SCREENING OF SELECTED MEDICINAL PLANTS FOR THEIR HEPATOPROTECTIVE ACTIVITY

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Under the guidance of

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CERTIFICATE

This is to certify that the thesis entitled **"SCREENING OF SELECTED MEDICINAL PLANTS FOR THEIR HEPATOPROTECTIVE ACTIVITY"**, submitted by **Mrs. PADMA R,** to The Tamil Nadu Dr. M. G. R. Medical University, Chennai, for the award of the Degree of Doctor of Philosophy in Pharmaceutical Sciences, is a record of the independent research work carried out by her at J.S.S. College of Pharmacy, Ootacamund, under my supervision, during 2006–2009. I also certify that the thesis or any part thereof has not formed the basis for the award of any other research degree, of this or any other University, previously.

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DECLARATION

I hereby declare that the thesis entitled **"SCREENING OF SELECTED MEDICINAL PLANTS FOR THEIR HEPATOPROTECTIVE ACTIVITY"** submitted by me to The Tamil Nadu Dr. M. G. R. Medical University, Chennai, for the award of Degree of Doctor of Philosophy in Pharmaceutical Sciences, is the result of my original and independent work carried out at J.S.S. College of Pharmacy, Ootacamund, under the supervision of **Dr. P. Vijayan,** Professor, J.S.S. College of Pharmacy, Ootacamund. The thesis or any part thereof has not formed the basis for the award of any degree, diploma, associateship, fellowship, or any other similar title, of this or any other University, previously.

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INTRODUCTION

1.1. Use of plants in health and disease management.

Food and medicine have been the inseparable companions of mankind from the beginning of man's existence and man had to contend with diseases that affected his life. Among the different sources of medicines, plants have been widely used for their healing abilities and have provided mankind with a large variety of potent drugs to alleviate suffering from various diseases. The ancient traditional Indian system of medicine, Ayurveda, is predominantly plant based, making use of native plants in spite of powerful contemporary modern medicine.

Though the usage of plants and plant based products in various contemporary and traditional system of medicine has significantly increased, most of the traditional knowledge about medicinal plants was in the form of oral knowledge that had been lost with persistent invasions and cultural adaptations without any written document and regulations. There was no uniform or standard procedure for maintaining the inventory of these plants and the knowledge about their medicinal properties. Therefore, it is essential that such uses of natural products be documented and studied for systematic regulation and wide-spread application.

Hence, the most urgent need is to rescue and record all traditional knowledge on plants and prepare inventories of medicinal plants along with their traditional uses, also taking into account the tribal knowledge. An inventory of medicinal plants compiled by the WHO in 1978 covered only ninety member countries and contained 20,000 species, of which only about 250 were of widespread use, and some of which had been analyzed to identify their main active chemical compound. In India, the Department of Biotechnology (DBT), Ministry of science and Technology, Government

of India has sponsored a project on inventorizing medicinal plants, wherein literatures on ethnobotanical, chemical, pharmacological and toxicological details of around 1700 plants are being collated[1].

1.2. Utility of plants in contemporary medicine.

Even though a large number of very specific pharmaceutical synthetic products are available to treat almost every disease, plants still have a definite place in today's therapy. The reasons may be:

- Many of the lead molecules of modern therapy are originated from the plant sources. The anti-malarial drugs artemisin from ***Artemisia annua,*** quinine from the species of cinchona[2], cardiotonic drug digitoxin from ***Digitalis purpurea*** and ***Digitalis lanta*** and anticancer drug vincristine from ***Cathranthrus roseus*** have been few of the representatives. Plants would be useful to treat viral diseases as well, example, Neem / Phillanthus.
- A number of plant based drugs such as taxol, reserpine, ergotine, opioids, ephedrine, colchicine, rutin, coumarins, anthraquinones, etc., are still a part of standard therapy. Most of these do not have any synthetic substitutes.
- Phytochemicals are a major source of dyes, flavours, sweeteners[3], aromas, perfumes, insecticides[4], antiparasitic drugs, and many other substances.
- Though some natural products suffer from certain disadvantages such as their occurrence in minute quantities in the source material, poor solubility, low stability, poor absorption, incorrect distribution, failure to reach the target

etc., a structural modification improves the efficiency of these drugs, the modified form being called the `pro-drug`, example, vindesine, an anticancer alkaloid, is a pro-drug of vinca (***Catharanhus roseus***) alkaloids[5]. Vincristine, the antileukaemia drug which is in great demand, suffers from the disadvantage of very low yields from the source material, and so is very expensive. Vinblastine, another anticancer drug from the same plant is present at levels of 1,000 times higher than vincristine and the cost is one third of vincristine. Vinblastine is now being used as the parent drug to obtain through structural modifications, the pro-drug vincristine.

Thus there are several methods of improving the performance of native drugs.

Several other plant products are used in formulations that are sold over the counter (OTC) in several countries. All these potential justifies the broadest and most exhaustive phytochemical research.

Though giant strides made by analytical and synthetic chemistry, electronics and science in general, have immensely contributed to the development of the science on biomedicine that has achieved miracles in medical practice, unfortunately, has resulted in sky-rocketing medical costs putting it beyond the reach of the vast majority of the world's population and has not been able to cure all the sickness in the world. Scientific (or standard) medicine generally serves only a minority (about 30 to 35 percent) of the total population in the developing countries[6,1].

The rest of the population attends to its health needs through the traditional medicine, which is essentially based on the use of easily accessible low-cost medicinal plants. Several considerations make the use of medicinal plants desirable.

Recognizing the merits of a wider usage of medicinal plants and of further integrated research on them, the WHO since 1977 has encouraged the study of traditional medicine in the hope of deriving the possible benefits for the needy world population. At the same time the WHO has also cautioned against the harmful effects that this type of medicine can cause on an irrational use. In order to reach its goal of **'Health for all'** the WHO had suggested that governments incorporate the favourable aspects of traditional medicine, especially the use of medicinal plants, in their primary health care procedures[7].

1.3. Indian systems of medicine.

The Indian subcontinent is endowed with a rich expertise in local health traditions. Alternative medical systems are those that offer independent therapies for the full range of all the diseases, as Allopathy does. The traditional medicine in India functions through two social streams. One is the local folk stream which is prevalent in rural and tribal villages of India, which is called uncodified system of medicine. The carriers of these traditions are millions of housewives, thousands of traditional birth attendants, bone setters, practitioners skilled in acupressure, eye treatment or treatment of snakebites or the **vaidyas**, who are the traditional village level herbal physicians. These local health traditions thus represent an autonomous community supported system of healthcare at the village level which runs parallel to the state supported systems.

A second level of traditional health care system is the academic or classical system. This consists of codified and organized medical wisdom with sophisticated theoretical foundations and philosophical explanations, expressed in thousands of regional manuscripts, covering treatises on all

branches of medicine and traditionally systems like Ayurveda, Siddha, Unani, Yoga, Naturopathy and Amchi. Thus the term Indian systems of medicine (ISM) incorporates the systems which originated in Indian or which originated outside but got adapted in Indian in the course of time[8].

Herbs have provided the basis for the great medical systems in human history, of Hippocrates and Galen, the great Islamic medical eras, and the Charaka and Susruta of the Indian sub-continent. Herbal drugs constitute a major part in all the traditional systems of medicine. Herbal medicine is a triumph of popular therapeutic diversity. Plants, above all other agents have been used for medicine from the time immemorial because they have fitted with the immediate personnel need. About 3000 plants have been scientifically investigated for their beneficial effects based on leads from traditional medicine. Interest is traditional medicines / drugs is not new but has been spurred in recent years by methodological advances in phytochemistry, a growing number of ethnobotanical studies, and an upsurge of interest in renewable resources and traditional medicine[9].

In the last few decades there has been an exponential growth in the field of herbal medicine. It is getting popularized in developing and developed countries. In olden times, Vaidyas used to treat patients on individual basis, and prepare drug according to the requirement of the patient. Now, herbal medicines are being manufactured on a large scale in mechanical units, where manufacturers come across many problems such as availability of good quality raw materials, authentication of raw material, availability of standards, proper standardization methodology of single drugs and formulations, quality control parameters, etc.[10]

Chromatographic finger printing techniques are most significant methods which can be used for the routine herbal drug analysis and for

the quality assurance[11]. As herbal medicines are prepared from materials of plant origin they are prone to contamination, deterioration and variation in composition. This gives rise to inferior quality of herbal products with little therapeutic efficiency. Most often the desired biological response is due to not one constituent but a mixture of bioactive constituents and the relative proportion of active constituents can vary from plant to plant of the same species and also in different plant parts. It is therefore essential to establish internationally recognized guidelines for assessing quality. The World Health Organization has recognized the importance of traditional medicine and has created strategies, guidelines and standards for botanical medicines.

The World Health Assembly – in its resolutions, has emphasized the need to ensure the quality of herbal medicine by using modern control techniques and applying suitable standards[12]. It is therefore necessary to develop methods for rapid, precise and accurate identification and estimation of active constituents in order to bring out consistency of important constituents in the formulations. Hence before proceeding to clinical studies, scientists need a tool to authenticate plants and also to detect their potency[13].

1.4. Herbal medicines for liver diseases.

As per the global estimates, there are about 18,000 deaths every year due to liver cirrhosis, mainly caused by hepatitis. Although viruses remain the main cause of liver diseases, the liver lesions arising due to imbalance of enzymatic levels which results from xenobiotics, excessive drug therapy, environmental pollution and alcoholic intoxication are among the secondary reasons[14]. As modern drugs have very little to offer for the alleviation of hepatic ailments, the use of plant products which protect the

liver from damage or which helps in balancing enzyme levels in the body, is gaining wide acceptance.

A phytotherapeutic approach to modern drug development can provide many invaluable drugs from traditional medicinal plants[15]**.** Numerous plants and polyherbal formulations are used for treatment of liver diseases. Latest trends have shown increasing demand of phytodrugs and a few of them have proven for their hepatoprotective potential. Silymarin, a flavonol ligan mixture extracted from the ***Silybum marianum*** (milk thistle) is a popular remedy for hepatic diseases.

Several hundreds of plants have been examined for use in a wide variety of liver disorders. These plants include Silymarin from ***Silybum marianum,*** picroliv from ***Picrorrhiza kurroa,*** andrographolide and neoandrgropholide from ***Andrographis paniculata***, Phyllanthin and hypophyllanthin from ***Phyllanthus niruri,*** Wedelolactone from ***Eclipta alba***, glycyrrhizin from ***Glycyrrhiza glabra***, curcuminoids from ***Curcuma longa*** are proven hepatoprotective medicinal herbs, which have shown genuine utility in liver disorders[16].

These plants are used widely in hepatotprotective preparations and extensive studies have been carried out on them.

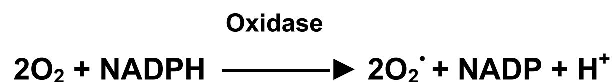
1.5. Antioxidants.

Cells in the human body use oxygen to break down carbohydrates, protein and fats that give them energy. Metabolically active cells produce by-products called free radicals. These are atoms or group of atoms that have at least one unpaired electron, which make them highly reactive. They promote beneficial oxidation that produces energy and kill bacterial invaders. If free radicals are at reasonable levels, the human body

produces enzymes to combat them for a useful immune system and antibacterial cell activity.

Excess free radicals attack DNA, a cell's genetic material leading to cancer and cardiovascular diseases. They are also implicated in arthritis, strokes, cataracts and degenerative health problems such as diabetes, Alzheimer's disease, retinal degeneration, ischemic dementia and aging. Fried foods, cigarette smoke, air and water pollution as well as toxins also create free radicals. When these free radicals are added to metabolic free radicals, they may lead to over exposure, which cause oxidative stress, a condition in which the body's natural defenses are overrun.

The living cell during several metabolic pathways generates reactive oxygen species (ROS) and reactive nitrogen species (RNS). Pathophysiological conditions enhance the generation of ROS and RNS and lead to oxidative stress. The generation of ROS begins with the rapid uptake of oxygen and activation of NADPH oxidase and the production of the superoxide free radical ($O_2^{\bullet}$)



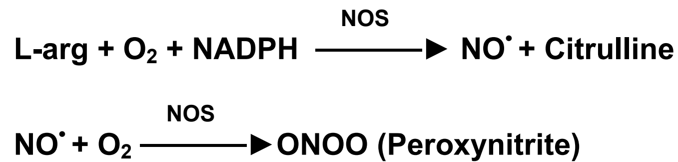
$$2O_2 + NADPH \xrightarrow{\text{Oxidase}} 2O_2^{\bullet} + NADP + H^+$$

ROS can also be generated through the Fenton (A) reactions (I) and Haber Weiss reaction (II)

I. $H_2O_2 + Fe^{+2} \longrightarrow Fe^{+3} + OH + OH$

II. $H_2O_2 + O_2 \longrightarrow O_2^{\bullet} + OH + OH$

The free radical nitric oxide ($NO^{\bullet}$), which, is known as endothelium-derived relaxation factor (EDRF), is formed from arginine by nitric oxide synthase (NOS)[2].



Peroxynitrite is a very strong oxidant, which reacts with aromatic amino acid residues to form nitrotyrosine, which can lead to enzyme inactivation. To escape ROS, RNS and lipid peroxidation dependence injury, biological structures have protective machinery in the form of endogenous antioxidants. Among different endogenous antioxidants, superoxide dismutase (SOD), reduced glutathione (GSH), catalase and glutathione peroxides (GPX) are important for counteractive oxidative stress.

Lipid peroxidation is a complex process that occurs in aerobic cells and reflects the interaction between molecular oxygen and unsaturated fatty acids. This produces and propagates the lipid radical ($L^\bullet$), uptake of O_2, generation of lipid alkoxyl ($LO^\bullet$), lipid peroxyl radicals ($LOD^\bullet$), rearrangement of double bonds, lipid hydroperoxide (LOOH) as well as a number of degradation products. Two paths are known for the formation of lipid peroxide ***in vivo***. One occurs through autoxidation of compounds such as catecholamines, quinines, thiols and redox reactions of myglobin and oxyhaemoglobin, and the other from active oxygen by the action of NADPH oxidase, xanthine oxidase, catalase, superoxide dismutase and glutathione peroxide. The disturbance of balance between the formation of free radicals (lipid radicals) and antioxidants leads to oxidative stress.

Antioxidants act as radical scavengers, hydrogen donors, peroxide decomposers, electron donors, enzyme inhibitors, singlet oxygen quenchers, synergist and metal chelating agents.

These are a broad group of compounds that destroy single oxygen molecules (free radicals) in the body, there by protecting against oxidative damage of cells. They are essential for good health and are found naturally in wide variety of foods and plants including many vegetables and fruits For e.g.; lemon, wild apricot, cherry laurel, sour cherry, water melon, melon, blue berry, grapes, strawberries, pineapple, peas, carrots, tomatoes, yams, garlic, leaves of the green leafy vegetables such as spinach, pumpkin, green tea, maize, wheat, corn, soybean, oats.

Plants also need to protect themselves from free radical damage, so they have evolved many different classes of phytochemicals to do so. The pigments in the barks, seeds, leaves, fruits and flowers are very active antioxidants because of the presence of phytochemicals including plant phenolics such as phenylpropanoids, flavonoids, coumarins and polyphenols like proantho cyandins and tannins, phytosterols, carotenoids, chlorophyll derivatives (pigments), essential oils, flavolignans, gums and resins.

The antioxidant activity of a compound or extract can be studied by 1,1-dipheny-1,2-picrylhydrazyl (DPPH) assay , thiobarbituric acid-reactive substances (TBARS) assay , superoxide dismutase (SOD) assay, assay of catalse (CAT), glutathione peroxide (GPX) activity , xanthione oxidase (XO) activity, deoxyribose degradation assay, reduced glutathione (GSH) assay methods. All these methods can be done either *in vitro or in vivo.* Among all the methods, DPPH is very rapid, simple, sensitive, and reproducible for the screening of large number of plant extracts.

