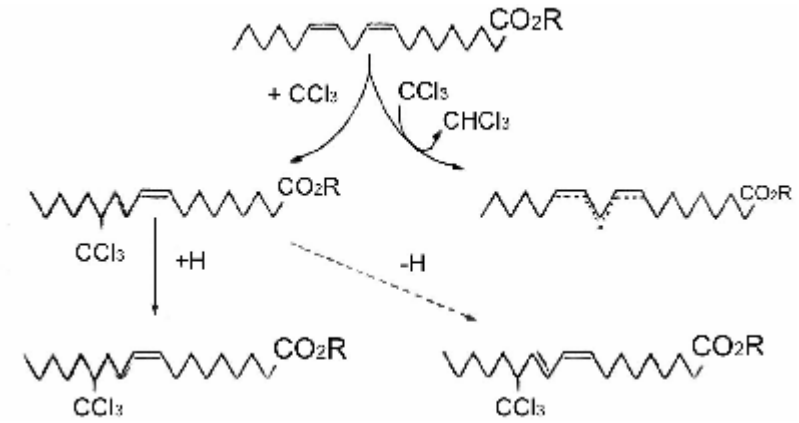
IV Alpha-1-antitrypsin deficiency :

Alpha-1 antitrypsin deficiency is an inherited disorder that can cause lung disease in adults and liver disease in adults and children[17-18].

Mechanism of Carbon tetrachloride induced Hepatotoxicity

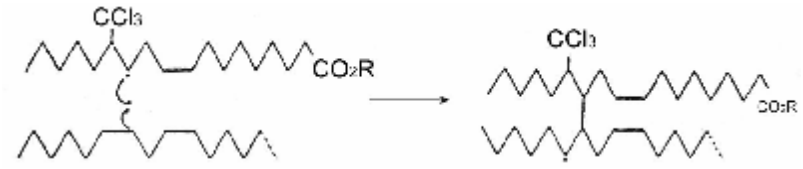
Toxic injury of the liver induced by Carbon tetrachloride is a model system of hepatotoxicity. Injury produced by CCl_4 seems to be mediated by reactive metabolite – trichloromethyl free radical ($CCl_3^{\bullet}$) formed by the homolytic cleavage of CCl_4 or by an even more reactive species trichloromethylperoxy free radical ($Cl_3 COO^{\bullet}$) formed by the reaction of $CCl_3^{\bullet}$ with O_2. This biotransformation is catalyzed by a cytochrome P_{450} dependent monooxygenase[19].

CCl_4 is reductively converted by P_{450} to the trichloromethyl radical the fate of this radical is of interest. First the radical add covalently to unsaturated fatty acids, trichloromethyl fatty acids, particularly of membrane phospholipids.



Recently these substituted fatty acids have been noted to be partially resistant to replace from endoplasmic reticular phospholipase A2.

This seems to be result of cross linking of trichloromethyl fatty acid radical, which adds to double bond of another adjacent fatty acids .



The physiologic significance of this cross linking on membrane structure and function may be of great importance, particularly if these phospholipids are transformed to other critical sites in the cell. Besides covalent binding to lipid, the cells can abstract an electron from unsaturated fatty acids, yielding $CHCl_3$ and or fatty acid radical. Either the trichloromethyl fatty acid radical or the fatty acid radical can react with oxygen to form peroxy radical, which initiates the lipid peroxidation chain reaction[20].

Table 2

Plants having hepatoprotective activity

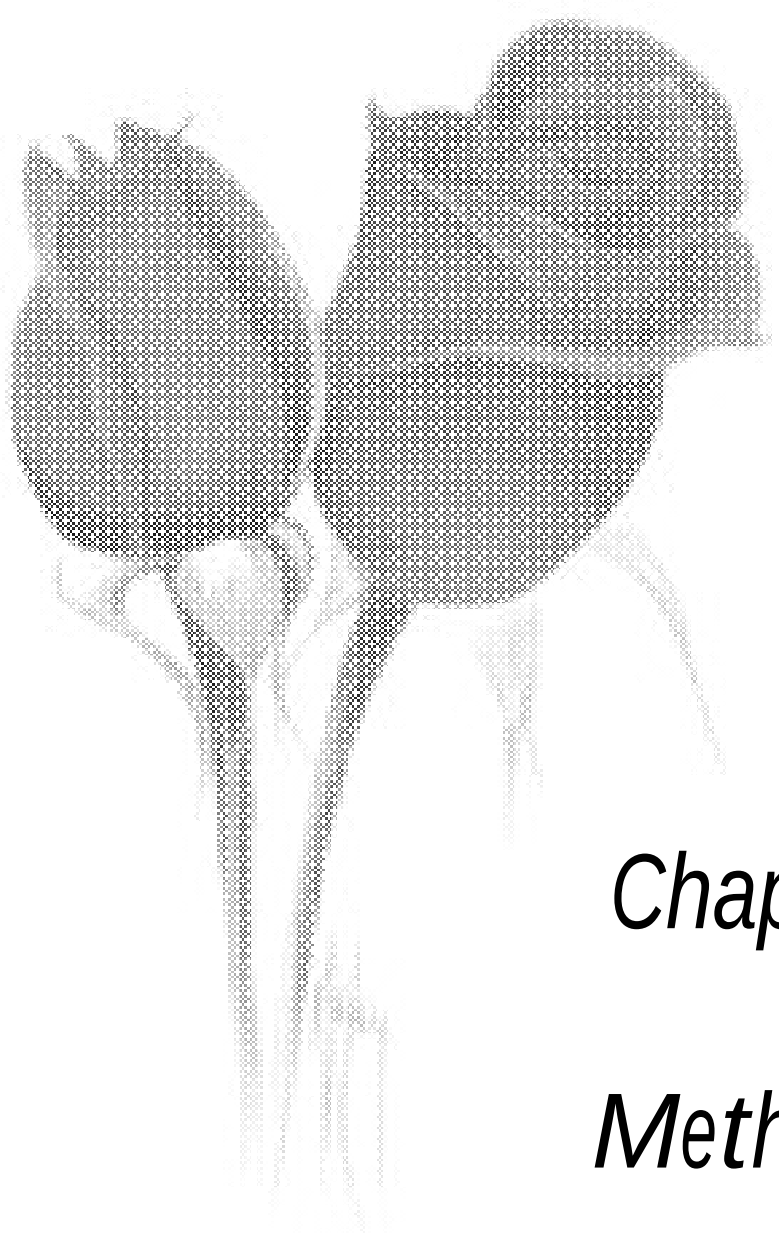
Sr. no.	Name of the plant	Family	Part used	Ref.
1	*Ambrosia maritima*	Asteraceae	Whole plant	21
2	*Andrographis paniculata*	Acanthaceae	Whole plant	22
3	*Beta vulgaris*	Chenopodiacae	Roots	23
4	*Boehmeria nivea var. nivea*	Urticaceae	Roots	24
5	*Boehmeria nivea var.tenacissia*	Urticaceae	Roots	24
6	*Boerhaavia diffusa*	Nyctaginaceae	Roots	25
7	*Cassia fistula*	Leguminosae	Leaves	26
8	*Cassia occidentalis*	Caesalpinaceae	Leaves	27
9	*Cochlospermum planchonii*	Cochlospermaceae	Rhizomes	28
10	*Curculigo orchioides*	Amaryilidaceae	Rhizomes	29
11	*Cyperus rotundus*	Cyperaceae	Tuberous roots	30
12	*Daucus carota*	Apiaceae	Roots	31
13	*Emblica officinalis*	Euphorbiaceae	Fruits	32
14	*Gundelia tourenfortii*	Compositae	Stalks	33
15	*Hedyotis corymbosa*	Rubiaceae	Roots	34
16	*Kalanchoe pinnata*	Crassulaceae	Leaves	35
17	*Psidium guajava*	Myrtaceae	Leaves	36
18	*Rubia cordifolia*	Rubiaceae	Roots	37
19	*Strychnos potatorum*	Loganiaceae	Seeds	38
20	*Trianthema portulacastrum*	Ficoidaceae	Aerial parts	39

Table 3

Proprietary hepatoprotective Multiherbal formulation

Trade marks	Herbal ingredients	Reference
Jigrine	*Careya arborea* *Cassia occidentalis* *Cichorium intybus* *Cusuta reflexa* *Foeniculum vulgare* *Phyllantus amarus* *Plantago major* *Tamarix dioica* *Solanum nigrum* *Rubia cordifolia* *Vitex negundo* *Rosa damascena* *Solanum xanthocarpum*	40
Liv-52	*Capparis spinosa* *Cassia occidentalis* *Cichorum intybus* *Mandur bhasma* *Solanam nigrum* *Terminalia arjuna* *Tamarix gallica*	41

Livex	*Aconitum heterophyllum* *Andrographis paniculata* *Cassia occidentalis* *Cichorium intybus* *Embelia ribes* *Tephrosia purpurea* *Piper longum* *Solanum nigrum* *Tamarix gallica*	42
Rhinax	*Withania somifera* *Asparagus racemosus* *Mucuna pruriens* *Phyllanthus emblica* *Terminalia chebula* *Glycyrrhiza glabra* *Myristica fragrans*	3



Chapter-4

Methodology

MATERIALS AND METHODS

Materials

Leaves of the plant *Rhododendron arboreum* were collected from the surrounding fields of Meghalaya. The identification of plant was made by Mrs. J.M.Q.Lyngdoh, Lecturer, Department of Botony, K.N.G.College, Jowai.The leaves were collected in the month of October.

1) Preparation of Extracts

The leaves of *Rhododendron arboreum* were washed thoroughly in tap water, shade dried and powdered. this powder was packed into soxhlet column and extracted with petroleum ether (60-80ºC) for 24 h. The same marc was successively extracted with chloroform (50-60ºC) and afterwards with ethanol for 24 h. The extracts were concentrated on water bath (bath temperature 50ºC). The dried extracts were stored in airtight container in refrigerator below 10ºC.