Phytochemicals that have been reported to possess antioxidant activity are silybin, dihydro quercetin, catechin, epicatechin, supermine, mansonone, ferulic acid, chromo-saponin, emblican A and B, punigluconin, pedunculagin, curcumin, gallic acid, and bengalenoside[17].

1.6. The liver and its function.

Location and size of the liver

The liver, the largest organ in the vertebrate body, composed of a spongy mass of wedge shaped lobes has many metabolic and secretary functions. It weighs about 1.5kg (3 to 4 pounds), lies immediately under the diaphragm and occupies most of the right hypochondrium and part of the epigastrium.

Liver lobes and lobules

The liver consists of two lobes separated by the **falciform** ligament (Fig. 1). The left lobe forms about one sixth of the liver and the right lobe makes up the remainder. The right lobe has three parts designated as the right lobe proper, the caudate lobe (a small oblong area on the posterior surface) and the quadrate lobe (a four-sided section on the undersurface). Each lobe is divided into numerous lobules by small blood vessels and by fibrous strands that form a supporting framework (the capsule of Glisson) for them. The capsule of Glisson is an extension of the heavy connective tissue capsule that envelops the entire liver.

The hepatic lobules (Fig. 2), the anatomical units of the liver, are tiny hexagonal or pentagonal cylinders about 2 mm high and 1 mm in diameter. A small branch of the hepatic vein extends through the center of each lobule. Around this central (intralobular) vein, plates or irregular walls radiating outward, are arranged in the hepatic cells. On the outer corners of each lobule, several sets of tiny tubes – branches of the hepatic artery of the portal vein (interlobular veins) and of the hepatic duct (interlobular bile ducts) are arranged. From these, irregular branches (sinusoids) of the interlobular veins extend between the radiating plates of hepatic cells to join the central vein. Minute bile canaliculiare formed by the spaces around each cell that collect bile secreted by the hepatic cells.

Blood enters a lobule from branches of the hepatic artery and portal vein. Arterial blood oxygenates the hepatic cells (Fig. 3), whereas blood from the portal system passes through the liver for “inspection”. Sinusoids in the lobule have many reticuloendothelial cells (mainly **Kupffer cells**) along their lining. These phagocytic cells can remove bacteria, worn RBCs, and other particles from the bloodstream. Ingested vitamins and other nutrients to be stored or metabolized by liver cells enter the hepatic cells that form radiating walls of the lobule. Dissolved toxins in the blood are also absorbed into hepatic cells, where they are detoxified (rendered harmless). Blood continues along sinusoids to a vein at the center of the lobule. Such central, intralobular veins eventually lead to the main hepatic veins that drain into the inferior vena cava. Bile formed by hepatic cells passes through canaliculi to the periphery of the lobule to join small bile ducts.

Bile Ducts

The small bile ducts within the liver join to form two larger ducts that emerge from the undersurface of the organ as the right and left hepatic ducts. These immediately join to form one hepatic duct. The hepatic duct merges with the cystic duct from the gallbladder, forming the common bile duct, (Fig. 4) which opens into the duodenum in a small raised area called the major duodenal papilla. This papilla is located 7 to 10cm (2 to 4 inches) below the pyloric opening from the stomach[18].

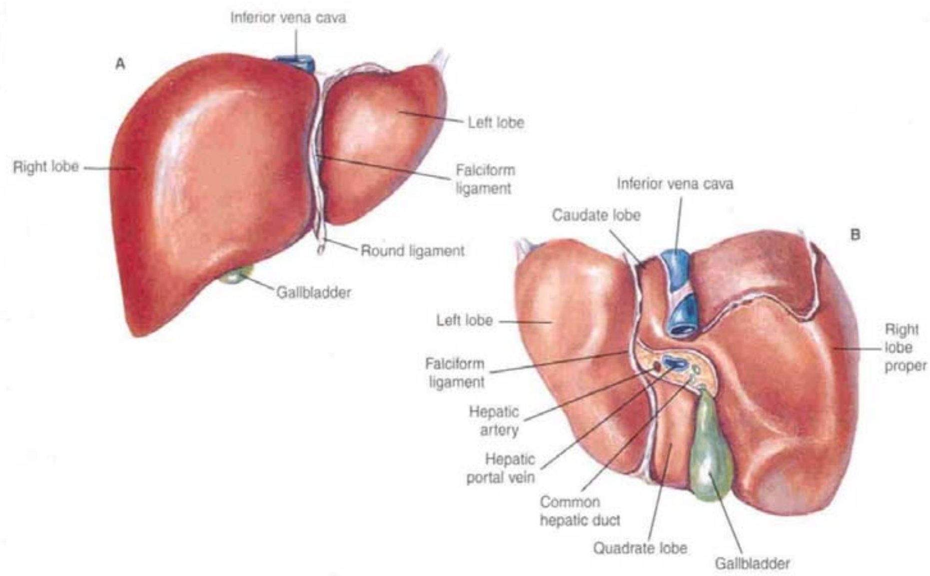


Fig. 1 Diagram of gross structure of the liver

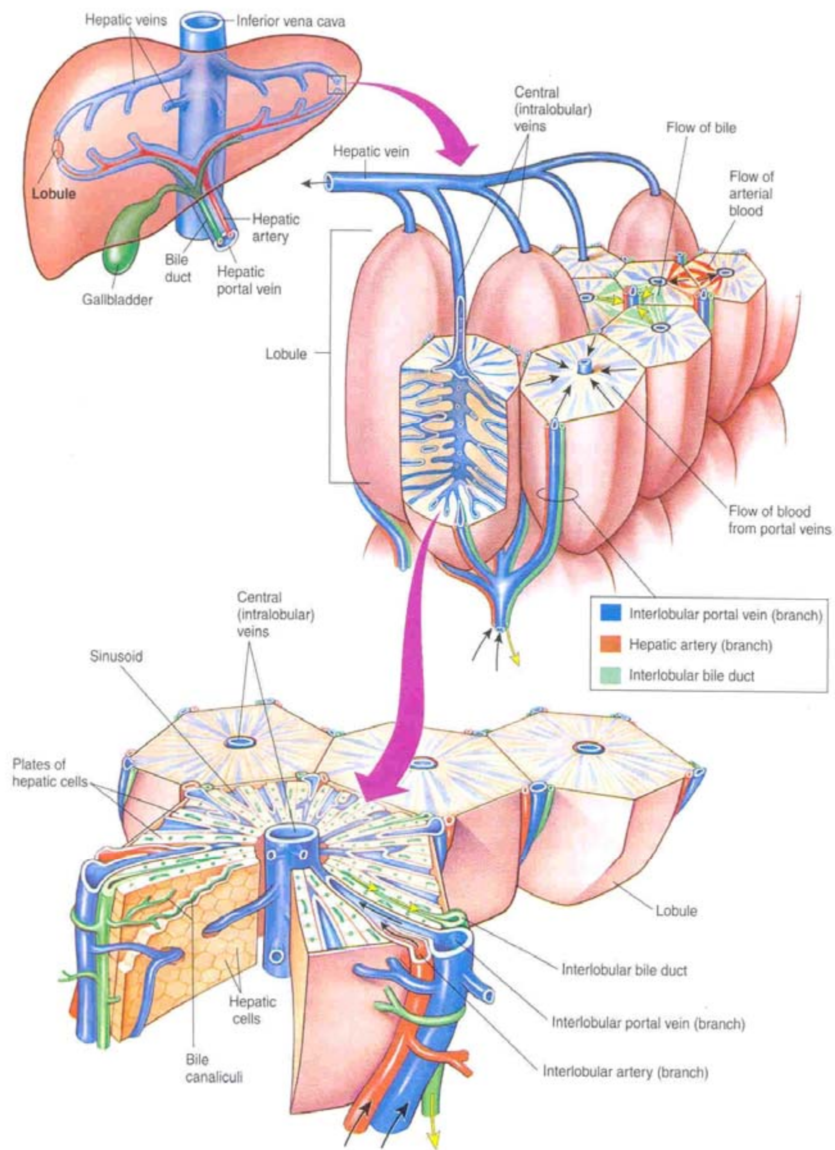


Fig. 2 Microscopic structure of the liver showing the location of liver lobules with hepatic blood flow

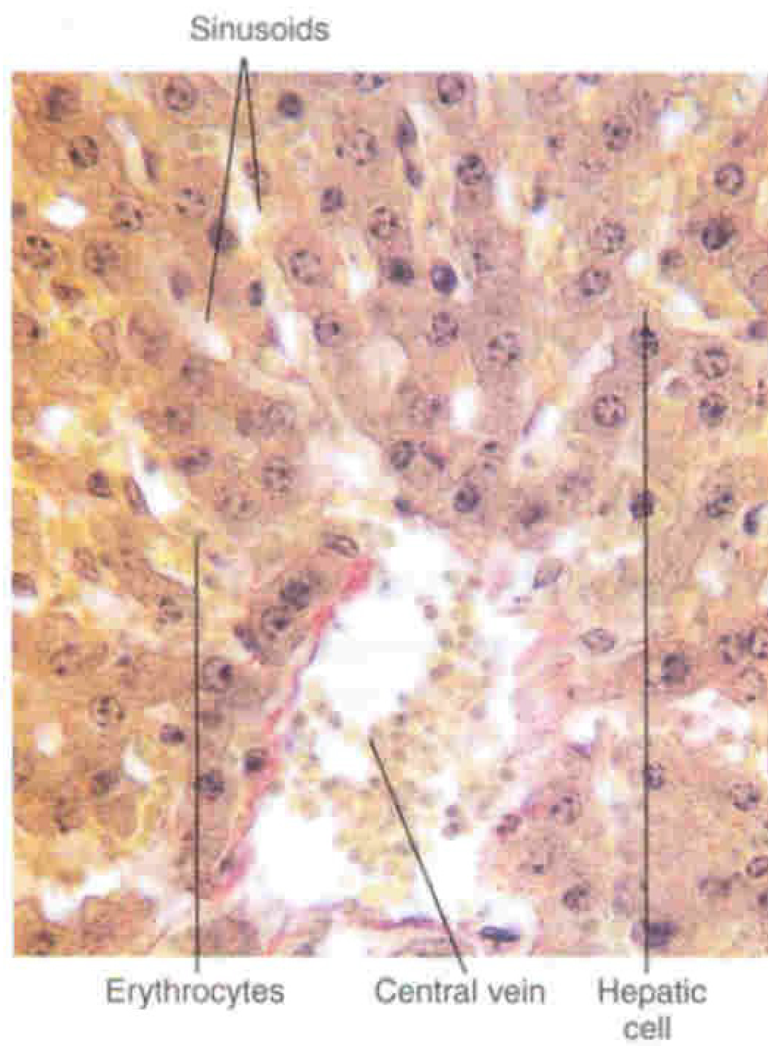


Fig. 3 Liver tissue showing hepatic cells

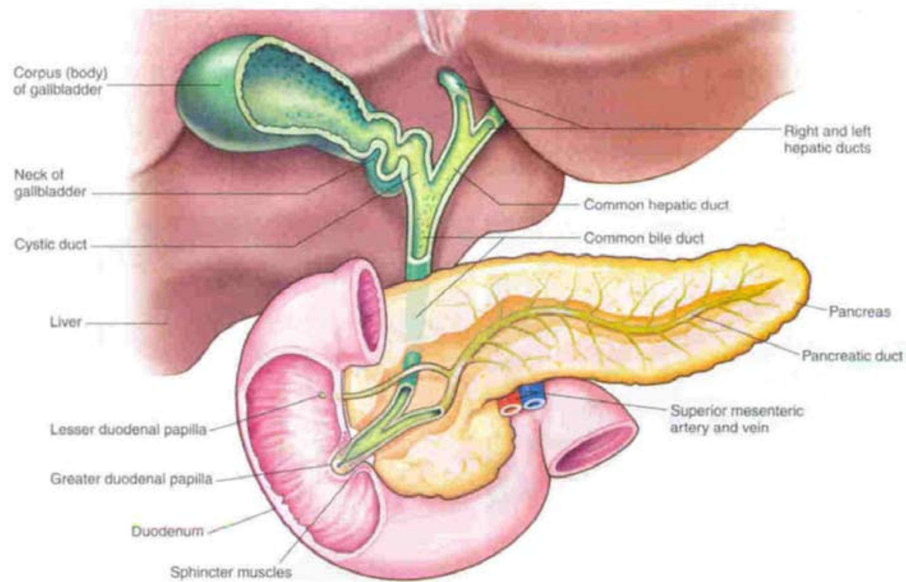


Fig. 4 Diagram of bile duct and its tributaries

Functions of the liver

Liver has three quite distinct functions:

- It supplies bile salts and bicarbonates to assist in digestion.
- It acts as a buffer between the gut and the systemic circulation maintaining stable levels of amino acids and glucose.
- It is the major excretory organ for the larger and more hydrophobic metabolities, foreign substances and drugs.

Besides secreting bile, which is needed for absorption of dietary fats, the liver performs many other vital functions. They are as follows:

- **Carbohydrate metabolism:**

The liver is especially important in maintaining a normal blood glucose level. When blood glucose is low, the liver can break down glycogen to glucose and releases glucose into the bloodstream. The liver

can also convert certain amino acids and lactic acid to glucose and it can convert other sugars, such as fructose and galactose, into glucose. When blood glucose is high as occurs just after eating a meal the liver converts glucose to glycogen and triglycerides for storage.

- **Lipid metabolism**: Hepatocytes store some triglycerides; break down fatty acids to generate ATP; synthesize lipoproteins, which transport fatty acids, triglycerides and cholesterol to and from body cells; synthesize cholesterol; and use cholesterol to make bile salts.
- **Protein metabolism**: Hepatocytes deaminate amino group from amino acids so that the amino acids can be used for ATP production or converted to carbohydrates or fats. The resulting toxic ammonia (NH_3) is then converted into the much less toxic urea, which is excreted in urine. Hepatocytes also synthesize most plasma proteins, such as alpha and beta globulins, albumin, prothrombin and fibrinogen.
- **Processing of drugs and hormones:** The liver can detoxify substance such as alcohol or excrete drugs such as penicillin, erythromycin and sulfonamides into bile. It can also chemically alter or excrete thyroid hormones and steroid hormones.
- **Excretion of bilirubin:** Bilirubin, derived from the heme of aged red blood cells, is absorbed by the liver from the blood and secreted into bile. Most of the bilirubin in bile is metabolized in the small intestine by bacteria and eliminated in feces.
- **Synthesis of bile salts:** Bile salts are used in the small intestine for the emulsification and absorption of lipids, cholesterol, phospholipids and lipoproteins.
- **Storage:** In addition to glycogen, the liver is a prime storage site for certain vitamins (A, B_{12}, D, E and K) and minerals (iron and copper), which are released from the liver when needed elsewhere in the body.

- **Phagocytosis:** The stellate reticuloendothelial (Kupffer) cells of the liver phagocytize aged red and white blood cells and some bacteria.
- **Activation of vitamin D:** The skin, liver and kidneys participate in synthesizing the active form of vitamin D.

Uncontrolled environmental pollution, poor sanitary conditions, xenobiotics, alcoholic intoxication and the indiscriminate use of potent drugs predispose the liver to a vast array of disorders. However, infection by virus still remains the major cause of liver disease[19].

1.7. Liver disorders

Liver disorders may be classified as hepatitis (inflammation of the liver), hepatotosis (non-inflammatory disorders or degeneration of the liver parenchyma), chronic hepatitis and liver cirrhosis. There is no strict hepatological delineation of these disorders and making a similar classification of the hepatoprotective agents is virtually impossible. Though a simple classification of hepatotoxins is difficult, various authors have categorized hepatotoxins based on factors like chemical nature, source of the agent, circumstances of exposure, mechanism of injury or type of liver damage (Table 1).

Table 1

Morphological changes induced by frequently encountered drugs

Morphology	**Drug class**	**Examples**
Necrosis	Mushroom	Amanite Phalloides
	Metals	Phosphorus
	Hydrocarbon	Carbon tetrachloride
	Analgesics	Paracetamol
	Anesthetics	Halothane

Hepatitis	Anesthetics	Halothane
	Antitubercular	Isonicotinic hydrazide
	Antithypertensive	Methyl dopa
	Chemotherapeutic	Nitrofurantoin
Steatonecrosis	Anticholesterol	Levostatin
	Sedative	Alcohol
	Chemotherapeutic	Methotrexate, tetracycline
	Cardiovascular	Animodarone
Cholestatis	Chemotherapeutic	Erythromycin
	Antipsychotic	Chloropromazine
	Hormones	Oral contraceptives
	Cardiovascular	Catopril, verapamil
	Anticonvulsant	Carbamazepine

Hepatitis: It is an inflammation of the liver that may be caused by viral and bacterial infections, chemical toxicity (primarily drugs), or autoimmune hypersensitivity reactions.

Viral hepatitis: Viral hepatitis is a major health concern worldwide. The categories of viral agents currently implicated in hepatitis are hepatitis-A (HAV), hepatitis-B (HBV), hepatitis-C (HCV), hepatitis-D (HDV), hepatitis-E (HEV) and hepatitis-F (HFV) virus[20].

Acute viral hepatitis: It is a self-limiting disorder[21]. No specific treatment is available. Drugs are of little value and do not alter the severity or time course of the disease. Areas of necrosis develop as groups of hepatocytes die and the eventual outcome depends on the size and number of these areas. Causes of the damage may be a variety of conditions, including:

- Viral infections
- Toxic substance
- Circulatory disturbances[22].

Chronic hepatitis: Chronic liver disease is defined as inflammation of the liver that persists for over six months. It is associated with permanent structural change within the liver which occur secondary to long-standing cell damage. The main causes of chronic liver disease are alcoholism, viral hepatitis, immunological diseases, vascular diseases, metabolic diseases due to genetic factors and infectious diseases. Chronic hepatitis represents a medical challenge. The goal of any treatment plan is to halt the progression to cirrhosis, liver failure and death.

Drugs induced hepatitis: Modern day medicines have become the leading cause of liver failure (30-50% of cases) and serious drug induced liver injury is the main reason that many drugs are withdrawn from the market. The recent withdrawal of nefazodone from the European market due to drug induced liver injury, once again focuses the attention of the pharmaceutical industry, regulators and doctors on the hepatotoxicity of drugs.

Two major types of hepatotoxicity have been noted. They are direct toxicity, also called predictable toxicity and idiosyncratic and non predictable toxicity. Idiosyncratic reactions are sometimes referred to as hypersensitivity reactions.

Disruption of metabolic process, toxic destruction of essential cell structures, induction of immunologic reactions, carcinogenic effect, transmission of infections, exacerbation of underlying disease are some of the basic mechanisms of drug induced hepatic injury. Table 1 shows the characteristic morphologic lesions seen in drug induced injury related to these mechanisms.

Jaundice: Jaundice is a general condition that results from abnormal metabolism or retention of a yellow coloured compound called bilirubin. Jaundice causes a yellow coloration of the skin, mucous membranes and sclera. The three principle types of jaundice are prehepatic, hepatic and posthepatic.

Prehepatic jaundice: This is the result of acute and chronic hemolytic anemia.

Hepatic jaundice: This includes disorders of bilirubin metabolism and transport defects. Levels of unconjugated bilirubin are elevated in this disorder.

Posthepatic jaundice: It is also known as cholestatic and obstructive jaundice. This is generally caused by biliary obstructive disease resulting from spasms of the biliary tract, ductal occlusions by stones or compression by neoplastic disease. The major increase is in the conjugated fractions.

Hepatic Injury: From a morphologic standpoint, the liver is an inherently simple organ, with a limited repertoire of responses to injurious events. Regardless of the cause, the general responses are necrosis, fatty liver, cholestasis and cirrhosis.

Necrosis involves the death of hepatocytes caused by progressive enzymatic degradation. It may affect group of cells or part of a structure of an organ. Fatty liver (steatonecrosis) is due to the microvesicular fat accumulation in the hepatocytes. Alcohol is the most common cause of this type of lesion. Cholestasis is due to the hepatic obstruction of biliary micelle by drugs.

Cirrhosis is characterized by a diffuse increase in the fibrous connective tissue of the liver, with area of both necrosis and regeneration of parenchyma cells imparting a nodular or glandular texture. In later stages,

cirrhosis leads to such deformity of the liver that it interferes with hepatobiliary function and the circulation of blood both to and from the liver.

1.8. Hepatotoxins and experimental models for evaluation of hepatoprotective activity

i. Intrinsic hepatotoxins

Two types of intrinsic hepatotoxins are:

a. Direct hepatotoxins
b. Indirect hepatotoxins

a. Direct hepatotoxins:

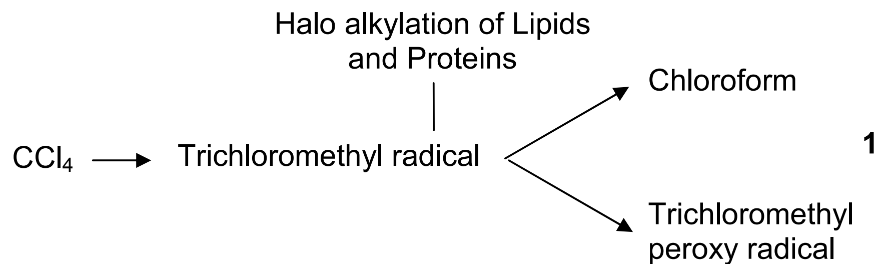
This group includes agents (or their metabolic products) that directly injure the plasma membrane, endoplasmic reticulum and other organelles of the hepatocyte. The prototype is CCl_4 which produces peroxidation of the lipids, denaturation of the proteins, or other chemical changes in the membranes which leads to their disruption. These changes, which begin almost immediately after the administration of the toxin, are the first stages in the injury, the culmination of which is hepatic necrosis, steatosis, or both[23].

1. Carbon tetrachloride:

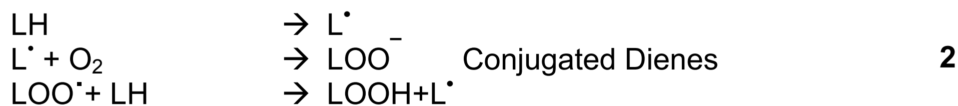
The prototype chemical inducing lipid peroxidation is carbon tetrachloride. Carbon tetrachloride initiates lipid peroxidation in experimental animals within a few minutes of administration which results in morphological and functional changes. CCl_4 is metabolically activated by the cytochrome P-450 to trichloromethyl radical ($CCl^{\bullet}_3$) which results in necrosis. The radical also binds covalently to proteins, DNA and lipids.

The $CCl^{\bullet}_3$ radical is oxidized to trichloromethyl peroxy radical ($CCl_3O_2^{\bullet}$) in the presence of molecular oxygen. The free radicals derived

from CCl_4 initiate lipid peroxidation within the endoplasmic reticulum by hydrogen abstraction from unsaturated fatty acids (LH). $CCl_3^{\bullet}$ is thereby reduced to the unreactive trichloromethane (Chloroform). Lipid peroxidation is a chain reaction[24].



Lipid peroxidation



Oxidation of the carbon centred lipid radical $L^{\bullet}$ yields the hydroperoxy radical $LOO^{\bullet}$ which in turn abstracts a hydrogen atom from another intact, molecule of fatty acid. Thus, a new $L^{\bullet}$ radical is created and while $LOO^{\bullet}$ is transformed to a lipid hydroperoxide LOOH.

2. Chloroform ($CHCl_3$)

Chloroform is a less potent hepatotoxin than carbon tetrachloride. Fewer studies have focused on the hepatotoxic effects of chloroform than those of carbon tetrachloride.

Chloroform induces centrizonal hepatic necrosis and steatosis, accompanied by renal and myocardial injury. Prolonged administration leads to cirrhosis and hepatic carcinoma. Pathogenesis of necrosis is similar to carbon tetrachloride. Steatosis results from impaired exit of lipid

from the liver. The mechanism of injury results from the conversion to a hepatotoxic metabolite[23].

3. Phosphorus

The white allomorph of elemental phosphorus produces its hepatic injury by a direct attack on cell constituents. The injury consists of hepatic steatosis and necrosis. But the mechanism of hepatotoxicity remains unclear. The accumulation of fat leads to impaired exit of fat from the liver.

Impaired protein synthesis occurs after two hours of a dose of phosphorus, and deficient production of apolipoproteins leads to steatosis. The basis for necrosis remains obscure[23].

b] Indirect hepatotoxin

1. Paracetamol

It is the active metabolite of phenacetin. Paracetamol is an effective to aspirin as an analgesic and antipyretic, but a weak anti-inflammatory agent. Paracetamol is a mild analgesic that is harmless in therapeutic doses and a potent hepatotoxin in overdose. It has been proved to be an elegant model for the exploration of mechanisms of hepatotoxicity.

Paracetamol is largely converted to conjugates of glucuronate and sulfate. A minor amount, about 25%, is converted to the active metabolite which binds promptly to glutathione (GSH), the resulting compound being converted to mercapto uric acid and cystine. When the amount of active molecule formed exceeds the GSH available for binding, and then it causes necrosis[25].

Paracetamol causes hepatic necrosis only when the dose is sufficiently large to deplete hepatic glutathione by 85% or more.

2. Galactosamine

Hepatic injury by galactosamine results from the metabolism of galactosamine in the liver and consequent effect on nucleic acid

metabolism. Galactosamine enters the pathways for galactose metabolism after which there are two hypothetical alternative pathways, both leading to the production of UDP-glucosamine. The latter compound, unlike the UDP-glucose, which is produced by galactose metabolism, cannot serve as an uridylate donor in the uridyl transfer reaction. The formation of UDP-hexosamine is a metabolic bind alley which leads to trapping of the uridylate. It leads to a decrease in UDP-glucuronate. The toxicity occurs as a form of high output failure of uridylate biosynthesis[26].

3. Orotic acid

Orotic acid produces fatty liver. Steatosis results from the reduction in the transport of lipid from the liver. Other evidences of steatosis are ATP deficiency, inhibition in the synthesis of phospholipids, proteins, RNA and ultrastructural abnormalities of nucleoli, nuclei and cytoplasmic organelles[27].

4. Ethionine

Single or repeated doses rapidly lead to hepatic steatosis with little or no necrosis in the rat. Prolonged administration can lead to a proliferation of cells and bile ducts resulting in cirrhosis and /or hepatic carcinoma[28].

ii. Host idiosyncracy

a) Hypersensitivity

Individual susceptibility of patient has long been attributed to hypersensitivity in which the responsible drug or metabolite has been assumed to act as a hapten. E.g. Para amino salicylic acid (PAS)[23].

Acute and chronic hepatic injury from PAS have been reported as clinical features of hepatic injury, which has a high mortality rate, with ascites, hepatomegaly and splenomegaly often with other evidence of fatal hypertension. Hepatotoxic effects of PAS are due to its toxic metabolites.

b) Metabolic idiosyncracy

It is due to the aberration of the enzymatic machinery for biotransformation of the incriminated drug. E.g. Isonicotinic hydrazide (INH). INH is converted into its principal metabolite acetyl INH, which is converted into monoacetyl hydrazine. This is speculated to cause hepatotoxicity.

Experimental hepatotoxicity permits the study of accidental and industrial toxicity screening of medicinal agents for potential hepatotoxic effects, study of hepatic physiology, histopathology and the regeneration and the development of diagnostic tools (Table 2).

Table 2

Classification of hepatotoxic agents and the major characteristics of each group

Category of agents	Mechanism	Histological lesion	Examples
1. INSTRINSIC TOXINS			
a) Direct	Direct physiochemical distortion and destruction of structural basis cell metabolism	Necrosis (zonal) and / or steatosis	Carbon tetrachloride, Chloroform, Phosphorus
b) Indirect			
i) Cytotoxic	Interference with specific metabolic pathways leading to structural injury	Steatosis or Necrosis	Paracetamol, Ethionine, Ethanol, Galactosamine, Natural hepatotoxins
ii) Cholestatic	Interference with hepatic excretory pathways leading to cholestasis	Bile casts	Interogenin, Anabolic and Contraceptive steroids
2. HOST IDIOSYNCRACY			
a) Hyper sensitivity	Drug allergy	Necrosis or Cholestasis	Sulfonamides, Para amino salicylic acid, Halothane
b) Metabolic abnormality	Production of hepatotoxic toxic metabolites	Necrosis or Cholestasis	Isonicotinic hydrazide, Halothane

Types of experimental models

Both *in vivo* and *in vitro* models[23] are available for the evaluation of anti-hepatotoxic properties of a drug. In the *in vivo* methods, whole animals are used for the demonstration of an injury by an exogenous agent on the liver with its physiological significance. *In vitro* models are employed to elucidate specific aspects of the mechanism of injury.

***In vitro* models**

A number of *in vitro* models have been developed for the study of hepatotoxicity. These include the perfused liver, liver homogenates and slices, suspensions of hepatocytes freshly isolated from the liver or isolated organelles from hepatocytes.

The animals are treated with the agent under study prior to their sacrifice and then the liver is removed for the *in vitro* studies, or by adding the agent under study to the perfusate or to the medium containing hepatic tissue, cells or organelles.

1. Liver perfusate

Studies of the effect of a number of toxic agents on metabolic activity, bile flow or excretion and lipid secretion by the isolated liver into the perfusate or into the bile, all have served to elucidate the mechanism of injury. The nature of hepatic injury also has been studied by observing the effect of hepatotoxic agent on leakage of intracellular enzymes into the perfusate.

Liver perfusion has also been useful in the study of effects of drugs on the liver. Anabolic steroids, phenothiazines, erythromycin estolate and other drugs have been demonstrated to interfere with bile flow and BSP (Bromosulfalein Sulfobromo phthalein) clearance by *in vitro* perfused rat liver.

2. Tissue homogenates and slices

Homogenates of liver tissue have generally served to test some of the adverse effects of toxic agents or functions of liver that cannot be measured directly *in vivo.* Metabolism of drugs and uptake of labeled amino acids by these tissue preparations as a measure of protein synthesis serves to demonstrate the effects of toxic injury. Slices of the liver tissue have also been used to measure the inhibition of lipid secretion by the toxin damaged liver and to demonstrate an adverse effect on plasma membrane of the hepatocytes by the study of potassium or enzyme leakage into the medium.

3. Hepatocyte suspension

This method has been developed by the isolation of rat hepatocytes that have retained sufficiently intact metabolic functions to mimic that of the normal liver suspensions of rat hepatocytes. Suspensions of rat hepatocytes and of tissue culture grown cells have been used in studying mechanisms of injury induced by phenothiazines, tricyclic antidepressants, aflatoxins, chlorinated hydrocarbons and galactosamine.

4. Organelles

Organelles isolated from hepatocytes or normal animals or from animals pretreated with hepatotoxic agents, have served to pose some important questions. The adverse effects of hepatotoxic agents such as carbon tetrachloride, ethionine, phosphorus, thioacetamide, mycotoxins and others agents on the protein-synthetic functions of the liver have been studied by measuring the ability of ribosomes isolated from animals pretreated with the respective agent to incorporate labeled amino acid. Also, the integrity of lysosomes, the functional status and integrity of mitochondria and the chemical changes in the nucleus and nucleoli, isolated from the liver of animals pretreated with hepatotoxic agents, have

been the subject of considerable study by hepatotoxicologists. The adverse effects of adding hepatotoxic agents *in vitro* to isolated ribosomes, mitochondria, lysosomes, plasma membrane and golgi apparatus also have provided information.