The yield of the ethanolic extract was found to be 12.5% (w/w). For administration, the crude extract was dissolved in distilled water, to required concentrations.

2) Animals

Wistar albino rats (150-200 g) of either sex and albino mice (20-25g) procured from Bioneeds animal house, Dhavas pet, Tumkur. were used for the study. The animals were kept in polypropylene cages and maintained at a temperature of 26 ± 2ºC. They were fed with standard diet supplied by Pranav agro industries Ltd. Sangli. The study has got the approval (No. 997/c/06/CPCSEA) from the Institutional Animal Ethical Committee (IAEC) of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). After procuring the animals were acclimatized for 10 days under standard husbandry conditions as follows:

Room temperature – 26 ± 2ºC

Relative humidity – 45 - 55%

12 h light and dark cycle[37]

3) Chemicals and equipments used by the study

Table – 3 List of chemicals and equipments used during experiments

1	Activated charcoal (Universal Laboratories, India)
2	Anaesthetic ether (KFC, Bangalore)
3	Analytical balance (Oriental Balance, India)
4	Ascorbic acid (LOBA Chemical, India)
5	Auto Analyser (Metrolab Pvt. Ltd., Chennai)
6	Carbon tetrachloride (S.D. Fine Chemicals, Mumbai)
7	Chemicals Kit for cholesterol estimation (TECHO Diagnostic, USA)
8	Chemicals Kit for ALP estimation (TECHO Diagnostic, USA)
9	Chemicals Kit for Bilirubin (BIT & BID) estimation (TECHO Diagnostic, USA)
10	Chemicals Kit for SGOT estimation (TECHO Diagnostic, USA)
11	Chemicals Kit for SGPT estimation (TECHO Diagnostic, USA)
12	Chemicals Kit for Total protein estimation (TECHO Diagnostic, USA)
13	Chemicals Kit for Triglycerides estimation (TECHO Diagnostic, USA)
14	Ethanol (Nice, Cochin)
15	Formalin (Nice, Cochin)
16	Heating Metal (Sigma Instrument , Chennai)
17	Petroleum ether (S.D. Fine Chemicals, Mumbai)
18	pH Meter (Elico , India)

19	Silymarin (Microlab ,Bangalore)
20	Soxhlet apparatus (TECHO Scientific , Mumbai)
21	Sulphuric acid (Universal Laboratories, India)
22	Trichloroacetic acid (SRL, Mumbai)

All the chemicals used for the study were of analytical grade.

Methods:

1) Preliminary Phytochemical Screening

The preliminary phytochemical Screening was carried out on the petroleum ether, chloroform, and ethanolic extracts of leaves of *Rhododendron arboreum* for qualitative identification. Tests for common phytochemicals were carried out by standard methods described in practical pharmacognosy[43].

2) Determination of Acute Toxicity (LD_{50})

The albino mice of 20-25g body weight of either sex were selected to find out the acute toxicity study of ethanolic extract of *Rhododendron arboreum* leaves. The dose of 5, 50, 300 and 2000 mg/kg were selected based on the Fixed dose (OCED Guideline No. 420) method of CPCSEA[44]. The extract was administered by intraperitonially. The animals were continuously observed for 24 h to detect changes in autonomic or behavioural responses. Mortality in each group was observed for 7 days.

3) Assessment of Hepatoprotective activity

Albino rats weighing between 150-200 g were selected and divided into 6 groups of each containing six animals.

Group I - Served as negative control group (Received distilled water, 5 ml/kg, orally)

Group II - Served as positive control group (Received CCl_4 (Olive Oil 1:1), 1ml/kg, s.c.)

Group III - Served as extract treated group (Received ethanolic extract of leaves of *Rhododendron arboreum*, 40 mg/kg, orally.)

Group IV - Served as extract treated group (Received ethanolic extract of leaves of *Rhododendron arboreum*, 60 mg/kg, orally.)

Group V - Served as extract treated group (Received ethanolic extract of leaves of *Rhododendron arboreum*, 100 mg/kg, orally.)

Group VI - Served as standard group (Received Silymarin, 100mg/kg, orally)

Procedure of Hepatoprotective activity

The animals were fasted for 24 h prior to Carbon tetrachloride treatment. Group I was maintained as negative control received distilled water 5 ml/kg orally. All other groups received Carbon tetrachloride diluted with Olive oil (1:1) at dose of 1 ml/kg, subcutaneously for two successive days (2^{nd} and 3^{rd} day). Group II animals were maintained as Carbon tetrachloride control without any drug treatment (positive control). Group III animals were treated with 40 mg/kg ethanolic extract, orally. Group IV animals were treated with 60 mg/kg ethanolic extract, orally. Group V animals were treated with 100 mg/kg ethanolic extract, orally. Group VI animals were treated with Silymarin (100 mg/kg, orally) which served as standard group. The vehicle or drug treatment was carried out orally from 1^{st} day to 5^{th} day with concurrent administration of Carbon tetrachloride on 2^{nd} and 3^{rd} day. During the period of drug treatment the rats were maintained under normal diet and water *ad libitum*. On 6^{th} day the blood was collected from carotid artery under ether anaesthesia. Serum was prepared by allowing the blood samples to coagulate on ice for 1 min followed by centrifugation (3000 rpm for 15 min) and subjected for determination of biochemical parameters. Livers were removed and preserved in 10% formalin solution for histopathological studies[45].

I The Biochemical parameters includes:

- Serum Glutamate Pyruvate Transaminase (SGPT)
- Serum Glutamate Oxaloactetate Transaminase (SGOT)

- Serum direct Bilirubin
- Serum total Bilirubin
- Serum Alkaline Phosphatase (ALP)
- Serum cholesterol
- Serum triglycerides

Estimation of SGPT

(UV-KINETIC METHOD)

Principle:

SGPT catalyses the transfer of amino group from L-alanine to 2-Ketoglutarate with the formation of pyruvate and L-glutamate. The pyruvate so formed is allowed to react with NADH to produce lactate. The rate of this reaction is monitored by an indicator reaction coupled with LDH in the presence of NADH (nicotinamide adenine dinucleotide). The oxidation of NADH in this reaction is measured as a decrease in the absorbance of NADH at 340 nm, which is proportional to SGPT activity[46].

L-Alanine + 2-Ketoglutarate $\xrightarrow{ALT}$ Pyruvate + L-Glutamate

Pyruvate + NADH + H^+ $\xrightarrow{LDH}$ Lactate + NAD^+ + H_2O

ALT: Alanine aminotransferase

LDH: Lactate dehydrogenase

Estimation of SGOT

(UV-KINETIC METHOD)

Principle:

SGOT catalyses the transfer of amino group from L-aspartate to 2-oxoglutarate forming oxaloacetate and L-glutamate. The rate of this reaction is monitored by an indicator reaction coupled with malate dehydrogenase (MDH) in which the oxaloacetate formed is converted to malate in the presence of reduced nicotinamide adenine dinucleotide (NADH). The oxidation of NADH in this reaction is measured as a decrease in absorbance of NADH at 340 nm, which is proportional to SGOT activity[46].

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

Oxaloacetate + NADH + H^+ $\xrightarrow{MDH}$ L-Malate + NAD^+ + H_2O

AST : Aspartate aminotransferase

MDH : Malate dehydrogenase

LDH : Lactate dehydrogenase

Estimation of Serum Bilirubin

(Diazo method, End point)

Principle:

Bilirubin reacts with diazotised sulphanilicacid in acidic medium to form pink coloured azobilirubin with absorbance directly proportional to Bilirubin concentration. direct Bilirubin, being water soluble directly reacts in acidic medium. However, indirect or unconjugated Bilirubin is solubilised using a surfactant and then it react similar to direct Bilirubin[47].

Estimation of Serum ALP

(KINETIC METHOD)

Principle:

Serum Alkaline phosphatase hydrolyzes p-nitrophenyl phosphatase into p-nitrophenol and phosphate in the presence of oxidizing agent Mg^{2+}. This reaction is measured as absorbance is proportional to the ALP activity[48].

p-Nitro phenyl phosphate + H_2O $\xrightarrow{ALP}$ p-Nitrophenol + Phosphate

Estimation of Serum Cholesterol

(Enzymatic, Cholesterol Oxidase – peroxidase method)

Principle:

The estimation of cholesterol involves the following enzymatic reactions.

a) Cholesterol esters $\xrightarrow{\text{Cholesterol esterase}}$ Cholesterol + Fatty acids

b) Cholesterol + O_2 $\xrightarrow{\text{Cholesterol oxidase}}$ Cholesten-3-one+H_2O_2

c) H_2O_2 + 4-amino antipyrine + phenol $\xrightarrow{POD}$ Quinoneimine + H_2O

POD: Peroxidase

The absorbance of Quinoneimine measured spectrometrically at 505 nm was proportional to cholesterol concentration in the specimen[49].

Estimation of Serum Triglycerides

Principle:

The enzymatic reaction sequence employed in the assay of tryglycerides is as follows:

Triglycerides + H_2O $\xrightarrow{LPL}$ Glycerol + Free fatty acids

Glycerol + ATP $\xrightarrow{GK}$ Glycerol – 3 – phosphate + ADP

Glycerol – 3 – phosphate + O_2 $\xrightarrow{GPO}$ DHAP + H_2O_2

$$2H_2O_2 + 4AAP \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 4H_2O$$

GK - Glycerol kinase

GPO - Glycerol – 3 – phosphate oxidase

DHAP - Dihydroxyacetone phosphate

ATP - Adenosine triphosphate

AAP - Aminoantipyrine

LPL - Lipoprotein lipase

The present procedure involves hydrolysis of triglycerides by lipase. The glycerol concentration is then determined by enzymatic assay coupled with Trinder reaction that terminates in the formation of quinoneimine dye. The amount of the dye formed, determined by its absorption at 520 nm, is directly proportional to the concentration of triglycerides in the samples[50, 51].