5. Nuclear components

Study of effects of known hepatotoxins on DNA, RNA, nuclei and synthesizing enzymes has provided key information on mechanism of injury. This fact has been approached by examining the various components isolated from the liver after pretreatment of the experimental animals with toxic agents or by isolating the respective compounds from normal animals and examining the effects of the particular toxic agent *in vitro* on the nucleic acid or enzymes. Thus, demonstration of breaks in the DNA chain or of alkylation of purines or pyrimidines, or of inhibition of nucleic acid synthesizing or repair enzymes has provided important information of hepatotoxic and hepatocarcinogenicity.

6. Microorganisms

Microorganisms have served as useful models for the study of the mechanism of injury of a number of hepatotoxins. Ethionine, an agent that injures the liver of many species, interferes with growth of several bacterial and protozoan species by a mechanism that is apparently related to its hepatotoxic effects.

***In vivo* models**

a] Whole animal as experimental model

The studies performed during the past 100 years have employed a variety of species, the most popular being rats because of their size and relatively low cost. Accordingly, most of the accumulated information bearing on experimental hepatotoxicity and on modifiers of susceptibility, such as age, sex, stage of development, diet and exposure to other toxic

substances, applies to the rat. The general employment of relatively uniform experimental model permits comparison of results obtained in widely separated laboratories. To a varying degree mice, hamsters, guinea pigs, rabbits, dogs, cats, cattle, horse, sheep and several species of birds have been employed, and studies of any particular chemical may include any of these or other species, during recent years, primates have come into use, for the obvious reason of the greater presumed relevance to the disease of humans.

b] Parameters of injury

Measure of hepatic injury includes lethality, histological changes seen by light and electron microscopy, chemical changes seen in the liver, and physiological and biochemical test that measure the functional status or that reflect the type or intensity of hepatic injury.

1. Lethality

Death as a measure of hepatotoxic potency is applicable mainly to known hepatotoxins. Employment of the LD_{50} or other measures of lethal potency permits comparison of hepatotoxic agents.

2. Histology

Toxic hepatic injury can be categorized by using light microscopy, electron microscopy and scanning electron microscopy.

a) Light microscopy

The traditional method of demonstrating toxic hepatic injury and categorising its type is studied by light microscopy. It provides the yardstick against which other abnormalities can be measured. However, light microscopy provides only a crude estimation for the quantification of the degree of injury.

b) Electron microscopy

It provides a much earlier demonstration of hepatocyte injury and permits the recognition of damage. It is also useful in differentiating lesions that appears to be similar in light microscopy. For e.g., hepatic injury induced in rats by galactosamine resembles that of viral hepatitis when examined by light microscopy, but not when examined by electron microscopy. It provides evidence of injury and may yield clues to the mechanisms of injury.

c) Scanning electron microscopy

This approach to ultra structural studies appears to have added new dimensions to the study of structural changes induced by toxic agents. Studies of cholestatic effects of hepatotoxins utilizing the scanning technique provide the data base for a new hypothesis for the development of cholestasis.

3. Measurement of serum enzyme levels

Measurement of serum enzymes levels has permitted detection of hepatic toxicity with far less labour than those required for histologic analysis. It is useful for detection of early damage for demonstration of the hepatic injury without sacrificing animals.

The employment of serum enzymology to test for hepatotoxicity requires that the enzyme test be hepatospecific. The transaminase levels e.g. alanine aminotransferase (ALT), aspartate aminotransferase (AST) are the most widely employed and generally accepted measures of hepatic injury despite the equal or even greater hepatospecificity of several other serum enzymes.

Levels of ALT, AST or both have been employed as evidence of hepatic injury or as guides to the time course or intensity of injury. In all

mammalian species, the AST is a sensitive measure of acute hepatic necrosis. However, the AST is an insensitive measure of steatosis.

1.9. Alteration in chemical constitution of liver.

In addition of producing elevation in serum enzyme activities and altering hepatocytes transport process, chemical hepatotoxicants can produce changes in structural and functional hepatic constituents. Hepatic lipid content, lipid peroxidation, hepatic glucose-6-phosphate activities, triglycerides and hepatic collagen contents have been found useful for detecting and qualifying the degree of liver damage produced.

Liver function tests

i) Aspartate aminotransferase (AST)

It is found practically in every tissue of the body, including RBCs, its concentration is particularly high in cardiac muscle and liver, intermediate in skeletal muscle and kidney, and much low in other tissues. In the hepatocyte, the different isoenzymes of AST exist in mitochondria and the cytosol. This enzyme catalyzes the transfer of the γ-amino groups of aspartate to the γ-keto group of ketoglutarate, leading to the formation of oxaloacetic acid and pyruvic acid. The measurement of the serum AST level is helpful for the diagnosis and in the cases of myocardial infarction, hepatocellular diseases and skeletal muscle disorders. Highest levels are found in association with conditions causing extensive hepatic necrosis, such as severe viral hepatitis, toxin-induced liver injury, or prolonged circulatory collapse. Lesser elevations are encountered in mild acute viral hepatitis as well as in both diffuse and focal chronic liver diseases (e.g., chronic active hepatitis, cirrhosis and hepatic metastases). However, the absolute levels of aminotransferases correlate poorly with severity of liver injury or prognosis and serial determinations are usually most helpful.

Thus, in the patient with massive hepatic necrosis, there may be marked elevations in the early phase (i.e., 24 to 48 h) but by the time the patient is tested 3 to 5 days later, the levels may be in the range of 3.34 to 5.8 μkat/L (200 to 350 U/L). Minimal elevations of AST and ALT (less than 1.67 μkat/L) also may be found in association with biliary tract obstruction; higher levels suggest the development of cholangitis with resultant hepatic cell necrosis.

In general AST and ALT levels parallel each other with a couple of exceptions. In alcoholic hepatitis the AST/ALT ratio may be greater than 2; this appears to result from a reduction in hepatic ALT content due to a deficiency in the cofactor pyridoxine-5-phosphate. An increase in the ratio of AST/ALT (>1) also can be seen occasionally in patients with the fatty liver associated with pregnancy. Reference interval: 6-25 U/L.

ii) Alanine aminotransferase (ALT)

The concentration of ALT is not really as great as for AST. It is present in moderately high concentration in liver, but is low in cardiac and skeletal muscles and other tissues. This enzyme catalyzes the transfer of the γ-amino groups of alanine to the γ-keto group of ketoglutarate, leading to the formation of oxaloacetic acid and pyruvic acid. Its use in clinical purpose is primarily for the diagnosis of liver diseases and to resolve some ambiguous increase in serum AST and ALT. The liver is the primary source of these enzymes. In the hepatocyte, ALT is found exclusively in the cytosol. If the serum AST is elevated while ALT remains within normal limits, it is a case of suspected myocardial infarction. Reference Interval: 3-30 U/L.

iii) Alkaline phosphatase (ALP)

Human serum contains several forms of alkaline phosphatase, a plasma membrane-derived enzyme of uncertain physiologic function which

hydrolyzes synthetic phosphate esters at pH 9. Eleven different isoforms of this enzyme have been identified in serum. These activities arise from bone, intestine, liver and placenta. A number of different assays have been developed which utilize different substrates.

In the absence of bone disease or pregnancy, elevated levels of alkaline phosphatase activity usually reflect impaired biliary tract function. The increased levels reflect increased synthesis of the enzyme by hepatocytes and biliary tract epithelium rather than regurgitation of enzyme due to obstruction. Bile acids may play a role both by inducing synthesis and by promoting solubilization of the membrane-associated enzyme activity.

Slight to moderate increases in alkaline phosphatase (1 to 2 times normal) occur in many patients with parenchymal liver disorders such as hepatitis and cirrhosis; transient increases may occur in all types of liver disease. However, the most striking increases in alkaline phosphatase (3 to 10 times normal) occur with extrahepatic biliary tract (mechanical) obstruction or with intrahepatic (functional) cholestasis, as in drug-induced cholestasis or primary biliary cirrhosis. Conversely, it is unusual for the serum alkaline phosphatase to remain normal when there is obstructive jaundice, and a normal enzyme level argues strongly against the presence of cholestasis. The alkaline phosphatase is usually mildly elevated in metastatic or infiltrative liver disease (e.g., leukemia, lymphoma and sarcoid). The enzyme may be elevated in the presence of incomplete biliary obstruction or when there is obstruction of only one hepatic duct, conditions in which the serum bilirubin is often normal or only slightly elevated. Serum alkaline phosphatase is also elevated in nonhepatic disorders most notably in some bone disorders (e.g., Paget`s disease,

osteomalacia and metastases to bone) and sometimes with malignancy. Reference interval: 40-223 U/L.

iv) Lactate dehydrogenase (LDH)

Moderate LDH elevations are common in acute viral hepatitis, cirrhosis, and metastatic carcinoma to the liver. Biliary tract disease also may produce slight elevations. Marked elevations of LDH in association with abnormal results of other liver function tests may reflect a hematologic malignancy such as lymphoma. Lactate dehydrogenase is localized in the cytoplasm of cells and this is extruded into the serum when the cells are damaged or necrotic. When only a specific organ, such as liver is known to be involved, in the measurement of total LDH is useful. Reference interval: 125-290 U.L.

v) Total cholesterol

Serum cholesterol comprises two forms, free cholesterol and esterified cholesterol. In jaundice and parenchymatous liver disease, serum cholesterol level will fall. Reference interval <200 mg/dl.

vi) Triglycerides (TGL)

Immediately after the administration of carbon tetrachloride, the level of triglycerides in the liver is elevated. The defect in the transport of triglycerides into the plasma is the cause for accumulation of lipids in the liver during carbon tetrachloride intoxication. Within 3-5 hours after administration of carbon tetrachloride, decrease in serum triglyceride level occurs in rats. Carbon tetrachloride intoxication evokes a defect in the secretary mechanism of triglycerides in the liver, resulting in accumulation of lipid in liver. A reduction in the synthesis of lipoproteins will result in the lower transport of triglycerides, which is associated with lipoprotein transport. Reference Interval: 35-200 mg/dL.

vii) Total protein (TP)

A healthy functioning of the liver is required for the synthesis of the serum proteins, except for the gamma globulins. The proteins synthesized in the liver are usually decreased in hepatocellular disease, but the immunoglobulins are increased in viral hepatitis and chronic liver infections. Reference Interval: 6-8.2 g/dL.

viii) Albumin

Albumin is quantitatively the most important serum protein synthesized by the liver. Albumin has a fairly long half-life (14 to 20 days) with less than 5 percent turnover daily. Albumin is decreased in chronic liver diseases and is generally accompanied by an increase in the beta and gamma globulins as a result of production of IGM and IGA in biliary or alcoholic cirrhosis, respectively. There is a substantial reserve of hepatic albumin synthesis; thus adequate synthesis may continue until there is extensive hepatocellular injury. Serum levels are influenced by a variety of nonhepatic factors, most notably nutritional status, hormonal factors and plasma oncotic pressure. Reference interval: 3.5-5.2 g/dL.

ix) Total bilirubin (TB)

Bilirubin has been used to evaluate chemically induced hepatic injury. It is the principle pigment in the bile, and is derived from the breakdown of hemoglobin by the process of phagocytosis of the RBCs. As most of the liver diseases are accompanied by jaundice, the differential diagnosis of jaundice plays an important role in elucidating hepatic dysfunction. An elevated level of serum bilirubin may be produced. It shows severe parenchymal injury. Reference interval: < 1 mg/dL.

x) Direct bilirubin (DB)

Bilirubin appears in the urine only after it is converted to a water-soluble form. Generally this involves conjugation with polar glucuronide

groups which enhance water solubility. Unconjugated bilirubin is not water-soluble. It is transported in the blood stream bound to albumin. It accounts for 30-50% of bilirubin rise in hepatocellular disease or cholestasis. Unconjugated hyperbilirubenemia is most often due to either haemolysis, or Gilbert’s syndrome, an inherited abnormality of bilirubin metabolism. Bilirubinuria occurs with even minimal degree of jaundice and may be detected before jaundice is evident Reference Interval: <0.25 mg/dL.

1.10. Currently available modern medicine for liver disorders and their limitations

Only a few modern drugs are available for treating liver diseases. Drugs such as tricholine citrate, trithioparamethoxy phenyl propene, essential phospholipids, combination of l-ornithine l-asparatate and pancreatin, silymarin and ursodesoxy cholic acid are generally prescribed for hepatitis, cirrhosis and other liver diseases. However, these modern medical treatments are still far from satisfactory.

Hepatitis C is an infectious viral disease of the liver. The main form of treatment of hepatitis C virus (HCV) infection is through drugs like interferon, amantadine and ribavarin. A combination therapy of interferon and ribavarin for 6 months is most commonly used for HCV treatment. The combination of interferon and ribavirin used for the treatment of hepatitis C is very expensive.

Ribavarin is the drug of choice along with interferon for HCV treatment, but it is not used in conditions like pregnancy, heart and kidney problems and psychological illnesses.

Interferon alpha have been widely used to treat chronic hepatitis C virus infection. These include recombinant interferons, or purified natural

leucocyte or lymphoblast interferon. Interferon alpha is usually administered by subcutaneous or intramuscular injection.

Therapy for chronic hepatitis B and C is evolving and may include interferon antiviral and immune-modulating drugs. Autoimmune hepatitis is usually treated with corticosteroids, such as prednisone.

With several grams of choline per day, some people will experience abdominal discomfort, diarrhoea or nausea. Supplementing choline in large amounts (over 1,000 mg/day) can lead to a fishy body odour. Depression has been reported as a side effect in people taking large amounts of choline, such as 9 grams per day.

Usage of many folklore remedies mainly plant products, is also quite common throughout India. In spite of such widespread use of medicinal herbs for different disorders interest in hepatoprotective activity is kindled because in India more than 18 million people are affected with liver disorders. Liver disease is still a world wide health problem. Unfortunately, conventional or synthetic drugs used for the treatment of liver diseases are inadequate and sometimes can have serious side effects. This is one of the reasons for many people the world over, including those in developed countries turning to complimentary and alternative medicine (CAM). Many traditional remedies employ herbal drugs for the treatment of liver ailments.

Based on the above facts and the need for the development of newer plant based hepatoprotective drugs, it was felt to carry out the hepatoprotective activity of the plants ***Cuscuta reflexa*** **Roxb** of Convolvulaceae / Cuscutaceae family and ***Cassytha filiformis*** **Linn** of Lauraceae family based on the ethnomedical claims.

Scope & Plan of Work

SCOPE AND PLAN OF STUDY

2.1. Scope

Ayurveda and other traditional medical practitioners of Siddha and Unani have claimed for centuries that extracts of plants can be effectively used for the alleviation of different types of liver diseases. Except for the use of appropriate vaccine for the treatment of hepatitis caused by viral infection, there are few effective plants that cure liver diseases. Therefore, it is not surprising that a considerable interest has been developed in the examination of those numerous worldwide traditional plant remedies, which are used for such treatment[29]. In the recent years, investigations are carried out to provide experimental evidence, which confirms that many of these plants do indeed have hepatoprotective properties. Recent progress in the study of such plants has resulted in the isolation of several phytoconstituents from plants belonging to various families, which exhibit hepatoprotective activity.

Liver diseases are among the important health disorders affecting mankind and remain one of the serious health problems[30]. No remedy is available to majority of them at present. In the absence of suitable liver protective drugs in allopathic medical practices, herbs play important role in the management of various liver disorders. A number of medicinal plants have been advocated in traditional system of medicine, especially in Ayurveda for treating liver disorders. This usage is in vogue since centuries and are quite often claimed to offer significant relief.

As there is an ever-increasing need for new hepatoprotective drugs, the present study aims to screen some plants for their hepatoprotective activity. A number of reviews have been published stating the importance

of plant drugs in diseases of the liver. Some of the plants mentioned in ethnobotany had been selected for investigating their hepatoprotective activity.

The present study therefore aims the following plants for hepatoprotective activity.

1. ***Cuscuta reflexa*** **Roxb** (Convolvulaceae / Cuscutaceae)
2. ***Cassytha filiformis*** **Linn** (Lauraceae)

A thorough survey of the journal literature of the selected plant showed that no work has been done as yet to justify the claims of this plant in the treatment of liver disorders. In this context evaluation of the antihepatotoxic activity of the various extracts of these plants were carried out.

2.2. Plan of work

The work of plan is divided into following phases:

Phase I

The collection of plant material and extraction

- Identification, authentication of the plant material.
- Collection, shade drying and powdering of the different parts of the plants that are claimed to be used in liver disorders.
- Extraction of the powdered material with suitable solvents, preferably with polar solvents like water or alcohol in the form of extracts.

Phase II

Pharmacognostical studies

The following pharmacognostical parameters were studied.

- Morphological profile of the plant.

- Microscopical analyses & anatomical standardisation of the plants.
- Determination of physicochemical constants such as total, acid insoluble, water-soluble and sulphated ash values.
- Determination of extractive values.
- Fluorescence analysis.
- Estimation of total phenolic content.
- Estimation of flavonoid content.

Phase III

Preliminary phytochemical analysis

- Preparation of various extracts.
- Qualitative analytical tests for detection of various plant constituents namely alkaloids, glycosides, tannins, steroids, flavanoids, triterpenoids, fixed oils, fats, saponins, carbohydrates, sugars and phenolic compounds in the extracts.

Phase IV

Isolation and characterization of active phytoconstituents / chemical components

- Isolation of compounds by bioactivity guided extract fraction using column chromatography technique.
- Qualitative chemical test for the identification of presence of the various phytoconstituents in the fractions.
- Thin layer chromatography (TLC) analysis for the isolated compounds.
- UV, IR, NMR and mass spectral studies to identify the structures of the isolated compounds.

Phase V

In vitro **antioxidant studies**

- DPPH method
- Reducing power ability activity
- Nitric oxide radical inhibition activity
- Phosphomolybdate method (total antioxidant activity).

Phase VI

Pharmacological studies

- *In vivo* screening for the evaluation of hepatoprotective activity.
- Biochemical estimations of parameters like Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphates (ALP), Total protein (TP) and Total bilirubin (TB)
- Histopathological studies.
- Statistical analysis of the Biochemical parameters.

Plant Profiles
&
Review of Literature

PLANT PROFILES AND REVIEW OF LITERATURE

3.1. *Cuscuta reflexa* Roxb

3.1.1. Plant introduction

Botanical Name	**:**	*Cuscuta reflexa* **Roxb**
Family	**:**	**Convolvulaceae/Cuscutaceae**.

Vernacular names

English	:	Dodder.
Sanskrit	:	Asparsa, Amaravela, Amar-bela Akashavalli.
Hindi	:	Akasbel, Akasbela, Amarabela.
Tamil	:	Sadadari, Kodiyagundal, Akashavalli.
Telugu	:	Sitamapurgonalu, sitammapogunalu, Lanjasavaramu, savarapukada, sitasavaramu.
Malayalam	:	Akasavalli, Mutillattali, Akasagarudakkoti.
Kannada	:	Amaraballi, Akasaballi, Badanike.
Bengal	:	Algusi, Hadialgusilutta.
Marathi	:	Nirmuli, Amarvela, Akashvel.

Distribution

Afghanistan through India, Sri Lanka, China, Siam and Malaysia. Balaghat; less on the (sub) coastal plains.

Habit and habitat

Profusely branched and spreading vine occurring as large yellowish patches often hanging from tress. *Flowers* December onwards. *Fruits* February onwards.

Botanical characters

Flower subsessile, in racemes. *Calyx* lobes 5, thick, 2 x 1.5 mm, thick, subacute. *Corolla* cream, tubular-campanulate; lobes 5, oblong-lanceolate, 8 x 2 mm, united from above the middle, chartaceous, subacute. *Stamens* 5, subsessile; anthers 1.3 mm. Scale 5, near the base of tube. *Ovary* 2 mm; styles 2, stout, 2 mm, divergent, shortly decurrent to ovary; stigmas acute. *Capsule* succulent, 5 mm across[37] (Fig. 5).

Parts used

Whole plant, stem, seeds and fruits.

Botanical Name : ***Cuscuta reflexa*** **Roxb**

Family : **Convolvulaceae/Cuscutaceae**.

Fig. 5 *Cuscuta reflexa* Roxb – whole plant

3.1.2. Ethnomedical information

A decoction of aerial part of akashilota *(Cuscuta reflexa* Roxb) is used in the treatment of Jaundice by traditional groups of upper Assam[39].

The fresh juice extracted by crushing the whole plant of *Cuscuta reflexa* called as Raven nari is given as dose of two teaspoons twice daily to cure jaundice by the Bodo tribe in Kamrup districts of Assam[40].

Decoction or extract of stem is administered orally against jaundice by the folk people of northern part of Maharashtra. The skin and eyes of a patient suffering from jaundice turn yellow. Urine also becomes yellow. Hence, yellow–coloured stem of this taxon is conceived useful to treat the said disease[41].

Vapour of boiled plants is inhaled 2-3 times a day for 5 days to reduce body swelling and to treat fever by the rural people of Palpa district in Nepal[42].

The entire plant is used to treat common fever and bronchitis[43].

- For treating common fever, entire plant of akashbanwaria (*Cuscuta reflexa*) mixed with stem-bits of “guruch” (*Tinospora cordifolia* Miers) are crushed and squeezed to obtain the juice and taken in the dose of 10 ml three times a day, for 3 days.
- In case of bronchitis, the decoction of 100 g plant is taken three times a day, for 5 days. The paste of the plant applied externally to cure swelling of testicle, headaches and for boils. The plant in combination with other plant drugs is used to treat general swelling of body and for rheumatism, gout, jaundice and tetanus

The latex of *Calotropis procera (*Ait*)* R. Br and whole plant of *C. reflexa* Roxb. (1:3 ratio) are burnt in a clay pot to prepare ash. About 10 g of ash in honey is given thrice a day to cure cold, cough by local inhabitants in Bharatpur district of Rajasthan[44].

The plant paste is applied for tongue ulcers by the tribals of Singbhum district in Bihar[45].

About 50 g powdered plant and one burnt bulb of garlic (*Allium sativa* L.) are mixed with 100 ml mustard oil (*Brassica campestris*). The preparation is used for massage, twice a day, in sun to treat body aches due to injuries in folk medicines of Nepal[46].

The whole plant is softened in hot water and after removing water, a dense paste is prepared and tied on affected parts with the help of a cotton bandage in cases of painful swellings due to accident and sprain by people of Baratpur in Rajasthan[47].

The entire plant is crushed with raisins ‘munnaka’ and half glass juice is taken to remove intestinal worms. It also controls heart beat in

weakness. Ladies apply juice of plant on hair to promote hair growth by the folk people of Solan district in Himachal Pradesh[48].

An aqueous paste of 20 cm long stem with little lime given as one teaspoonful once in the morning for four to five days to induce abortion at early stage of pregnancy (i.e.) less than four months by some tribal and rural communities of Pach Marchi forest in Madhya Pradesh[49].

The juice of whole plant given to women once only after menses claims to make the women barren forever as a birth control practice among rural and tribal women of Chatarpur district of Madhya Pradesh[50].

The plant and black peppers (2:1) are crushed and the aqueous extract is taken orally twice a day for spermatorrhoea. It is continued for a week. This practice is among the tribal population of Phulbani district in Orissa[51].

The powder of whole plant is given orally in pruritus by the Bondo tribe of Malkangiri district, in Orissia[52].

A decoction and poultice made from whole plant are used to cure foot and mouth disease in cattle by the people of Sun Sari district of eastern Nepal[53].

The stem extract of *Cuscuta reflexa* Roxb were used to cure epilepsy by Bhil tribe of Bibdod in Madhya Pradesh[54].

A stem decoction with honey is taken every morning for 7 days to cure epilepsy by the tribes called Kandhas of Kandhamal district of Orissa[55].

Cuscuta reflexa Roxb is used in spermatorrhoea, epilepsy, diarrhoea and also the whole plant is boiled in tin oil and is applied on hair for darkness and shine by the tribes of Kalahandi district in Orissa[56].

The plant juice causes depression with nausea, vomiting and abortion. Tribals mix the plant with fodder to kill enemy's live stock. The plants are found in Aravelli hills of Rajasthan[57].

3.1.3. Chemical review

Uddin SJ, et al., reported two novel tetrahydrofuran derivatives, named swarnalin and cis-swarnalin along with a known coumarin, 5,6,7-trim ethoxycoumarin, from the methanol extract of the aerial parts of the plant[58].

Tripati VJ, et al., reported a new compound, reflexin, 5-hydroxy-8-methoxy-6- (2-3- epoxy-3-methylbutyl)-flavonone[59].

Loffler C, et al., reported that soluble phenolic constituents mainly caffeic acid, was isolated from the ethyl acetate fraction of the aqueous portion in the methanol extract. The eight major phenolic compounds were identified as 3.4-dicaffeoylquinic acid; 3,5-dicaffeoylquinic acid; 3-caffeoylquinic acid (Chlorogenic acid), quercetin and its O-glycosides quercetin 3-O-β-galactoside and quercetin 3-O-β-glucoside; Kaempferol 3-O-β-galactoside and kaempferol 3-O-β-glucoside[60].

Loffler C, et al., reported that phenolic constituents can be used as taxonomic markers in the genus *Cuscuta* were nine species of the genus *Cuscuta* yielded same characteristic patterns of soluble phenolic constituents. The phenolic compounds identified include the caffeic acid depsides, chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoyl quinic acid and the flavanoids like quercertin, quercertin-3-O-galactoside, quercertin-3-O-glucoside, kaempferol-3-O-galactoside and kaempferol-3-O-glucoside[61].

Tewari KK, et al., reported acid soluble and acid insoluble inorganic poly phosphates[62].

Bhavsar GC, et al., reported cardiac glycosides in ethanol extract of *Cuscuta reflexa* Roxb grown on *Neruim indicum* Linn[63].

Bhavsar GC, et al., reported accumulation of vasicine and vasicinone in *Cuscuta reflexa* Roxb grown on *Adhatoda vasica* Ness[64].

Anis E, et al., reported two new compounds, 7'-(3',4'-dihydroxphenyl)–N-[(4-methoxyphenylethyl) Propenamide and 7'-(4'-hydroxy,3'-methoxyphenyl)–N-[(4-butylphenyl) ethyl] propenamide along with five known compounds, 6,7-dimethoxy-2H-1-benzopyran-2-one, 3-(3-4-dihydroxyphenyl)-2-propen-1-ethanoate, 6,7,8-trimethoxy-2H-1-benzopyran-2-one, 3-(4-O-β-D-glucopyranoside-3,5-dimethoxyphenyl)-2-propen-1-ol, 2-3-(3-hydroxyl-4-methoxyphenyl)-3,5-dihydroxy-7-O-β-D-glucopyranoside-4H-1-benzopyrane-4-one isolated for the first time in *Cuscuta reflexa*[65].

Chatterjee U, et al., reported the isolated and purified carboxymethyl cellulose from *Cuscuta reflexa* and studied its physico-chemical properties[66].

Anis E, et al., reported sterols and sterol glycosides like stigmast-5-en-3-O-beta-D-glucopyranoside tetra acetate along with known compounds stigmast-5-en-3-O-beta-D-glucopyranoside, stigmast-5-en-3-yl-acetate and β-sitosterol were isolated from the stems of *Cuscuta reflexa*[67].

Anis E, et al., reported long chain esters of oleane series, as 3-beta-hydroxyolean-12(13)-ene tridecanoate and 3-beta-hydroxyolean-12(13)–ene-heptadecanoate isolated from the stems of *Cuscuta reflexa*[68].

Mattoo RL, et al., reported the stimulating effect of detergents like Tween 20, Tween 80 and Triton 100 on the alkaline phosphatase activity of *Cuscuta reflexa*[69].

Srivastava S, et al., reported a purified starch phosphorylase enzyme from *Cuscuta reflexa* filaments, which was not like often starch phosphorylase enzyme. This enzyme not been inhibited by aromatic amino acids[70].

Srivastava S, et al., reported two multiple forms of pectin methyl esterase called A and B forms from the filaments of *Cuscuta reflexa* by DEAE Cellulose chromatography[71].

3.1.4. Pharmacological and biological review

Yadav SB, et al., reported antioxidant activity of the flavanoids like apigenin 7-O-glucoside, kaemferol 3-O-α-rhamnoside and myricetin 3-O-α-rhamnoside which were isolated from ethyl acetate fraction of the ethanol extract of the stems of *Cuscuta reflexa*[72].

Yadav SB, et al., reported antioxidant activity of various fractions like Benzene, chloroform, ethyl acetate from the ethanol extract of the stems of *Cuscuta reflexa*[73].

Pal DK, et al., reported antibacterial activity of the various extracts of *Cuscuta reflexa* stem[74].

Mazumder UK, et al., reported increase in the carbonic anhydrase activity in the uterus of mice with the methanol extracts of *Cuscuta reflexa* stem[75].

Gupta M, et al., reported delay in the onset of reproductive maturity (puberty) and ovarian steroidogenesis with methanol extract of *Cuscuta reflexa* stem[76].

Gupta M, et al., reported anti-steroidogenic activity of methanol extract of *Cuscuta reflexa* Roxb stem in mouse ovary[77].

Pandit S, et al., reported the effect of *Cuscuta reflexa* Roxb on androgen-induced alopecia[78].

Babu BH, et al., reported cytotoxic and tumour reducing activity of the ethanol extract of the stems of *Cuscuta reflexa*[79].

Poudel RC, et al., reported anti-hepatitis herbal formulations of traditional healers in mild hills of central Nepal which include plants like *Cuscuta reflexa*[80].

3.2. *Cassytha filiformis* Linn

3.2.1. Plant introduction

Botanical Name	**:**	***Cassytha filiformis*** **Linn**
Family	**:**	Lauraceae

Vernacular names

English	:	Dodder-Laurel, Love-vine
Sanskrit	:	Akashavalli / Akasha-Vadi
Hindi	:	Amarbeli / Amor-beli
Tamil	:	Erumaikkottan, Kottan, Kodikkottan, Indiravalli.
Telugu	:	Nulutega, Pachitige, Antaravallitige.
Malayalam	:	Akashavalli, Akash-bullee, Moodillathali.
Kannada	:	Akashaballi / Akasaballi, Beluballi.
Bengal	:	Akasbel
Marathi	:	Amarvela

Distribution

Sri Lanka, India, Bangladesh to Malacca, Arabia, Mascarene Isl., tropical E. Asia & Isls., Australia, Polynesia, tropical Africa, America.

Habit and habitat

Plain from the coast, in scrub jungles, common; less on hills to 500 (800) m. Parasitic twiner seen in dense clustered yellowish masses on thickets, *flowers* yellowish, almost with two peaks during September-October and January-March. *Fruits* green, through the year.