Histopathological studies:

The rats were sacrificed and the liver of each rat was isolated. The liver was washed in normal saline and blotted with filter paper. The isolated liver was cut in to small pieces and preserved in 10% formalin. It was subjected to histopathological examination.

Estimation of Ascorbic acid in rat urine

Calibration curve for Ascorbic acid

1 mg/ml standard solution of ascorbic acid was prepared by dissolving 10 mg of ascorbic acid in 10 ml of diluted water. By using this different concentration of ascorbic acid 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 and 150µg/ml were prepared with 4% TCA. 10 ml of each of these were taken and mix well with 0.375 gm of activated Charcoal and filtered. 4% TCA was used for the blank. From the filtrate 1ml was taken into the test tube and a drop of thiourea was added to that.Then to those test

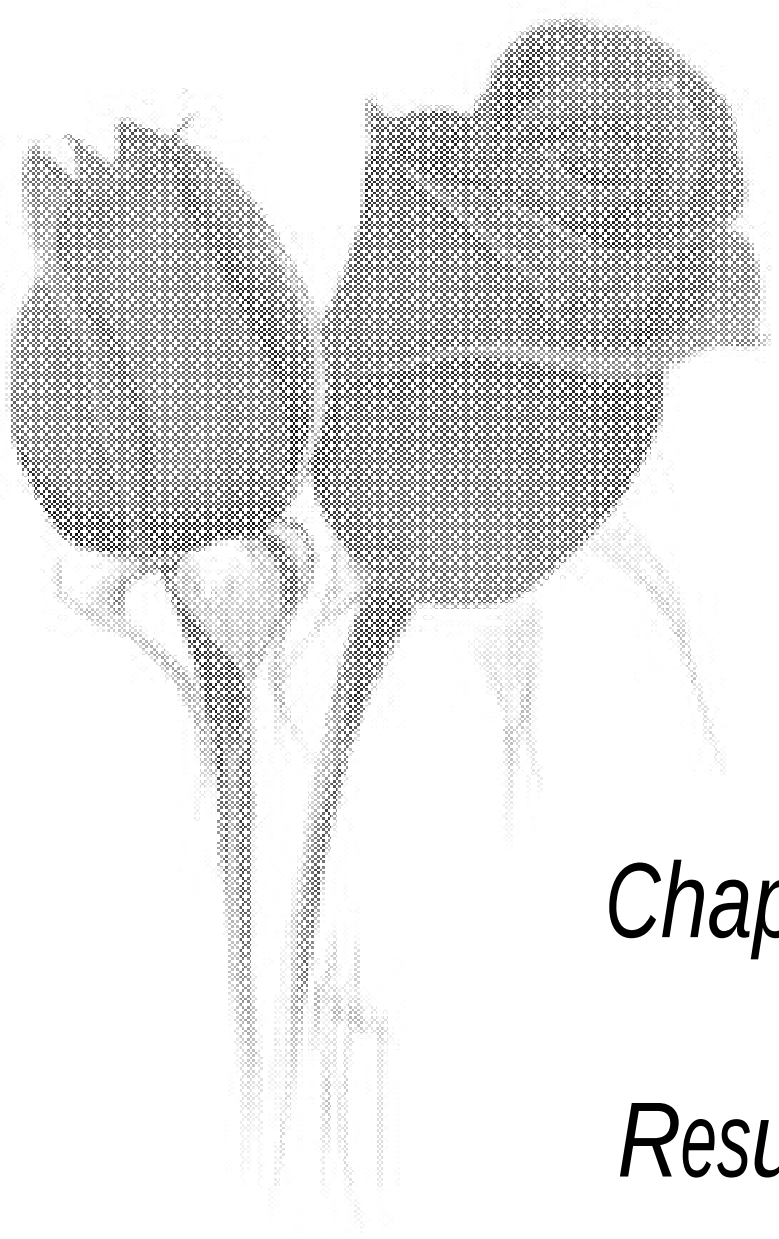
tubes 1 ml of 2, 4-DNP was added and kept in incubator for about 3 h, maintained at 37 ºC. The tubes were then placed in the beaker containing ice and 5 ml of 95 % sulphuric acid was added drop by drop within one minute interval with intermittent mixing. Finally they were shaken and kept aside for 30 min. Then optical density was measured at 550 nm using spectrophotometer. Standard curve was plotted by taking concentration of ascorbic acid on X-axis and optical density on Y- axis.

Procedure for estimation of Ascorbic acid in urine:

Wistar albino rats of either sex (150-250 g) were divided into six groups each consisting of six animals. They were kept in a metabolic cages for collection of urine. They were supplied with standard diet and water ***ad libitum***, one week before and during the experimental period. 24 h urine sample were collected separately for each group in 5 ml of oxalic acid solution and analyzed for ascorbic acid by the method of **Roe** and **Kuether** and their average value were taken as controls (negative control). Then the rats of groups II, III, IV, V and VI were treated with 1 ml/kg of Carbon tetrachloride for two days (2^{nd} and 3^{rd} days). Group III, IV, and V were treated orally with ethanolic extract of *Rhododendron arboreum* 40, 60, 100 mg/kg respectively and group VI were orally treated with Silymarin 100 mg/kg. Then optical density was measured at 550 nm using spectro- photometer[52, 53].

3) Statistical analysis

Results are presented as Mean ± SEM and percentage degree of reversal against hepatotoxin by test. The percentage was calculated by considering enzyme level difference between CCl_4 and normal rats as 100% degree of reversal. Difference among means has been analysed by applying Dunnet's *'t'* test.



Chapter-5

Results

RESULTS

(I) Preliminary Phytochemical Screening:

Preliminary phytochemical investigation of different extracts of leaves of *Rhododendron arboreum* were studied. The petroleum ether extract contains phytosterols, saponins and fixed oils. The chloroform extract contains proteins. The ethanolic extract contains carbohydrates, saponins, flavonoids, phytosterols, tannins and phenolic compounds.

(II) Acute Toxicity studies:

Acute toxicity study of ethanolic extract of leaves of *Rhododendron arboreum* was carried out for determination of LD_{50} by adopting fixed dose method of CPCSEA (OECD Guideline 420). In the acute toxicity study ethanolic extract of leaves of *Rhododendron arboreum* were found to be toxic (2/3 mice died) at a dose of 300 mg/kg, intraperitoneally. Hence, LD_{50} cut off value of ethanolic extract was fixed as 300 mg/kg body weight. So, that $1/7^{th}$, $1/5^{th}$ and $1/3^{rd}$ of the LD_{50} cut off value i.e. 40, 60 and 100 mg/kg body weight were selected as screening dose for hepatoprotective activity.

(III) Hepatoprotective activity

a) Biochemical parameters

The results obtained in the experiments are reported in Table-10.

The leakage of SGPT, SGOT, total and direct bilirubin, ALP, triglycerides and cholesterol activities in the blood reflects indirectly the failure of liver function due to CCl_4-induced hepatotoxicity. In Table 10, SGPT (216.27 ± 2.44), SGOT (214.07 ± 2.69), total bilirubin (1.4334 ± 0.0048), direct bilirubin (0.3357 ± 0.002), ALP (319.03 ± 2.89), triglycerides (280.82 ± 1.30) and cholesterol (284.74 ± 2.96) activities were significantly

increased after the administration of CCl_4 as compared with the negative control group ($P < 0.01$). Pretreatment with 60 and 100 mg/kg of ethanolic extracts of leaves of *Rhododendron arboreum* significantly reduced the elevation of SGPT, SGOT, (total and direct) bilirubin, ALP, triglycerides and cholesterol ($P < 0.01$).

The ethanolic extract of leaves of *Rhododendron arboreum* at the dose of 40 mg/kg produced very less reduction in elevated SGPT (5.21%), SGOT (4.67%), ALP (2.71%), total bilirubin (4.17%), direct bilirubin (1.75%), triglycerides (3.84%), cholesterol (12.74%).

Figure-2 shows a magnification of the changes of liver histopathology from the negative control. Under the electronic microscope, normal cellular architecture with distinct hepatic cells, sinusoidal spaces and a central vein were observed in the negative control group (Figure-2). However, CCl_4-intoxicated treatment exhibited severe histopathological changes, such ascentrilobular hepatic necrosis, fatty change, kupffer cell, ballooning degeneration, and infiltrating lymphocytes (Figure-3). Pretreatment with 60 and 100 mg/kg of ethanolic extract of *Rhododendron arboreum* prevented these histopathological changes associated with the hepatotoxicity from CCl_4-intoxicated treatment (Figures-6 and 7). But the dose of 40 mg/kg treatment did not reverse these histopathological changes, when compared to positive control group (Figures-4 and 5).

b) Estimation of Ascorbic acid in urine

The daily excretion of ascorbic acid by different groups of rats before and after treatment is summarized in Table-13. A dose of 1 ml/kg Carbon tetrachloride produced significant reduction in ascorbic acid excretion. Ethanolic extract of *Rhododendron arboreum* at 60 and 100 mg/kg prevented Carbon tetrachloride induced reduction in

ascorbic acid. The standard reference drug Silymarin prevented Carbon tetrachloride induced reduction in ascorbic acid significantly ($P < 0.01$) as summarized in Table-13.