Botanical characters

The plant is a leafless *twiner. Spikes* terminal or axillary; peduncle 2-4.5 cm; bracteoles 3, ovate, 1.2 mm, ciliate. *Flowers* (sub) sessile, in spikes, 3-merous, *bisexual*, 3 mm across. *Tepals* 6, free, unequal, 3+3, truncate, obtuse; outer lobes ovate-orbicular, 1.2 mm, ciliate; inner lobes obovate, 2.5 mm. Fertile *stamens* 9; filaments 1.5 mm; glands sessile; anthers 2-celled, 1.5 mm; staminodes 3, to 1 mm. *Ovary* 1.5 mm; style 0.5 mm; stigma capitate. *Drupe* globose, enclosed within inflated perianth, crowned by lobes; seed 1, globose, conform to *fruit*: testa thin[37] (Fig. 6).

Botanical Name : ***Cassytha filiformis* Linn**

Family : Lauraceae

Fig. 6 ***Cassytha filiformis*** **Linn – whole plant**

3.2.2. Ethnomedical information

"Paachi-teega (*Cassytha filiformis*-Lauraceae) is pounded in human urine and banded over the affected part in the case of bone fracture (Emuka viruguta) by the folk people of Nalgonda district in Andhra Pradesh for some common veterinary diseases[81].

Ethno medico botanical studies in Guntur district of Andhra Pradesh reveals that paachi-teega (*Cassytha filiformis*) whole plant (50 g) crushed with turmeric (50 g) is applied externally for body pains[82].

3.2.3. Chemical review

Wu YC, et al., reported two novel aporphinoid alkaloids cathafiline and cathaformine possessing a N-methoxy carbonyl group which was isolated from the chloroform fraction of methanol extract, along with six known alkaloids actinodaphnine, cassythine, isoboldine, cassameridine, cassamedine and lysicamine[83].

Chang FR, et al., reported three new compounds, an aporphine alkaloid cassyformine, an oxoaporphine alkaloids filiformine and a lignan(+)–diasyring aresinol along with 14 known compounds like (+) cassyformine, (+)-N-methylactinodaphnine, predicentrine and ocoteine which are aporphine type; one oxoaporphines thalicminine; two proaporphines stepharine and pronuciferine; one morphinandienone O-methyl flavinantive; two lignans (+)-yangambin (+)- syringaresinol; two aromatic aldehydes, Vanillin and isovanillin one glycerol ester; and two phytosterols as well as a mixture of β-sitosterol and stigmasterol and the mixture of β-sitosterol-D-glucoside and stigmaterol-D-glucoside[84].

3.2.4. Biological review

Tsai TH, et al., reported two new aporphine alkaloids, isofiliformine and cassythic acid along with other known compounds like cassythine, neolitsine and dicentrine were isolated from the ethanol extract of the whole plant of *Cassytha filiformis* had potent vaso relaxing effects on precontracted rat aortic preparation compounds like 1,1,2-methylene dioxy–3,10,11-(-)-salutaridine, isohamnetin-3–O-β-glucoside and isohamnetin 3-O-rutinoside exerted moderate vessel relaxing activities[85].

Babayi HM, et al., reported an oral administration of aqueous extract of whole plant of *Cassytha filiformis* showed certain effects on haematograms and plasma biochemical parameters in rats[86].

Chang CW, et al., reported ocoteine, isolated from *Cassytha filiformis*, was found to be an α_1-adrenoreceptor blocking agent in rat thoracic aorta as revealed by its competitive antagonism of phenylephrine induced vaso constriction[87].

Khan MR, et al., reported antibacterial activity of acidic, basic and neutral fraction of petroleum ether and chloroform extracts of some Tanzanian medicinal plants like *Cassytha filiformis* was reported[88].

Akar PA, et al., reported the aqueous extracts of a Nigerian medicinal plant species like *Cassytha filiformis* showed an abortifacient activity[89].

Hoet S, et al., reported alkaloids from *Cassytha filiformis* and its related aporphines showed antitrypansomal activity and cytotoxicity[90].

Materials & Methods

MATERIALS AND METHODS

4.1. Materials

4.1.1. Plant materials

The following plant materials were selected for the present study.

1. ***Cuscuta reflexa*** **Roxb** (Convolvulaceae / Cuscutaceae) – whole plant
2. ***Cassytha filiformis*** **Linn** (Lauraceae) – whole plant

4.1. 2. Instruments

Auto-analyser: Merck Microlab 200 manufactured by M/s Vital scientific N. V., the Netherlands, was used to estimate various biochemical parameters viz. SGOT, SGPT, alkaline phosphatase (ALP), total protein and albumin.

Centrifuge: Remi centrifuge, R-8c Laboratory centrifuge and Elvenjan homogenizer, Remi motors Ltd, Mumbai, India were used to separate serum from blood.

^{13}C NMR: DAM X – 400 m/z – Bruker (India) Pvt Ltd., Hyderabad, India, was used for ^{13}C NMR studies.

Elisa Reader: BioRad Laboratories Inc, California, USA, model 550 was used in *in vitro* antioxidant assays.

FTIR: Perkin Elmer, 1600 Series FTIR, Perkin Elmer (India) Pvt Ltd., Nand champers, L.B.S Marg, Thane, India.

Grinding Mill: Junior Grindwell, Chowthry, J.U.C, Mumbai, India was used for powdering of plant materials.

HPTLC: CAMAG Linomat lV, No-022.78.6, twin trough chamber No-022-5155 and TLC Scanner-3, No.027-6480 Switzerland, were used for HPTLC studies.

Incinerator: Ambassador, model-2265, Matri, Pondicherry, India, was used for determination of ash values of plant materials.

Mass spectroscopy: MALDI, Altraflex, TOF, Bruker, Bruker Daltonics, Germany, was used for mass spectroscopy studies.

Melting point apparatus: Lab India melting point apparatus, India was used for determination of melting point.

Proton NMR: DDR X-500m/z-Bruker (India) Pvt. Ltd., Hyderabad, India, was used for proton NMR studies.

Rotary Microtome: Leica RM 2135, Leica Microsystem GmbH, Germany was used for sectioning of the paraffin embedded specimens of plants and animal tissues.

Rotary evaporator: Superfit, India rotary evaporator was used for concentration of plant extracts.

Spectrophotometer: Shimadzu 160-A UV-VIS Spectrophotometer manufactured by Shimadzu, Japan was used to estimate total phenol content, flavanoids content and biochemical estimations.

4.1.3. Source of chemicals.

Pyrocatechol was purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai. Ascorbic acid and Folin-Ciocalteu reagent was obtained from SD Fine Chemicals Pvt. Ltd, Mumbai. All other drugs and chemicals used in the study were obtained commercially and were of analytical grade. 2,2-dipheny-1-picryl hydrazyl (DPPH), rutin and quercetin were procured from Sigma Chemical Company, California, USA. Aluminium backed HPTLC plates coated with 0.2 mm layers of silica gel 60 F_{254} plates, Ecoline diagnostic kits were from E-merck Ltd., Mumbai, India.

4.2. Methods

4.2.1. Collection and authentication of plant materials

The plant materials, ***Cuscuta reflexa*** **Roxb** (Convolvulaceae/ Cuscutaceae) – whole plant, ***Cassytha filiformis*** **Linn** (Lauraceae) – whole plant, were collected from different parts of Chennai, Tamil Nadu, India. The plants were authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Centre, Chennai, Tamil Nadu, India. The voucher specimens are preserved in our laboratory for further reference. After authentication, the plant materials were dried under shade. After optimum drying the materials were coarsely powdered separately and stored in well closed containers till further use.

4.2.2.a. Extraction of plant material

The term extraction involves separation of therapeutically required portions of the plant material from the inactive components by using selective solvents (menstrum). The extract thus obtained may be ready to use as such in the form of tinctures or fluid extracts or it may be processed to be incorporated in dosage forms like tablets or capsules or may be fractionated to isolate individual chemical compounds which are used as modern drugs.

General methods of extraction

1. Maceration
2. Infusion
3. Digestion
4. Hot extraction (Decoction)
5. Cold extraction (Percolation)
6. Hot continuous extraction (Soxhlet)

Parameters essential for the selection and standardization of the extraction methods

- Authentication of the plant material and removal of foreign matter.
- Use of right plant part, time and place of collection
- Proper drying and powdering of plant material
- Nature of phytochemicals
  i. Solvent used for extraction depends upon the nature of the active constituents. If the activity lies in the non polar constituents, non polar solvent may be used.
  ii. If the constituents are thermolabile, then cold extraction methods are preferred. For thermostable constituents, Soxhlet extraction can be used.
  iii. In case of hot extraction, higher than required temperature should be avoided.
- Time and number of extractions
- Proper quality of the solvent used for extraction
- Proper concentration and drying of extract to ensure the safety and stability of active constituents
- Analytical parameters of the final extract e.g. HPTLC fingerprinting and analytical limits should be set to monitor the quality of different batches of the extracts.

4.2.2.b. Preparation of the extracts

The plant material which was powdered and stored was used for extraction. A weighed quantity of each of the plant powdered material was extracted by cold maceration with ethanol and 50% methanol for 72 hrs with intermediate heating at 40°C one time in a day. The extract was filtered using Whatmann filter paper and then the filterate was concentrated under reduced pressure and controlled temperature (40-50°C). The marc was dried and weighted.

This marc was again extracted with water by cold maceration for 72 hrs to yield aqueous extracts (Table 3).

Table 3

Yield and nature of the extracts

S. No.	Plant material	Quantity used for extraction in grams	Type of the extract	Nature of the extract	Yield	
					G	%
1	***Cuscuta reflexa* Roxb**	1000	50% methanol extract	Dark brown solid	132.9	13.3
2	***Cuscuta reflexa* Roxb**	850	Aqueous extract	Brown solid	93.27	10.9
3	***Cassytha filiformis* Linn**	1000	Ethanol extract	Greenish brown solid	58.9	5.9
4	***Cassytha filiformis* Linn**	1000	50% methanol extract	Greenish brown solid	29.1	2.9
5	***Cassytha filiformis* Linn**	920	Aqueous extract	Light brown powder	21.2	2.3

4. 2. 3. Pharmacognostical studies

Most of the raw materials used by the herbal industry are procured from wild sources and this result in inconsistency in quality, adulteration and substitution. Hence, pharmacognostical evaluation of crude drugs is most

important in herbal drug industry. Pharmacognostical studies include authentication, determination of foreign matter, organoleptic evaluation, macroscopic and microscopical examination, volatile oil content determination and evaluation of ash values and extractive values.

Crude drugs when supplied in intact from can be identified by morphological characteristics. The same can be investigated for histological characteristics to confirm the identity of the supplied drugs. Microscopical techniques provide detailed information about the crude drugs by virtue of their two main analytical uses. Firstly, their property of magnification permits the fine structures of minute objects to be visualized and thereby confirm the structural details of the plant drugs under evaluation. Secondly, these techniques can be used in the determination of the optical as well as micro-chemical properties of the crude drug specimens under study. The powdered crude drugs can be identified based on the form, the presence or absence of different cell types based on their cytomorphological characters e.g. parenchyma, collenchyma, fibres, stone cells, trichomes, secretary cells, epidermal cells etc. The same may some times be achieved by evaluating the cell inclusion characteristics for some unorganized crude drugs like starch grains, aleurone grains, gums, mucilage, calcium oxalate crystals etc. Ash values are helpful in determining the quality and purity of the crude drugs, especially in powdered form. The objective of ashing vegetable drugs is to remove all traces of organic matter, which may otherwise interfere in an analytical determination[91]. Extractive value determines the amount of active constituents in a given amount of medicinal plant material when extracted with solvents. Further, these values indicate the nature of the constituents present in a crude drug.

4.2.3.a. Plant anatomical studies

Preparation of specimens

The fresh, healthy samples were cut and removed from the plant and fixed in FAA (formalin 5 ml + acetic acid 5 ml + 70% ethyl alcohol 90 ml). After 24 hrs of fixing, the specimens were dehydrated with gradual series of TBA (tertiary butyl alcohol) as per the schedule given by Sass[92]. Infiltration of the specimens was carried out by gradual addition of paraffin wax (m.p. 58-60°C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

Sectioning

The paraffin embedded specimens were sectioned with the help of Rotary **Microtome**. The thickness of the sections was 10-12 µm. De-waxing of the sections was done by customary procedure given by Johansen[93]. The sections were stained with **Toluidine blue** method of O'Brien[94]. Since **Toluidine blue** is a polychromatic stain, the staining results were remarkably good; and some cytochemical reactions were also obtained. The dye rendered pink colour to the **cellulose** mass, blue to the **lignified cells**, dark green to **suberin**, violet to the **mucilage**, blue to the **protein** bodies etc. Wherever necessary, sections were also stained with **safranin** and **fast-green** and potassium iodide (for starch).

For studying the stomatal morphology, venation pattern and trichrome distribution, paradermal sections (sections taken parallel to the surface of leaf) as well as clearing of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey's maceration fluid[92] were prepared. Glycerin mounted temporary preparations were made for macerated / cleared materials. Powdered materials of different parts were cleared with sodium hydroxide and mounted in glycerine medium after staining. Different cell component were studied and measured.

Photomicrographs

Microscopic descriptions of tissues are supplemented with micrographs. Photographs of different magnifications were taken with **Nikon labphoto 2** microscopic unit. For normal observations **bright field** was used. For the study of **crystals**, **starch grains** and **lignified cells**, **polarized light** was employed. Since these structures have **birefringent property**, under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale-bars. Descriptive terms of the anatomical features are given as in the standard anatomy books[95].

4.2.3.b. Determination of physicochemical constants

Ash values

Ash values are helpful in determining the quality and purity of crude drugs in powdered form. The values vary within fairly wide limits and are therefore an important parameter, for the purpose of evaluation of crude drugs. The total ash usually consists of inorganic radicals like carbonates, phosphates, silicates and silica of sodium, potassium, magnesium and calcium. Sulphated ash involves the treatment of sample with dilute sulphuric acid before ignition. In this, all oxides and carbonates are converted to sulphates and the ignition is carried out at a higher temperature. Sometimes inorganic variables like calcium oxalate, silica and carbonate contents of crude drugs affect total ash values. Such variables are then removed by treating with acid (as they are soluble in HCI) and then, acid insoluble ash value is determined. For the determination of various ash values viz. total ash, acid insoluble ash and water-soluble ash all the powdered plant materials were passed through sieve no 40 and used[96].

Determination of total ash

About 3 g of the powdered material was accurately weighed in a silica crucible, which was previously ignited and weighed. The powdered drug was

spread as a fine even layer at the bottom of the crucible. The crucible was incinerated at a temperature not exceeding 450°C until free from carbon.

The crucible was cooled and weighed and the procedure was repeated to get the constant weight[96].

Determination of acid insoluble ash

The ash obtained as described in the total ash was boiled with 25 ml of 2M hydrochloric acid for five min. The insoluble ash was collected on an ash less filter paper and washed with hot water. The residue was transferred into pre-weighed silica crucible, ignited, cooled and weighed and the procedure was repeated to get the constant weight [96].

Determination of water-soluble ash

The ash obtained as described in the total ash was boiled in 25 ml of chloroform water for five min. The insoluble matter was collected in a Gooch crucible or ash less filter paper and washed with hot water. The residue was transferred into pre-weighed silica crucible, ignited for 15 min at a temperature not exceeding 450°C, cooled and weighed and the procedure was repeated to get the constant weight[96]. The weight of the insoluble matter was subtracted from the weight of the total ash. The difference of weight was considered as the water-soluble ash.

Determination of sulphated ash

A silica crucible was heated to redness for about 10 min, allowed to cool in a desiccator and weighed. About 1 g of powdered sample was accurately weighted and taken in the crucible. The crucible was ignited gently first until the sample was thoroughly charred. The crucible was cooled and the residue was moistened with 1 ml of sulphuric acid, heated gently until the while fumes were no longer evolved and ignited at 800°C until all black particles disappeared. The ignition was conducted in a place protected from air currents. The crucible was

allowed to cool, few drops of sulphuric acid was added and again heated. The ignition was carried out as before, allowed to cool and weighed[96].

Extractive values

The amount of extractive sample yield to a given solvent is often an approximate measure of a certain constituents or a group of related constituents the sample contains. In some cases, the amount of sample soluble in a given solvent is an index of its purity. The solvent used for extraction should be in a position to dissolve appreciable quantities of the substances desired.

Water-soluble extractive

5 g of coarsely powdered material was mixed with 100 ml of chloroform water and kept in a closed flask for 24 h, shaking frequently during the first 6 h and then allowed to stand for 18 h. Thereafter, it was filtered rapidly, taking precautions against loss of the solvent. 25 ml of the filtrate was evaporated to dryness in a tarred flat-bottomed shallow dish, dried at 105°C and weighed[96].

Ethanol soluble extractive

5 g of coarsely powdered material was mixed with 100 ml of 95% ethyl alcohol in a closed flask and kept for 24h, shaking frequently during the first 6 h and then allowed to stand for 18 h. Thereafter, it was filtered rapidly taking precautions against loss of the solvent. 25 ml of the filtrate was evaporated to dryness in a tarred flat-bottomed shallow dish, dried at 105°C and weighed[96].

4.2.3.c. Fluorescence analysis of the plant powders with various chemical reagents.

The organic molecules absorb light usually over a specific range of wavelength, and many of them re-emit such radiations. This phenomenon is called as luminescence. When the re-emission of the absorbed light lasts only whilst the substance is receiving the exciting rays, the phenomenon is known as fluorescence. A very small quantity of powdered drug was kept in a watch glass in an accumulated form. Then 2-3 drops of respective reagent was added and the

fluorescence character of the plant powders was studied both in daylight and UV light as such and after treatment with reagent like sodium hydroxide, picric acid, acetic acid, hydrochloric acid, nitric acid, iodine, ferric chloride etc. Similarly the fluorescence analysis of the plant extracts was observed under visible and UV lights[97,98].

4.2.3.d. Phytochemical studies

The therapeutic potentials of plant and animal origin are being used from the ancient times in the form of crude drugs or galenicals prepared from them without the isolation of the pure compounds. The pharmacological action of crude drug is determined by the nature of its constituents. To obtain these pharmacological effects, the plant materials are used as such in their crude form or they were extracted with suitable solvents to take out the desired components and the resulting principles employed as therapeutic agents.

The development of phytochemistry took its turn, when modern isolation methods for herbal ingredients found their way. Percolation process was used for the extraction of crude drugs from 1817 onwards.

Qualitative phytochemical analysis

General screening of the raw plant powder, alcoholic and aqueous extracts of the plant material is carried out for qualitative determination of the groups of organic compounds present in them[99,100].

Test for alkaloids

(a) **Dragendorff's test:** Dissolve a few mg of alcoholic or aqueous extract of the drug in 5 ml of distilled water, add 2 M hydrochloric acid until an acid reaction occurs, then add 1 ml of Dragendorff's reagent, an orange or orange-red ppt. is produced immediately.

(b) **Hager's test:** To 1 ml of alcoholic extract of the drug taken in a test tube, add a few drops of Hager's reagent. Formation of yellow ppt. confirms the presence of alkaloids.

(c) **Wagner's test:** Acidify 1 ml of alcoholic extract of the drug with 1.5% v/v of hydrochloric acid and add a few drops of Wagner's reagent. A yellow or brown ppt is formed.

(d) **Mayer's test:** Add a few drops of Mayer's reagent to 1 ml of acidic aqueous extract of the drug. White or pale yellow ppt is formed.

Test for carbohydrates

(a) **Benedict's test:** To 0.5 ml of aqueous extract of the drug add 5 ml of Benedict's solution and boil for 5 mins. Formation of a coloured ppt is due to the presence of carbohydrates.

(b) **Fehling's test:** To 2 ml of aqueous extract of the drug add 1 ml of a mixture of equal parts of Fehling's solution 'A' and Fehling's solution 'B' and boil the contents of the test tube for few mins. A red or brick red ppt is formed.

(c) **Molisch's test:** In a test tube containing 2 ml of aqueous extract of the drug add 2 drops of a freshly prepared 20% alcoholic solution of naphthol and mix, pour 2 ml concentrated sulphuric acid through the sides so as to from a layer below the mixture. Carbohydrates, if present, produce a red-violet ring which disappears on the addition of an excess of alkali solution.

(d) **Barfoed's test:** To 2 ml of extract solution, add 2 ml of Barfoed's reagent. Mix well. Heat for 1-2 minute in boiling water bath and cool. Formation of a red precipitate indicates the presence of carbohydrates.

(e) **Anthrone test:** To 2 ml of anthrone test solution, add 0.5 ml of aq extract of the drug. A green or blue colour indicates the presence of carbohydrates.

Test for glycosides

(a) **Legal test:** Dissolve the extract in pyridine and add sodium nitroprusside solution and then make it alkaline by adding 10% solution of sodium

hydroxide. The formation of pink red colour shows the presence of glycosides.

(b) **Baljet test:** To 1 ml of the test extract add 1 ml of sodium picrate solution, an yellow to orange colour reveals the presence of glycosides.

(c) **Borntrager's test:** Add a few ml of dilute sulphuric acid to 1ml of the extract solution. Boil, filter and extract the filtrate with chloroform. The chloroform layer is treated with 1ml of ammonia. The formation of red colour shows the presence of anthraquinone glycosides.

(d) **Keller Kiliani test:** Dissolve the extract in acetic acid containing traces of ferric chloride and transfer to a test tube containing sulphuric acid. At the junction, formation of a reddish brown colour, which gradually becomes blue, confirms the presence of glycosides.

Test for saponins

(a) In a test tube containing about 5 ml of an aqueous extract of the drug add drop of sodium bicarbonate solution, shake the mixture vigorously and leave for 3 mins. Honey comb like froth is formed.

(b) About 1 ml of extract is diluted separately with distilled water to 20 ml, and shaken in a graduated cylinder for 15 minutes, a 1cm layer of foam indicates the presence of saponins.

Test for protein and amino acids

(a) **Biuret's test:** To 1 ml of hot aqueous extract of the drug add 5-8 drops of 10% w/v sodium hydroxide solution followed by 1 or 2 drops of 3% w/v copper sulphate solution. A red or violet colour is obtained.

(b) **Millon's test:** Dissolve a small quantity of aqueous extract of the drug in 1 ml of distilled water and add 5-6 drops of Millon's reagent. A white ppt is formed which turns red on heating.

(c) **Ninhydrin test:** Add two drops of ninhydrin solution (10mg of ninhydrin in 200ml of acetone) to 2 ml of the aqueous filterate. A characteristic purple colour indicates the presence of amino acids.

Test for flavanoids

Shinoda test / Magnesium and hydrochloric acid reduction:

In a test tube containing 0.5 ml of alcoholic extract of the drug, add 5-10 drops of dil hydrochloric acid followed by a small piece of magnesium. In the presence of flavanoids a pink, reddish pink or brown colour is produced.

Test for triterpenoids

(a) **Libermann-Burchard's test:** Add 2 ml of acetic anhydride solution to 1 ml of petroleum ether extract of the drug in chloroform followed by 1 ml of concentrated sulphuric acid. A violet colour ring is formed indicating the presence of triterpenoids.

(b) Dissolve two or three granules of tin metal in 2 ml thionyl chloride solution. Then, add 1 ml of the extract into the test tube. The formation of a pink colour indicates the presence of triterpenoids.

Test for steroids

(a) **Libermann-Burchard's test:** Add 2 ml of acetic anhydride solution to 1 ml of petroleum ether extract of the drug in chloroform followed by 1 ml of concentrated sulphuric acid. A greenish colour is developed which turns to blue.

(b) **Salkowski reaction:** Add 1 ml of concentrated sulphuric acid to 2 ml of chloroform extract of the drug carefully, from the side of the test tube. A red colour is produced in the chloroform layer.

(c) **Libermann's reaction:** Mix 3 ml extract with 3ml acetic anhydride, heat and cool, add few drops of concentrated sulphuric acid. Appearance of blue colour indicates the presence of steroids.

Test for tannins and phenolics

(a) **Ferric chloride test:** To 1-2 ml of extract of the drug add a few drops of 5% $FeCl_3$ solution. A green colour indicates the presence of gallotannins while a brown colour indicates the presence of tannins.

(b) To the extract add potassium dichromate solution, formation of a precipitate shows the presence of tannins and phenolics.

(c) **Gelatin test:** The extract (50mg) is dissolved in 5 ml of distilled water and 2 ml of 1% solution of gelatin containing 10% sodium chloride is added to it. White precipitate indicates the presence of phenolic compounds.

(d) **Lead acetate test:** The extract (50mg) is dissolved in distilled water and to this, 3 ml of 10% lead acetate solution is added. A bulky white precipitate indicates the presence of phenolic compounds.

(e) **Alkaline reagent test:** An aqueous solution of the extract is treated with 10% ammonium hydroxide solution. Yellow fluorescence indicates the presence of flavanoids.

Test for resins

Dissolve the extract in acetone and pour the solution into distilled water. Turbidity indicates the presence of resins.

Test for starch

Dissolve 0.015g of Iodine and 0.075g of Potassium iodide in 5 ml of distilled water and add 2–3 ml of an aqueous extract. A blue colour is produced.

Test for gums and mucilages

(a) The extract (100 mg) is dissolved in 10 ml of distilled water and to this add 25 ml of absolute alcohol with constant stirring. White or cloudy precipitate indicates the presence of gums and mucilages.

(b) Hydrolyse the test solution using dilute HCl. Perform Fehling's or Benedicts test. Red colour is developed.

(c) Powdered drug material shows red colour with ruthenium red.

(d) Powdered drug swells in water

Tests for fixed oils

(a) **Spot test:** Press a small quantity of extract between two filer papers. Oil stains on paper indicate the presence of fixed oils.

(b) **Saponification test:** To 1 ml of the extract add few drops of 0.5 N alcoholic potassium hydroxide along with drop of phenolphthalein. Heat the mixture on a water bath for 1-2 hrs. The formation of soap or partial neutralization indicates the presence of fixed oils.

4.2.3.e. Estimation of total phenolic content

Total soluble phenolics of the extract were determined with Folin-Ciocalteu reagent using pyrocatechol as the standard[101]. This test is based on the oxidation of phenolic groups with phosphomolybdic and phosphotungstic acids. After oxidation a green-blue complex is formed and its absorbance is measured at 765 nm.

Chemicals and reagents used

1. Folin-Ciocalteu Reagent: Folin-Ciocalteu reagent was diluted (1:10) with distilled water and used.
2. Sodium carbonate: 202.5 g of sodium carbonate was dissolved in 1ml of distilled water and used (0.7 M)

Procedure

An aliquot of 0.1 ml suspension of 1 mg of the extracts in water was totally transferred to a 100 ml Erlenmeyer flask and the final volume was adjusted to 46 ml by the addition of distilled water. Folin-Ciocalteu reagent (1 ml) was added to this mixture, followed by 3 ml of 2% sodium carbonate 3 min later. Subsequently, the mixture was shaken for 2 h at room temperature and the absorbance was measured at 760 nm. The concentration of total phenolic compounds in the extracts was determined as µg pyrocatechol equivalent by using the standard pyrocatechol graph.

4.2.3.f. Estimation of flavanoid content

Total soluble flavanoid content of the extracts was determined with aluminium nitrate using quercetin as the standard[101].

Chemicals and reagents used

1. Aluminium chloride - 10%

   10 gms of $AlCl_3$ is dissolved in 100 ml of distilled water, filtered and used.

2. Potassium acetate – 1 M

   98.1 g of Potassium acetate was dissolved in 1 litre of distilled water and used.

3. Methanol – Distilled

Procedure

One mg of the extract was added to 1ml of 80 % ethanol. An aliquot of 0.5 ml was added to test tubes containing 0.1 ml of 10 % aluminium nitrate, 0.1 ml of 1 M potassium acetate and 4.3 ml of 80 % ethanol. The absorbance of the supernatant was measured at 415 nm after incubation at room temperature for 40 min. The total flavanoid content in the extracts was determined as µg quercetin equivalent by using the standard quercetin graph.

4.2.4.a. Chromatography analysis

Chromatography is a physical method of separation in which the components to be separated are distributed between the two phases; one of these is a stationary phase bed and the other is a mobile phase which percolates through this bed. It occurs as a result of repeated sorption/desorption during the movement of the sample components along the stationary bed, and the separation is due to differences in distribution constants of the individual sample components. The stationary phase includes solid, liquid coated on a solid support. The mobile phase includes liquid and gas.

Based on the stationary and mobile phase, the chromatographic technique can be of the following types:

1. **Gas chromatography**: The mobile phase is a inert gas and the stationary phase is either an adsorbent or a liquid distributed over the surface of a porous, inert support.
2. **Liquid chromatography**: the mobile phase is a liquid of low viscosity that is caused to flow through a bed of sorbent. The sorbent may be immiscible liquid coated on to a porous support, or an inert sorbent of controlled pore size.
3. **Thin Layer Chromatography (TLC)**: a liquid mobile phase moves through a layer of sorbent by the action of capillary forces. TLC is a open bed technique as pressure is not required for the movement of the mobile phase.

GC and LC are closed bed techniques as pressure gradient is used in the movement of the mobile phase through the stationary phase.

The information obtained by the chromatographic experiment is called the chromatogram, a record of the concentration or the mass profile of the sample components as a function of the movement of the mobile phase. Information that can be extracted from a chromatogram include (a) an indication of the sample complexity or the number of components present based on the number of peaks. (b) qualitative identification of the samples based on the accurate measurement of the peak positions, (c) quantitative assessment of the relative concentration or the amount of substance present based on the peak size.

The common chromatographic techniques used are Column chromatography, Paper chromatography, Thin layer chromatography, Gas chromatography and High performance liquid chromatography.

4.2.4.b. Column chromatography

The principle underlying the separation of the compounds is their adsorption at the solid-liquid interface. For successful separation the compounds of the mixture should show different degrees of affinity for the solid support (or

adsorbent) and the interaction between adsorbent and the component must be reversible. As the adsorbent is washed with the fresh solvent, the various components move down the column and arrange themselves in the order of affinity to the adsorbent. Those with the least affinity move down the column at a faster rate than those with greater affinity.

Column chromatography of aqueous extract of *Cuscuta reflexa*

The aqueous extract of *Cuscuta reflexa* (8 g) was subjected to chromatography over silica gel 60-120 mesh (130 g) of column length 100 cm diameter of 3 cm. Elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 5 ml portions and monitored on TLC (silica gel G as adsorbent with suitable solvent system). The fractions that showed similar spots were mixed and the chromatographic details are given in Table 4.