Table - 5

Effects of ethanolic extract of leaves of *Rhododendron arboreum* on certain serum biochemical parameters in CCl_4 induced hepatotoxicity in rats :

	GROUP-I							GROUP-II						
Animal No.	**SGPT (IU/L)**	**SGOT (IU/L)**	**Bilirubin (mg/dl)**		**ALP (IU/L)**	**TGL (mg/dl)**	**Cholesterol (mg/dl)**	**SGPT (IU/L)**	**SGOT (IU/L)**	**Bilirubin (mg/dl)**		**ALP (IU/L)**	**TGL (mg/dl)**	**Cholesterol (mg/dl)**
			Total	**Direct**						**Total**	**Direct**			
1	46.39	38.16	0.1120	0.0216	108.59	178.44	198.13	206.11	209.81	1.4140	0.3385	309.55	278.58	278.30
2	48.28	39.18	0.1178	0.0260	109.86	176.25	198.98	224.51	220.78	1.4367	0.3290	325.54	283.11	290.79
3	42.43	38.23	0.1169	0.0281	108.63	179.96	196.23	215.07	207.75	1.4299	0.3326	319.03	278.20	279.28
4	40.38	41.56	0.1190	0.0291	109.85	177.21	194.00	216.12	206.80	1.4295	0.3318	311.99	277.77	278.44
5	46.38	39.29	0.1188	0.0295	106.85	174.86	198.20	218.89	219.81	1.4432	0.3428	327.12	285.72	295.40
6	46.12	41.23	0.1191	0.0298	104.29	175.67	192.12	216.92	219.45	1.4469	0.3397	320.97	281.56	286.24
Mean	45.16	39.61	0.1173	0.0274	108.01	177.07	196.28	216.27	214.07	1.4334	0.3357	319.03	280.82	284.74
± SEM	1.21	0.60	0.0011	0.0013	0.87	0.76	1.11	2.45	2.69	0.0048	0.0022	2.89	1.30	2.96
P value								**	**	**	**	**	**	**

Significant increase in control treated group as compared to normal treated group at P<0.05*, P<0.01** and P<0.001***.

Table - 6

Effects of ethanolic extract of leaves of *Rhododendron arboreum* on certain serum biochemical parameters in CCl_4 induced hepatotoxicity in rats :

	GROUP-II							GROUP-III						
Animal No.	**SGPT (IU/L)**	**SGOT (IU/L)**	**Bilirubin (mg/dl)**		**ALP (IU/L)**	**TGL (mg/dl)**	**Cholesterol (mg/dl)**	**SGPT (IU/L)**	**SGOT (IU/L)**	**Bilirubin (mg/dl)**		**ALP (IU/L)**	**TGL (mg/dl)**	**Cholesterol (mg/dl)**
			Total	**Direct**						**Total**	**Direct**			
1	206.11	209.81	1.4140	0.3385	309.55	278.58	278.30	207.06	204.86	1.3851	0.3340	312.40	278.99	269.95
2	224.51	220.78	1.4367	0.3290	325.54	283.11	290.79	207.54	206.75	1.4830	0.3272	313.66	273.50	276.32
3	215.07	207.75	1.4299	0.3326	319.03	278.20	279.28	204.05	204.84	1.3679	0.3270	311.38	273.79	270.81
4	216.12	206.80	1.4295	0.3318	311.99	277.77	278.44	207.25	204.30	1.2555	0.3282	312.59	277.70	268.02
5	218.89	219.81	1.4432	0.3428	327.12	285.72	295.40	210.25	208.96	1.3861	0.3345	313.50	278.61	277.93
6	216.92	219.45	1.4469	0.3397	320.97	281.56	286.24	208.00	205.80	1.3929	0.3311	316.34	278.45	277.77
Maen	216.27	214.07	1.4334	0.3357	319.03	280.82	284.74	207.359	205.917	1.3784	0.3303	313.31	276.84	273.47
± SEM	2.45	2.69	0.0048	0.0022	2.89	1.30	2.96	0.81	0.70	0.0297	0.0014	0.69	1.03	1.77
P value								ns	ns	ns	ns	ns	ns	ns

Non significant reduction in Extract treated group compared to Carbon tetrachloride treated group at P<0.05*, P<0.01** and P<0.001***.

Table – 7

Effects of ethanolic extract of leaves of *Rhododendron arboreum* on certain serum biochemical parameters in CCl_4 induced hepatotoxicity in rats :

	GROUP-II							GROUP-IV						
Animal No.	**SGPT (IU/L)**	**SGOT (IU/L)**	**Bilirubin (mg/dl)**		**ALP (IU/L)**	**TGL (mg/dl)**	**Cholesterol (mg/dl)**	**SGPT (IU/L)**	**SGOT (IU/L)**	**Bilirubin (mg/dl)**		**ALP (IU/L)**	**TGL (mg/dl)**	**Cholesterol (mg/dl)**
			Total	**Direct**						**Total**	**Direct**			
1	206.11	209.81	1.4140	0.3385	309.55	278.58	278.30	94.99	68.04	0.5019	0.1524	277.91	243.29	213.40
2	224.51	220.78	1.4367	0.3290	325.54	283.11	290.79	100.48	72.53	0.6062	0.1468	275.42	247.38	219.14
3	215.07	207.75	1.4299	0.3326	319.03	278.20	279.28	94.97	66.02	0.4933	0.1427	276.41	241.27	213.38
4	216.12	206.80	1.4295	0.3318	311.99	277.77	278.44	93.18	72.23	0.4009	0.1430	276.62	242.48	210.59
5	218.89	219.81	1.4432	0.3428	327.12	285.72	295.40	105.09	76.14	0.5145	0.1528	278.53	246.39	215.50
6	216.92	219.45	1.4469	0.3397	320.97	281.56	286.24	100.93	75.98	0.5083	0.1507	277.37	244.83	214.34
Maen	216.27	214.07	1.4334	0.3357	319.03	280.82	284.74	98.27	71.82	0.5042	0.1481	277.04	244.27	214.39
± SEM	2.45	2.69	0.0048	0.0022	2.89	1.30	2.96	1.88	1.68	0.0267	0.0019	0.46	0.96	1.16
P value								**	**	**	**	**	**	**

Significant reduction in Extract treated group compared to Carbon tetrachloride treated group at P<0.05*, P<0.01** and P<0.001***.

Table - 8

Effects of ethanolic extract of leaves of *Rhododendron arboreum* on certain serum biochemical parameters in CCl_4 induced hepatotoxicity in rats :

	GROUP-II							GROUP-V						
Animal No.	**SGPT (IU/L)**	**SGOT (IU/L)**	**Bilirubin (mg/dl)**		**ALP (IU/L)**	**TGL (mg/dl)**	**Cholesterol (mg/dl)**	**SGPT (IU/L)**	**SGOT (IU/L)**	**Bilirubin (mg/dl)**		**ALP (IU/L)**	**TGL (mg/dl)**	**Cholesterol (mg/dl)**
			Total	**Direct**						**Total**	**Direct**			
1	206.11	209.81	1.4140	0.3385	309.55	278.58	278.30	45.25	40.67	0.1159	0.0318	118.87	216.37	201.68
2	224.51	220.78	1.4367	0.3290	325.54	283.11	290.79	49.03	42.46	0.1208	0.0252	120.36	216.86	204.35
3	215.07	207.75	1.4299	0.3326	319.03	278.20	279.28	45.23	47.91	0.1080	0.0263	117.85	215.35	202.66
4	216.12	206.80	1.4295	0.3318	311.99	277.77	278.44	44.44	41.86	0.0133	0.0261	119.06	216.56	203.87
5	218.89	219.81	1.4432	0.3428	327.12	285.72	295.40	48.35	42.77	0.1269	0.0297	120.38	218.47	204.78
6	216.92	219.45	1.4469	0.3397	320.97	281.56	286.24	47.19	42.61	0.1198	0.0353	119.57	217.31	204.62
Maen	216.27	214.07	1.4334	0.3357	319.03	280.82	284.74	46.58	43.05	0.1008	0.0290	119.35	216.82	203.66
± SEM	2.45	2.69	0.0048	0.0022	2.89	1.30	2.96	0.77	1.02	0.0177	0.0016	0.40	0.42	0.50
P value								**	**	**	**	**	**	**

Significant reduction in Extract treated group compared to Carbon tetrachloride treated group at P<0.05*, P<0.01** and P<0.001***.

Table - 9

Effects of ethanolic extract of leaves of *Rhododendron arboreum* on certain serum biochemical parameters in CCl_4 induced hepatotoxicity in rats :

	GROUP-II							GROUP-VI						
Animal No.	**SGPT (IU/L)**	**SGOT (IU/L)**	**Bilirubin (mg/dl)**		**ALP (IU/L)**	**TGL (mg/dl)**	**Cholesterol (mg/dl)**	**SGPT (IU/L)**	**SGOT (IU/L)**	**Bilirubin (mg/dl)**		**ALP (IU/L)**	**TGL (mg/dl)**	**Cholesterol (mg/dl)**
			Total	**Direct**						**Total**	**Direct**			
1	206.11	209.81	1.4140	0.3385	309.55	278.58	278.30	45.25	40.67	0.1159	0.0318	118.87	216.37	201.68
2	224.51	220.78	1.4367	0.3290	325.54	283.11	290.79	49.03	42.46	0.1208	0.0252	120.36	216.86	204.35
3	215.07	207.75	1.4299	0.3326	319.03	278.20	279.28	45.23	47.91	0.1080	0.0263	117.85	215.35	202.66
4	216.12	206.80	1.4295	0.3318	311.99	277.77	278.44	44.44	41.86	0.0133	0.0261	119.06	216.56	203.87
5	218.89	219.81	1.4432	0.3428	327.12	285.72	295.40	48.35	42.77	0.1269	0.0297	120.38	218.47	204.78
6	216.92	219.45	1.4469	0.3397	320.97	281.56	286.24	47.19	42.61	0.1198	0.0353	119.57	217.31	204.62
Maen	216.27	214.07	1.4334	0.3357	319.03	280.82	284.74	46.58	43.05	0.1008	0.0290	119.35	216.82	203.66
±SEM	2.45	2.69	0.0048	0.0022	2.89	1.30	2.96	0.77	1.02	0.0177	0.0016	0.40	0.42	0.50
P value								**	**	**	**	**	**	**

Significant reduction in Extract treated group compared to Carbon tetrachloride treated group at P<0.05*, P<0.01** and P<0.001***.

Table – 10

Effects of ethanolic extract of leaves of *Rhododendron arboreum* on certain serum biochemical parameters in CCl_4 induced hepatotoxicity in rats :

Group	Treatment	Dose	Biochemical Parameters						
			SGPT (IU/L)	SGOT (IU/L)	Bilirubin (mg/dl)		ALP (IU/L)	TGL (mg/dl)	Cholesterol (mg/dl)
					Total	Direct			
I	Normal control group	-	45.16 ±1.25	39.61 ± 0.60	0.1173 ± 0.0011	0.0274 ± 0.0013	108.01 ± 0.87	177.07 ± 0.77	196.28 ± 1.11
II	CCl_4 treated control	1.0 ml/kg	216.27 ± 2.45**	214.07 ± 2.69**	1.4334 ± 0.0048**	0.3357 ± 0.0022**	319.03 ± 2.89**	280.82 ± 1.30**	284.74 ± 2.96**
III	Ethanolic extract + CCl_4	40	207.36 ± 0.81^{ns} (5.21%)	205.92 ± 0.704^{ns} (4.67%)	1.3784 ± 0.0297^{ns} (4.17%)	0.3303 ± 0.0014^{ns} (1.75%)	313.31 ± 0.69^{ns} (2.71%)	276.84 ± 1.03^{ns} (3.84%)	273.47 ± 1.79^{ns} (12.74%)
IV	Ethanolic extract + CCl_4	60	98.27 ± 1.88** (68.96%)	71.82 ± 1.68** (81.54%)	0.5042 ± 0.0267** (70.60%)	0.1481 ± 0.0019** (60.85%)	277.04 ± 0.46** (19.90%)	244.27 ± 0.96** (35.23%)	214.39 ± 1.16** (79.52%)
V	Ethanolic extract + CCl_4	100	46.58 ± 0.77** (99.17%)	43.05 ± 1.02** (98.03%)	0.1008 ± 0.0177** (101.25%)	0.0290 ± 0.0016** (99.45%)	119.35 ± 0.40** (94.63%)	216.82 ± 0.42** (61.69%)	203.66 ± 0.50** (91.66%)
VI	Silymarin + CCl_4	100	46.10 ± 1.08** (99.45%)	41.12 ± 1.03** (99.13%)	0.1330 ± 0.0182** (98.81%)	0.0315 ± 0.0022** (98.67%)	110.45 ± 0.42** (98.84%)	180.83 ± 0.54** (96.37%)	198.79 ± 1.45** (97.16%)

- Values are Mean ± SEM, (n = 6 in each group)
- Control group was compared with normal group (negative control group) and all values were significantly different ($p < 0.01$)
- Experimental groups were compared with control: *P<0.05, **P<0.01 and ***P<0.001.
- ns- non significant

Table – 11

Effects of ethanolic extract of leaves of *Rhododendron arboreum* on Ascorbic acid in CCl_4 induced hepatotoxicity in rats urine:

Animal Number	GROUP I		GROUP II		GROUP III		GROUP IV	
	Optical Density (550 nm)	Concentration of Ascorbic acid (µg/ml)	Optical Density (550 nm)	Concentration of Ascorbic acid (µg/ml)	Optical Density (550 nm)	Concentration of Ascorbic acid (µg/ml)	Optical Density (550 nm)	Concentration of Ascorbic acid (µg/ml)
1	46	126.80	64	84.32	59	96.12	44	131.52
2	40	140.97	57	100.84	50	117.36	45	129.16
3	49	119.72	67	77.24	57	100.84	44	131.52
4	45	129.16	54	107.92	57	100.84	45	129.16
5	41	138.61	63	86.68	67	77.24	51	115.00
6	40	140.97	57	100.84	64	84.32	46	126.80
Mean		132.70		92.98		96.12		127.20
±SEM		3.59		4.86		5.75		2.54
P Value				**		ns		**

Significant reduction in Extract treated group compared to Carbon tetrachloride treated group at P<0.05*, P<0.01** and P<0.001***.

Table – 12

Effects of ethanolic extract of leaves of *Rhododendron arboreum* on Ascorbic acid in CCl_4 induced hepatotoxicity in rats urine:

Animal Number	GROUP I		GROUP II		GROUP V		GROUP VI	
	Optical Density (550 nm)	Concentration of Ascorbic acid (µg/ml)	Optical Density (550 nm)	Concentration of Ascorbic acid (µg/ml)	Optical Density (550 nm)	Concentration of Ascorbic acid (µg/ml)	Optical Density (550 nm)	Concentration of Ascorbic acid (µg/ml)
1	46	126.80	64	84.32	47	124.44	49	119.72
2	40	140.97	57	100.84	49	119.72	43	133.88
3	49	119.72	67	77.24	45	129.16	34	155.13
4	45	129.16	54	107.92	44	131.52	49	119.72
5	41	138.61	63	86.68	40	140.97	49	119.72
6	40	140.97	57	100.84	43	133.88	43	133.88
Mean		132.70		92.98		129.95		130.34
±SEM		3.59		4.86		3.03		5.71
P Value				**		**		**

Significant reduction in Extract treated group compared to Carbon tetrachloride treated group at P<0.05*, P<0.01** and P<0.001***.

Table – 13

Effects of ethanolic extract of leaves of *Rhododendron arboreum* on Ascorbic acid in CCl_4 induced hepatotoxicity in rats urine:

Group	Treatment	Dose(mg/kg)	Mean (µg/ml) ± SEM
I	Normal control	-	132.70 ± 3.59
II	CCl_4 treated control	1.0 ml/kg	92.98 ± 4.86**
III	Ethanolic extract + CCl_4	40	96.12 ± 5.75ns (7.91%)
IV	Ethanolic extract + CCl_4	60	127.20 ± 2.54** (86.15%)
V	Ethanolic extract + CCl_4	100	129.95 ± 3.03** (93.08%)
VI	Silymarin + CCl_4	100	130.34 ± 5.71** (94.06%)

- Values are Mean ± SEM, (n = 6 in each group)

- Control group was compared with normal group (negative control group) and all values were significantly different ($p < 0.01$)

- Experimental groups were compared with control: *P<0.05, **P<0.01 and ***P<0.001.

- ns: non - significant

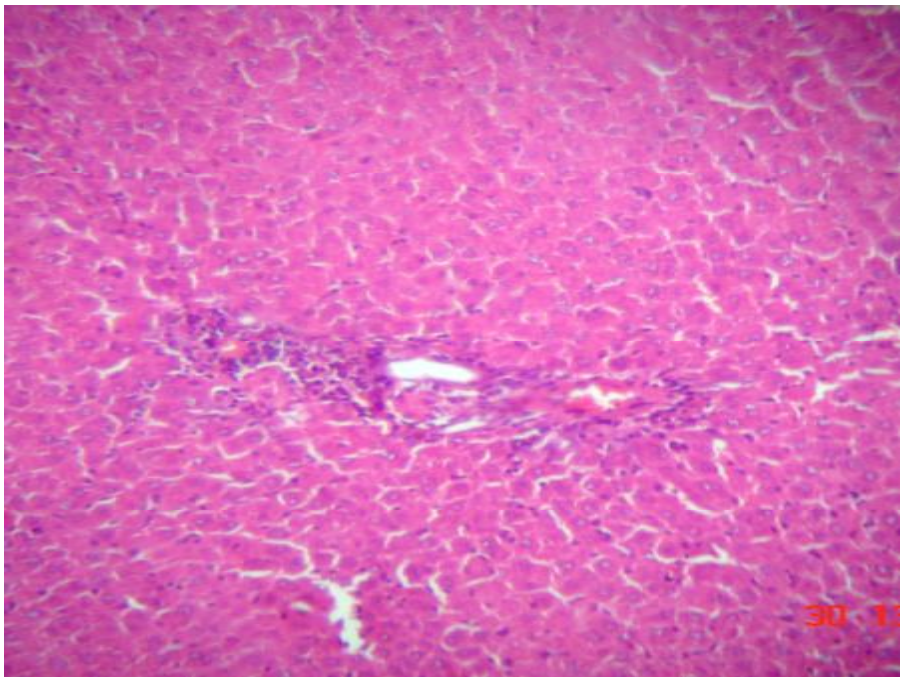


Figure: 2 Negative control group

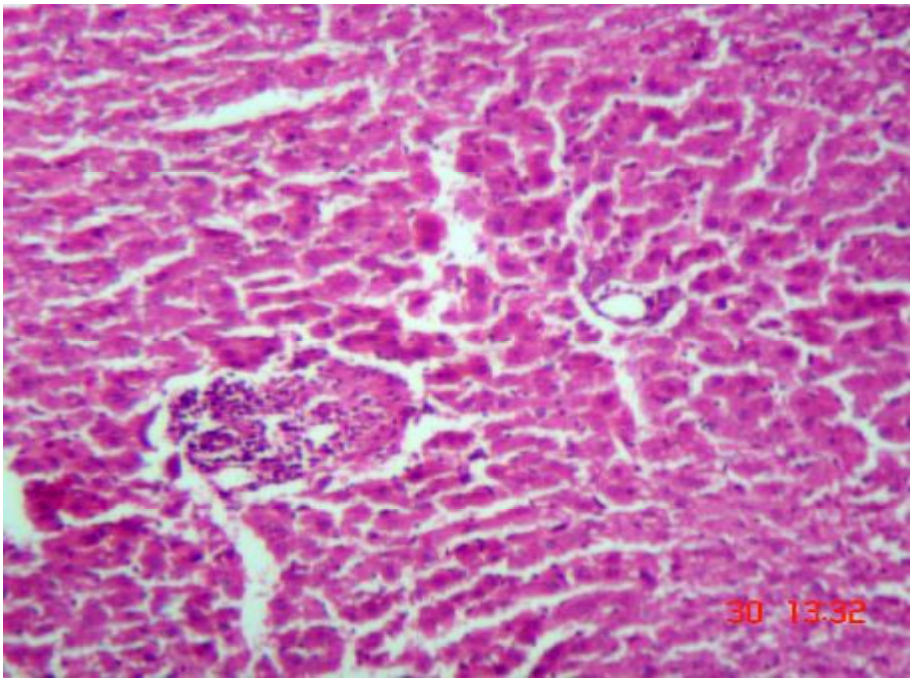


Figure: 3 Positive control group (CCl_4 treated)

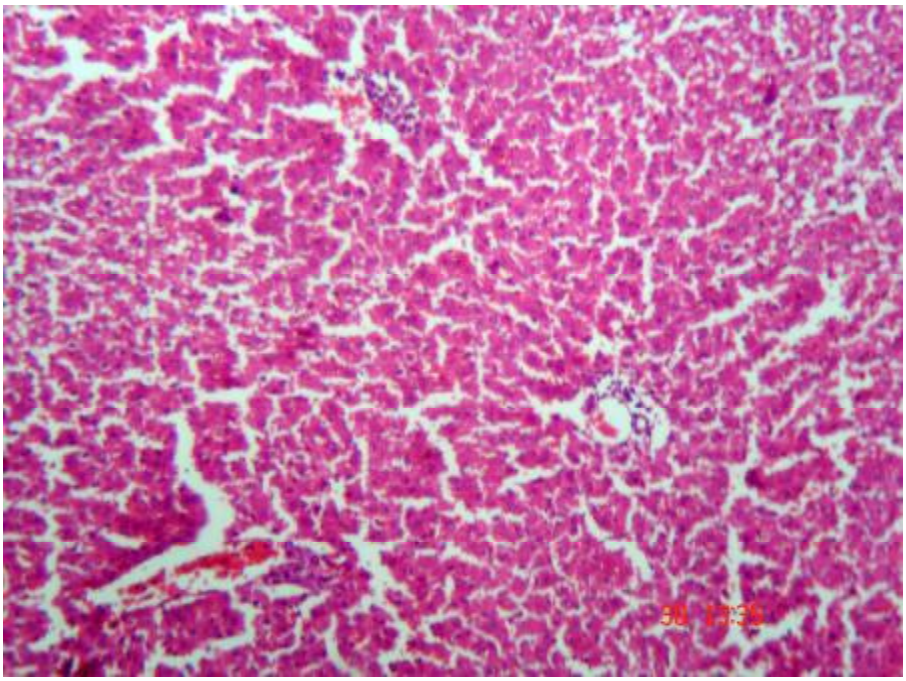


Figure: 4 Extract treated group (40 mg/kg)

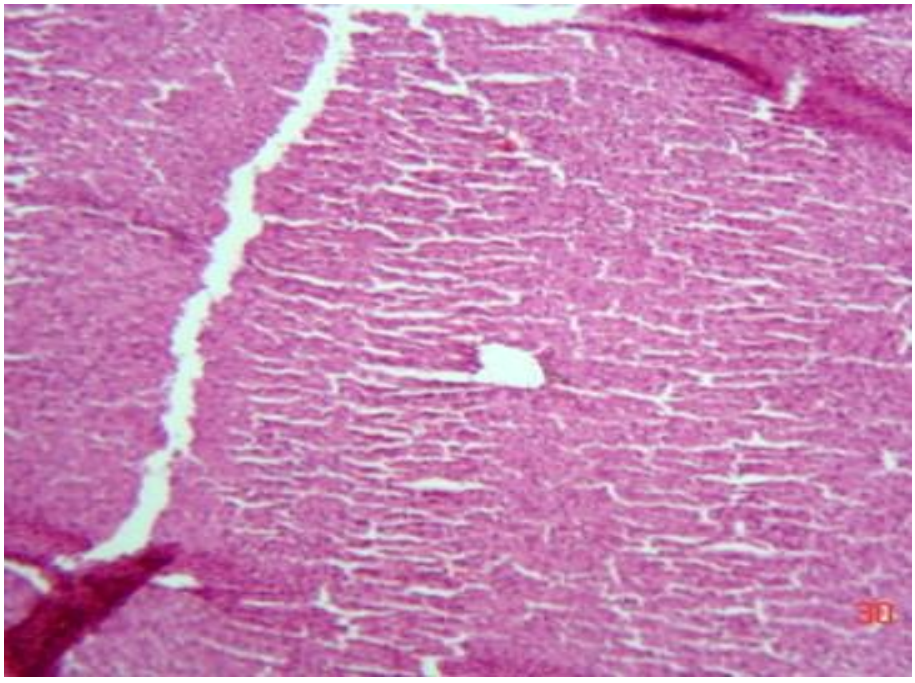


Figure: 5 Extract treated group (60 mg/kg)

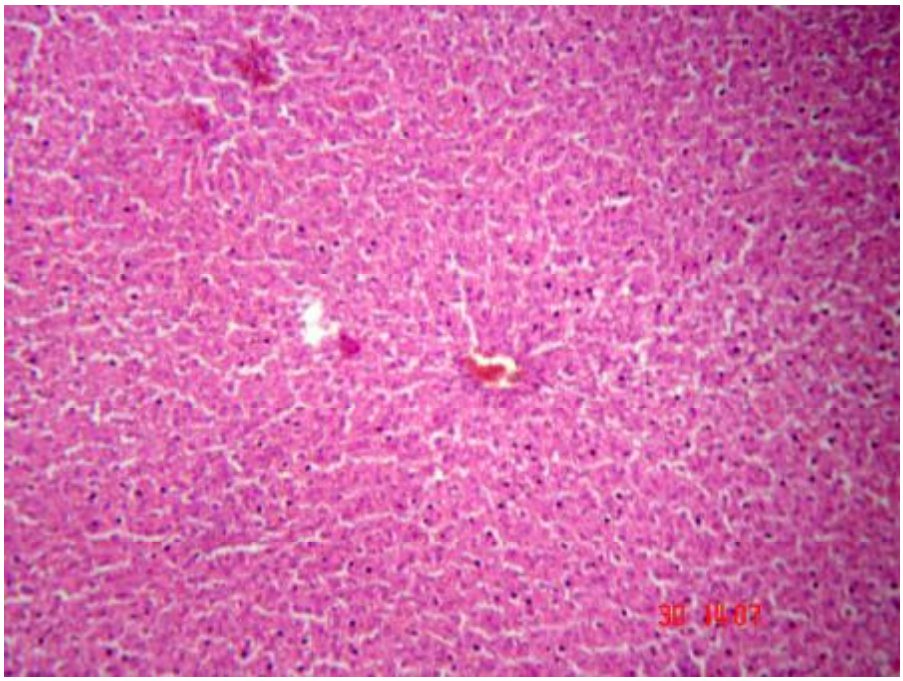


Figure: 6 Extract treated group (100 mg/kg)

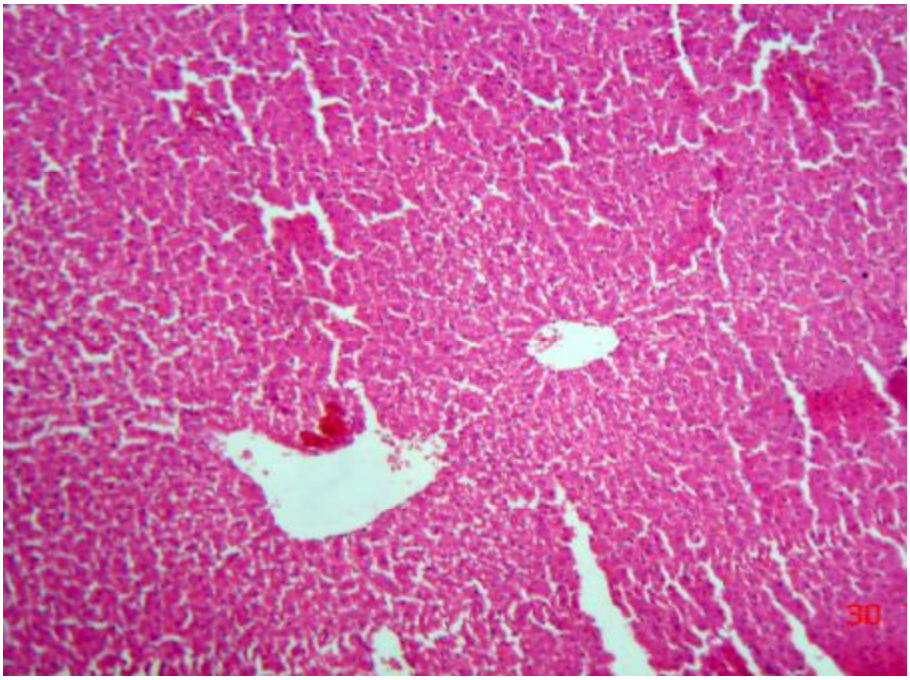
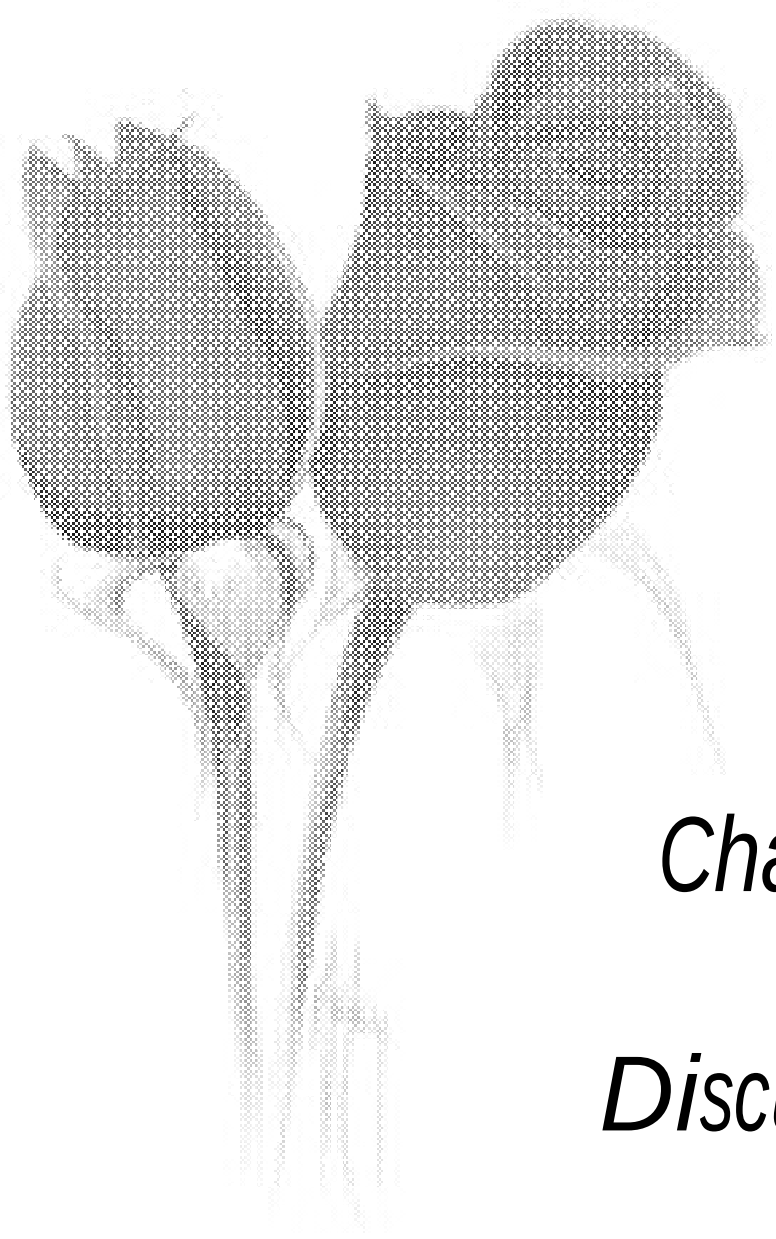


Figure: 7 Silymarin treated group (100 mg/kg)



Chapter-6

Discussion

DISCUSSION

The hepatotoxicity induced by CCl_4 is due to its metabolite $CCl_3^{\bullet}$, a free radical that binds to lipoprotein and leads to peroxidation of lipids of endoplasmic reticulum[54]. The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxin, is the index of its protective effects. Although serum enzyme levels and ascorbic acid in urine are not a direct measure of hepatic injury, they show the status of the liver. The lowering of enzymes level are definite indication of hepatoprotective action of the drug. Protection of hepatic damage caused by Carbon tetrachloride administration was observed by recording SGPT, SGOT, total and direct Bilirubin, ALP, triglycerides and cholesterol levels in treated, toxin control and normal groups because these parameters have been reported to be sensitive indicators of liver injury[55]. The disturbance in the transport function of the hepatocytes as a result of hepatic injury causes the leakage of enzymes from cells due to altered permeability of membrane[56]. This results in decreased levels of SGPT, SGOT, (total and direct) bilirubin, ALP, triglycerides and cholesterol levels in the hepatic cells and a raised level in serum.

The results presented in Tables - 10 explain that both the treatments 60 and 100 mg/kg of ethanolic extract of leaves of *Rhododendron arboreum*, offer hepatoprotection, but the ethanolic extract of leaves of *Rhododendron arboreum* at a dose of 100 mg/kg is more effective. It is also noticed that both the treatments with the different dose i.e. 60 and 100 mg/kg are particularly sensitive to the SGPT levels. A very important observation with this ethanolic extract of leaves of *Rhododendron arboreum* is that the

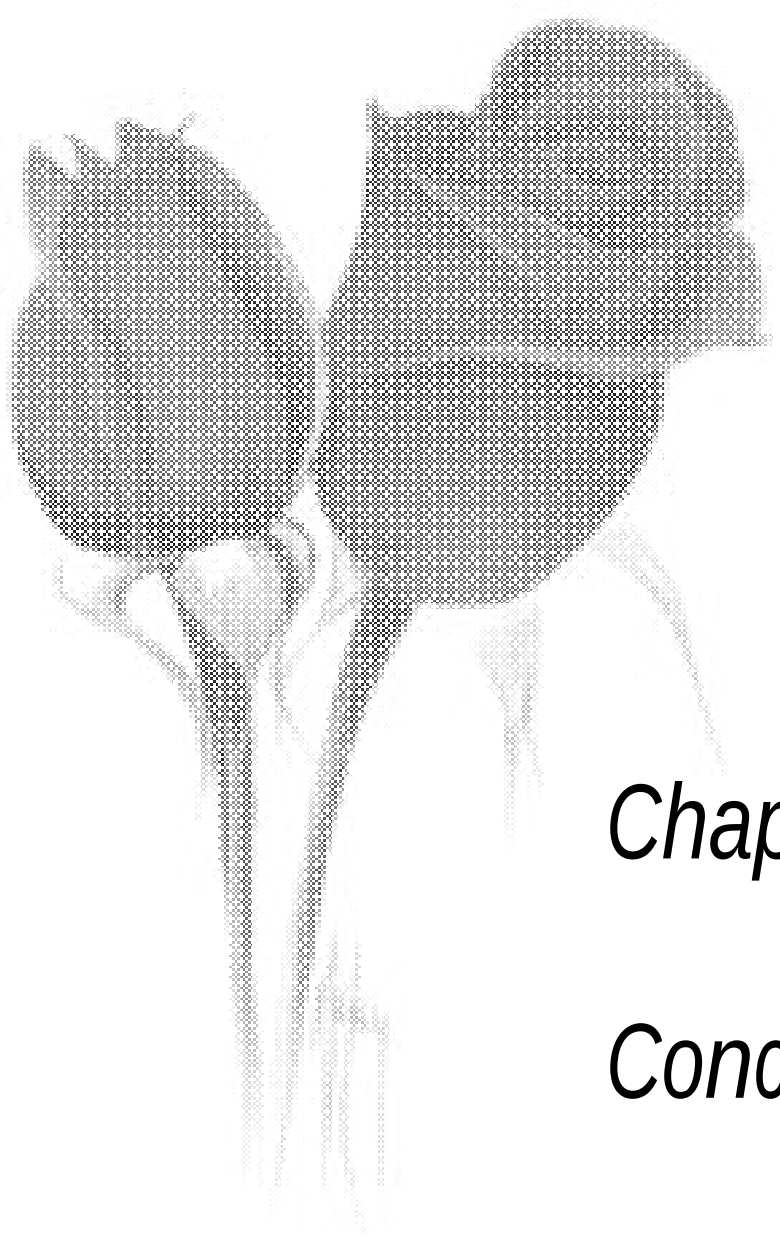
ethanolic extract at dose 100 mg/kg is highly effective in decreasing the elevated level of serum Bilirubin (101.25%). The decrease in serum Bilirubin suggests that the ethanolic extract can be used in jaundice.

The excretion of ascorbic acid by different groups of rats is shown in Table - 13. Ascorbic acid is formed as a metabolite of glucose and galactose in rat liver microsomes via the glucoronic acid pathway and is excreted in urine. Alteration in urinary ascorbic acid excretion appears to be reflecting ascorbic acid level in liver. Hence, the reduction in urinary ascorbic acid excretion can be used as an index for CCl_4 produced hepatotoxicity[53]. The results show that the both treatments with 60 and 100 mg/kg significantly prevented the decrease in ascorbic acid in CCl_4 treated rats as compared to positive control group.

The histopathological studies are direct evidence of efficacy of drug as protectant. Simultaneous treatment of ethanolic extract with CCl_4 exhibits less damage to the hepatic cells as compared to the rats treated with CCl_4 alone. Intralobular veins though are damaged but to a lesser extent. Endothelium is disrupted at places. Hepatic cells adjoining to intralobular vein show atrophy. The sections of the liver treated with ethanolic extract of leaves of ***Rhododendron arboreum*** and CCl_4 reveals better hepatoprotective activity. Almost negligible damage to a few hepatocytes present in the close vicinity of intralobular vein is observed. Endothelium lining is almost smooth except one or two places. Hepatocytes show normal appearance only some cells show higher numbers of vacuoles in the cytoplasm.

The results of histopathological study also support the results of biochemical parameters and explain the hepatoprotective activity of leaves of *Rhododendron arboreum*.

The remarkable results of this experiment are the decrease of the serum Bilirubin level by the ethanolic extract of leaves of ***Rhododendron arboreum*** at a dose of 100 mg/kg up to 101.25% and decrease of SGPT level by the ethanolic extract of leaves of ***Rhododendron arboreum*** at dose of 60 and 100 mg/kg up to 99.17% and 99.45%, respectively. These results strongly support the significant hepatoprotective activity of the drug because SGPT is more specific than SGOT as an indicator of hepatic damage since SGPT is a cytoplasmic enzyme found in very high concentrations in the liver and SGOT is present in the cytoplasm as well as in the mitochondria and is rapidly inactivated[57]. These data along with the histopathological studies clearly show the hepatoprotective activity of the ethanolic extract of leaves of ***Rhododendron arboreum.***



Chapter-7

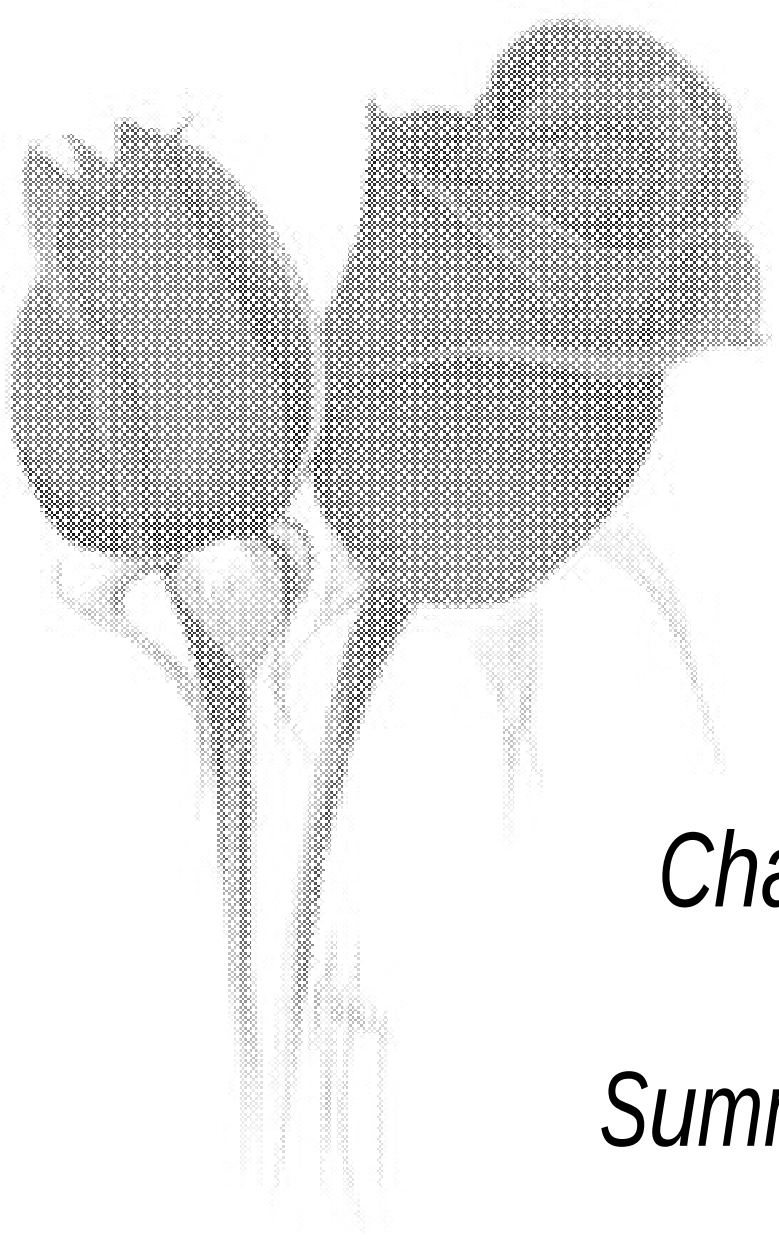
Conclusion

CONCLUSION

The following conclusions are drawn from the results and discussion described in the previous chapters:

- The coarse powder of leaves of *Rhododendron arboreum* was successively extracted with petroleum ether, chloroform, ethanol in soxhlet column.
- The ethanolic extract was subjected for preliminary phytochemical investigation and it contains carbohydrates, saponins, flavonoids, phytosterols, tannins and phenolic compounds.
- Acute toxicity study of ethanolic extract of leaves of *Rhododendron arboreum* was carried out for determination of LD_{50} and the dose 40, 60 and 100 mg/kg were selected for screening hepatoprotective activity.
- Rats treated with Carbon tetrachloride were developed significant hepatic damage as observed from elevated level of serum marker enzymes SGPT, SGOT, ALP, total and direct bilirubin, cholesterol and triglycerides were also significantly enhanced compared to normal control group.
- The ethanolic extract of leaves of *Rhododendron arboreum* at a dose of 40 mg/kg produced very less reduction in elevated serum levels of SGPT (5.21%), SGOT (4.67%), ALP (2.71%), total bilirubin (4.17%), direct bilirubin (1.75%), triglycerides (3.84%) and cholesterol (12.74%).
- Concominent treatment with ethanolic extract of leaves of *Rhododendron arboreum* (60 and 100 mg/kg, orally) significantly reduced the elevated serum levels of SGPT, SGOT, alkaline phosphatase, bilirubin, cholesterol and triglycerides compared to CCl_4 treated control group.

- Histopathological examination of the liver tissues supported the hepatoprotection as the hepatic damages in the rats treated with ethanolic extract were minimal with distinct preservation of structure and architectural frame of the hepatic cells.
- The dose of the extract (60 and 100 mg/kg, orally) prevent decrease in the excretion of ascorbic acid in rats urine due to the liver damage produced by Carbon tetrachloride.
- The results of ethanolic extracts of leaves of *Rhododendron arboreum* were comparable with the standard hepatoprotective agent Silymarin (100 mg/kg). At the dose of 100 mg/kg, of ethanolic extract of leaves of *Rhododendron arboreum* showed marked reduction in serum biochemical levels (98%).
- The present study demonstrates that the ethanolic extract of leaves of *Rhododendron arboreum* possess hepatoprotective property. In addition, the hepatoprotective property may be attributed to the antioxidant principles of the plant, namely quercetin related flavonoids, rutin and other phenolic compounds. Further study is warranted to isolate, characterize and screen the active principles from the leaves of *Rhododendron arboreum* that possess hepatoprotective property.

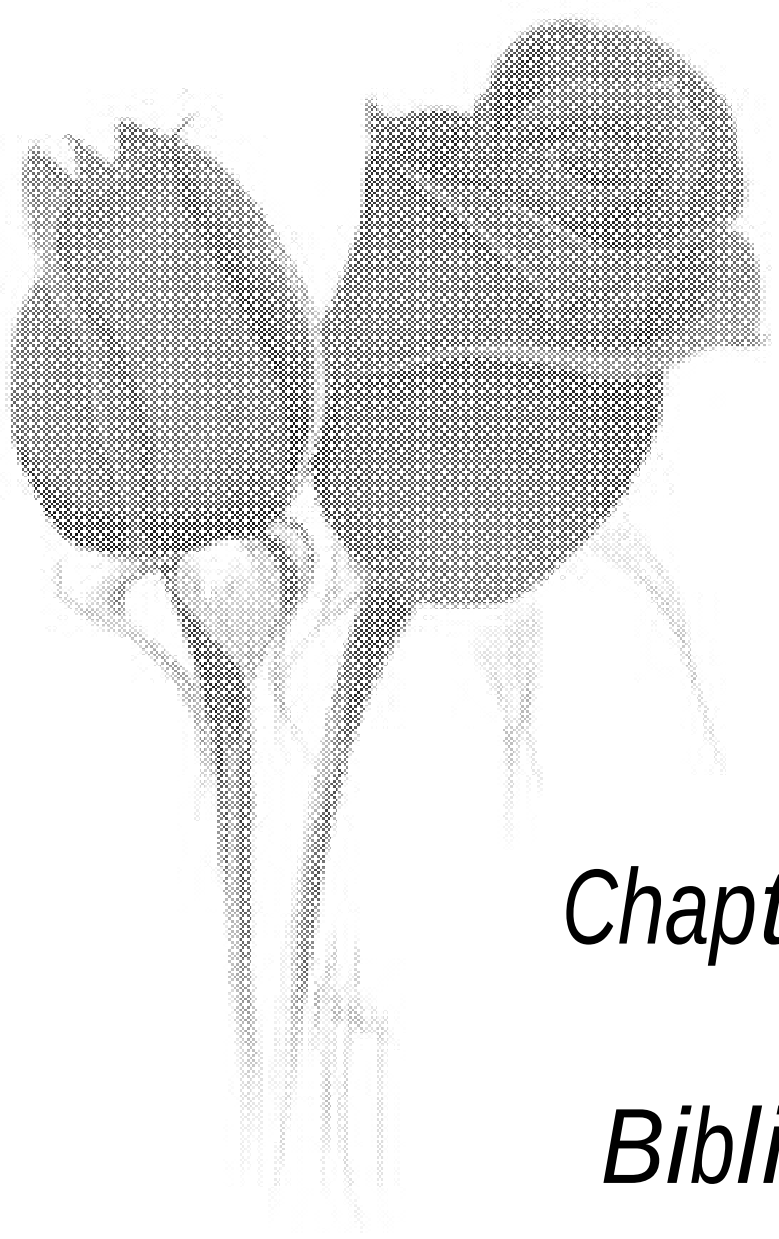


Chapter-8

Summary

SUMMARY

The present study was designed to investigate the effect of ethanolic extract of leaves of ***Rhododendron arboreum*** against Carbon tetrachloride induced hepatotoxicity. Preliminary phytochemical investigation of ethanolic extract of leaves of ***Rhododendron arboreum*** showed that it contains carbohydrates, saponins, flavonoids, phytosterols, tannins and phenolic compounds. Hepatoprotective activity of ethanolic extract of leaves of ***Rhododendron arboreum*** at a dose of 40, 60 and 100 mg/kg was investigated against Carbon tetrachloride induced hepatotoxicity in Wistar rats. The ethanolic extract of leaves of ***Rhododendron arboreum*** at 60 and 100 mg/kg and Silymarin produced a significant reduction in serum marker enzymes like SGPT, SGOT, ALP, (total and direct) bilirubin, triglycerides and cholesterol. Ethanolic extract at a dose of 60 and 100 mg/kg prevented the decrease in excretion of ascorbic acid in CCl_4 induced hepatotoxicity in rat urine. The results of histopathological study also support the results of biochemical parameters and explain the hepatoprotective activity of leaves of ***Rhodoendron arboreum***. The hepatoprotective property may be attributed to the flavonoids and phenolic compounds of the plant. However direct studies are needed to confirm this.



Chapter-9

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