Table 4

Column chromatography of aqueous extract of *Cuscuta reflex*

No. of Fraction	Eluent	Residue on Evaporation
1-20	n-Hexane - 100%	No residue
21-36	n-Hexane : petroleum ether (75:25)	No residue
37-54	n-Hexane : petroleum ether (50:50)	No residue
55-70	n-Hexane : petroleum ether (25:75)	White residue
71-92	Petroleum ether - 100%	No residue
93-118	Petroleum ether : Chloroform (90:10)	Colourless residue
119-135	Petroleum ether : Chloroform (80:20)	White residue
136-153	Petroleum ether : Chloroform (70:30)	No residue
154-161	Petroleum ether : Chloroform (60:40)	Colourless residue
162-183	Petroleum ether : Chloroform (50:50)	Brownish yellow residue
184-198	Petroleum ether : Chloroform (40:60)	No residue
199-210	Petroleum ether : Chloroform (30:70)	Cream residue
211-220	Petroleum ether : Chloroform (20:80)	Dark yellow residue
221-231	Petroleum ether : Chloroform (10:90)	Creamish white residue
232-245	Chloroform - 100%	No residue
246-259	Chloroform : Ethyl acetate (90:10)	No residue
260-274	Chloroform : Ethyl acetate (80:20)	Yellow residue

275-292	Chloroform : Ethyl acetate (70:30)	Yellowish cream residue
293-300	Chloroform : Ethyl acetate (60:40)	Yellow residue
301-325	Chloroform : Ethyl acetate (50:50)	Colourless residue
326-353	Chloroform : Ethyl acetate (40:60)	Green residue
354-387	Chloroform : Ethyl acetate (30:70)	Creamish yellow residue
388-399	Chloroform : Ethyl acetate (20:80)	Green residue
400-421	Chloroform : Ethyl acetate (10:90)	Dark green residue
422-439	Ethyl acetate -100%	Creamish green residue
440-457	Ethyl acetate : Methanol (90:10)	Light green residue
458-468	Ethyl acetate : Methanol (80:20)	Light brownish residue
469-480	Ethyl acetate : Methanol (70:30)	Dark green residue
481-501	Ethyl acetate : Methanol (60:40)	Brown Residue
502-535	Ethyl acetate : Methanol (50:50)	No residue
536-560	Ethyl acetate : Methanol (40:60)	No residue
561-579	Ethyl acetate : Methanol (30:70)	Light brown residue
580-599	Ethyl acetate : Methanol (20:80)	Light brown residue
600-699	Ethyl acetate : Methanol (10:90)	No residue
700-730	Methanol -100%	Dark brown residue

The fractions 260-300 yielded a yellowish coloured solid mass and the TLC profile showed two spots. This yellowish solid was designated as fraction-I. The fraction 400-480 yielded dark greenish coloured solid and the TLC profile showed three spots and it was designated as fraction-II.

Separation and purification of fraction-I

A cream coloured precipitate was separated when fraction-I was dissolved in methanol, which was then separated by filtration and the solid residue was treated with acetone. The precipitate was separated by filteration and washed with acetone to recrystallize. A colourless solid weighing 10 mg was obtained. It was designated as compound-I. The isolated compound-I was subjected to TLC physical, chemical and spectroscopic studies for its characterization.

Separation and purification of fraction-II

The fraction was washed with methanol, and the dark green coloured solid turned to light yellow colour and gave a single spot in TLC analysis. The light yellow colour solid 30 mg was subjected to recrystallization with acetone. The solid precipitate was designated as compound-II and was subjected to TLC physical, chemical and spectroscopic studies for its characterization.

Fractionation of the ethanol extract of *Cassytha filiformis*

The ethanol extract (50 g) was suspended in distilled water (100 ml) and was extracted successively in a separating funnel with n-hexane petroleum ether (60-80°C), chloroform, ethyl acetate and Methanol. The fraction was washed with distilled water, dried over anhydrous sodium sulphate and the solvent was removed by distillation under reduced pressure.

Yield of the various fractions

n-Hexane fraction	:	10 gms
Petroleum ether fraction	:	10 gms
Chloroform fraction	:	8 gms
Ethyl acetate fraction	:	9 gms
Methanol fraction	:	5 gms

Ethyl acetate fraction

The dark greenish residue of ethyl acetate fraction (8 g) was dissolved in methanol and was then loaded on a silica gel column (20 g). The column was eluted with 100% ethyl acetate followed by graded mixtures of 1%, 2%, 3%, 5% methanol in ethyl acetate. Elution of the different fraction was monitored by TLC Silica-Gel (Ethyl acetate : Methanol : water; 100 : 16.5 : 13.5) and visualized with UV/NH_3.

The eluate obtained with 100% ethyl acetate and 1% methanol in ethyl acetate showed the presence of a single spot on the TLC. The eluate on concentration deposited a yellowish brown colour compound

(8 mg). The compound was designated as compound-III, which was further purified and recrystallized with acetone. The solid precipitate was subjected to spectroscopic studies for its characterization.

4.2.4.c. Thin layer chromatography analysis

Thin-layer chromatography is a technique in which a solute undergoes distribution between two phases, a stationary phase acting through adsorption and a mobile phase in the form of a liquid. The adsorbent is a relatively thin, uniform layer of dry finely powdered material applied to a glass, plastic or metal sheet or plate. Glass plates are most commonly used. Separation may also be achieved on the basis of partition and adsorption, depending on the particular type of support, its preparation and its use with different solvent.

Identification can be effected by observation of spots of identical R_f value and about equal magnitude obtained, respectively, with an unknown and a reference sample chromatographed on the same plate. A visual comparison of the size and intensity of the spots usually serves for semi-quantitative estimation.

TLC is used for the separation of simple mixtures where speed, low cost, simplicity are required[102,103].

Preparation of plates

30 g of silica gel GF was weighed out and made in to a homogenous suspension with 60 ml of distilled water to form a slurry. The suspension was poured into a TLC applicator, which was adjusted to 0.25 mm thickness on a flat glass plate (20 x 10 cm and 20 x 5 cm). The coated plates were allowed to dry in air, followed by heating at 100–105°C for 1 h, cooled and protected from moisture. The plates were stored in a dry atmosphere. Whenever required the plates were dried in a hot air oven at 100°C for 30 min for activation.

Application

The extracts were dissolved in methanol (0.1-1% w/v). They were sucked within capillary tubes and spotted on the prepared TLC plate 2 cm above its

bottom end. The spots were equally sized as far as possible and had a diameter ranging from 2-5 mm.

Selection of mobile phase

The solvent systems were selected based on the chemical constituents identified in the qualitative phytochemical analysis. The solvent or mobile phase used depends upon various factors mentioned below

a. Nature of the substance to be separated
b. Nature of the stationary phase
c. Mode of chromatography (i.e., normal phase and reversed phase)
d. Separation to be achieved (i.e., analytical or preparative)

Pure solvents or mixture of solvents were used as mobile phase. The selection of solvent system for mobile phase is important in TLC, because the same solvent system can be used in HPTLC.

Several solvent systems were used as mobile phases for separation of various plant constituents from respective plants. The better solvent system, which gave good separation and more number of spots was selected for each extract. The R_f values were noted for each of the selected plant extracts, using different detecting agents viz., daylight, UV light, iodine vapour, with Dragendorff's reagent, Vanillin sulphuric acid and ninhydrin reagents.

4.2.4.d. High performance thin layer chromatography (HPTLC)

HPTLC is an advanced versatile chromatographic technique for quantitative analyses with high sample throughput and is complementary to HPLC/GLC. It provides a chromatographic drug fingerprint. It is therefore suitable for monitoring the identity and purity of drugs. In HPTLC the various steps involved are

a. Application of sample
b. Chromatographic development
c. Detection of spots

d. Quantification

e. Documentation

a. Application of sample

An automatic applicator (Linomat) is used for sample application. A known quantity of sample is dissolved in a known volume of solvent and the sample applied on precoated TLC plate either in the form of a spot or a band. However a band form is preferred because:

- Larger quantities of sample can be handled for application.
- Better separation because of rectangular area in which compounds are present on the plate
- Response of densitometry is better due to variable concentration of substances in a spot.

b. Chromatographic development (separation)

Development of the chromatogram is affected after the solvent of the applied sample is completely evaporated. Rectangular glass chambers or twin trough chambers are commonly used for TLC development.

c. Detection of spots

For densitometric scanning, detection under UV light is generally preferred. But post chromatographic derivatisation reactions are essentially required for detection when individual compounds does not respond to UV light or do not have intense fluorescence.

d. Quantification and documentation

Densitometry is *in situ* instrumental measurement of visible, UV absorbance and fluorescence quenching directly. The scanner converts the spot/band on the layer into a chromatogram consisting of peaks similar in appearance to HPLC.

The portion of the scanned peaks on the recorder chart is related to R_f values of the spots on the layer and the peak height or area is related to the concentration of the substance on the spot.

Application of sample

Commercially available pre-coated plates of silica gel GF_{254} were used for the study. The different fractions were applied on plates with bandwidth of 6mm. Application rate was maintained at 10µl/min, using Linomat IV applicator (automatic TLC applicator, Camag, Switzerland). A sample volume of 10 µl was applied.

Chromatogram development

The plates were developed in twin-trough chamber using the solvent system as used in the TLC of different fractions. After developing, the plates were air-dried and observed under UV light chamber (Camag UV chamber-3, model no: 022.9120).

Densitometric Scanning

The developed plates were scanned using densitometer at 254 and 366 nm (Camag TLC Scanner – 3, model no: 0227.6480, combined with integration software, CATS 4.06 (Switzerland)).

4.2.5. Antioxidant studies

Free radicals are chemical species possessing an unpaired electron that can be considered as fragments of molecules and which generally are extremely reactive and short lived. They are continuously produced during the body's normal functions such as respiration and some cell-mediated immune functions. They are also found or generated through environmental pollution, cigarette smoke, automobile exhaust fumes, radiation, air-pollutants, pesticides etc. Naturally, there is a dynamic balance between the amount of free radicals generated in the body and antioxidants to quench and / or scavenge them and protect the body against their deleterious effects. Any additional burden of free

radicals either from the environment or produced within the body, can impair the balance between free-radicals (Pro-oxidants) and anti-free-radicals (antioxidants) leading to oxidative stress, which may result in tissue injury and subsequent diseases[104].

There is an increasing evidence to support the involvement of free radical reactions in several human diseases. In recent years, it has become increasingly apparent that in man, free radicals play a role in a variety of normal regulatory systems. A majority of disease conditions like atherosclerosis, hypertension, ischaemic disease, Alzheimer's disease, Parkinson's disease, cancer and inflammatory[105,106,107] conditions are being caused primarily due to the imbalance between pro-oxidant and antioxidant homeostasis.

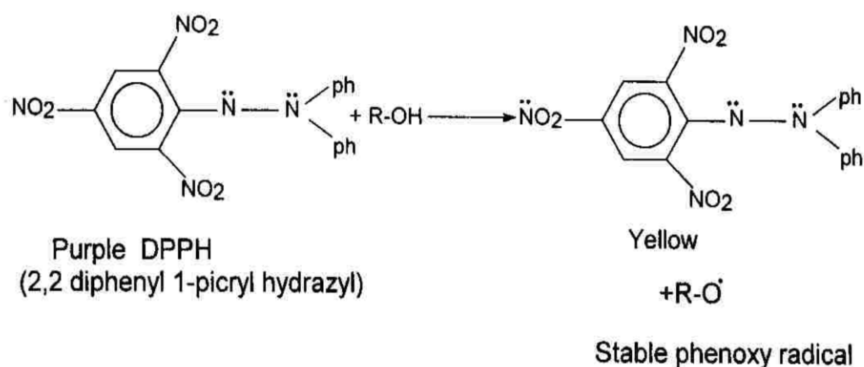
Anti-oxidant principles from natural resources possess multi face tenderness in their multitude and magnitude of activities and provide enormous scope in correcting the imbalance[104]. Active oxygen species and other free radicals have long been known to be mutagenic. These agents have more recently emerged as mediators of the other phenotypic and genotypic changes that lead from mutation to neoplasia. Therefore, free radicals may contribute widely to cancer development in humans. More interestingly, free oxygen radicals are increasingly discussed as important factors involved in the phenomenon of biological aging[108]. From the above discussions, it is proven that the free radical production in animal cells is inevitable and because they can be damaging and causative for a variety of diseases or disorders, they should be eliminated from the body.

Anti-oxidants are supposed to reduce the risk of cancer and other diseases by helping the body to get-rid of oxygen free radicals, which are thought to contribute to cancer development by damaging the DNA. Many plants and their extracts are rich sources of agents such as anti-oxidants, which can prevent the occurrence of cancer by reducing free-radical induced cell damage[108]. In the

present study, the antioxidant and hepatoprotective activities of the extract were carried out, based on the literature survey and the potential of these two plants.

***In vitro* antioxidant studies**

4.2.5.a. DPPH method



The antioxidant activity of various extracts of the two plants was assessed on the basis of the radical scavenging effect of the stable 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical[109,110]. The DPPH free radical is reduced to a corresponding hydrazine when it reacts with a hydrogen donor. The DPPH radical is purple in colour and upon reaction with hydrogen donor, it changes to yellow in colour. It is a discoloration assay, which is evaluated by the addition of antioxidant to a DPPH solution in ethanol or methanol and the decrease in absorbance was measured.

Chemicals and reagents

Diphenyl picryl hydrazyl solution (DPPH, 100 μM): 22 mg of DPPH was accurately weighed and dissolved in 100 ml of methanol. From this stock solution, 18 ml was taken and diluted to 100 ml using methanol to obtain 100 μM DPPH solution.

Procedure

About 0.3 mM solution of DPPH in 100% ethanol was prepared and 1 ml of this solution was added to 3 ml of the extract dissolved in ethanol at different concentrations (25-400 µg/ml). The mixture was shaken and allowed to stand at room temperature for 30 minutes and the absorbance was measured at 517 nm using a spectrophotometer. The percentage scavenging activity at different concentrations was determined and the IC_{50} value of the extracts was compared with that of ascorbic acid, which was used as the standard.

4.2.5.b. Measurement of Reducing power ability activity

The reducing power was investigated by the Fe^{3+}-Fe^{2+} transformation in the presence of the extracts as described by Fejes, et al[111].

Chemicals and Reagents

1. Potassium ferricyanide ($K_3Fe(CN)_6$, 1%): 1 g of potassium ferricyanide was weighed accurately and dissolved in distilled water to make up to the volume to 100 ml in a volumetric flask.
2. Trichloro acetic acid (TCA, 10%): 10 g of TCA was weighed accurately and dissolved in distilled water to make up the volume to 100 ml in a volumetric flask.
3. Ferric chloride ($FeCl_3$) solution (0.1%): 0.1 g of $FeCl_3$ was weighed accurately and dissolved in distilled water to make up the volume to 100 ml in a volumetric flask.
4. Phosphate buffer pH 6.6: 46.00 g of potassium dihydrogen phosphate and 60.50 g of dipotassium hydrogen phosphate and disodium edetate (100 ml of 0.02 M) were dissolved in 1ml distilled water. The pH was adjusted to 6.6 with KH_2PO_4.

Procedure

The Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm[112]. One ml of the extract (50-800 μg/ml), 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide were incubated at 50°C for 30 min and 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged for 10 min at 3000g. About 2.5 ml of the supernatant was diluted with 2.5 ml of water and is shaken with 0.5 ml of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm. Butylated hydroxy toluene (50-800 μg/ml) was used as the standard. All tests were performed in triplicate and the graph was plotted with the average of the three determinations

4.2.5.c. Nitric oxide radical inhibition activity

Sodium nitroprusside in aqueous solution at physiological pH, spontaneously generates nitric oxide, which interact with oxygen to produce nitrite ions, which can be measured by using a modified Griess-IIIosvoy method[113,114]. Sodium nitroprusside spontaneously generates nitric oxide in aqueous solution. Nitric oxide generated in this manner was converted into nitric acid and nitrous acids in contact with dissolved oxygen and water. The librated nitrous acid is estimated using Griess reagent, which forms a purple azodye in the presence of a test compound likely to be the scavenger and the amount of nitrous acid will decrease. The degree of decrease in the formation of purple azodye will reflect the extent of scavenging.

Chemicals and regents

1. Sodium nitroprusside solution: Weighed accurately 0.2998 g of sodium nitroprusside and dissolved in distilled water to make up the volume to 100 ml in a volumetric flask (10mM).
2. Naphthyl ethylene diamine dihydrochloride (NEDD, 0.1%): weighed accurately 0.1 g of NEDD and dissolved in 60 ml of 50% glacial acetic

acid by heating and made the volume to 100 ml in a volumetric flask with distilled water.

3. Sulphanilic acid (0.33% w/v) reagent: Weighed accurately 0.33 g of sulphanilic acid and dissolved in 20% glacial acetic acid by heating and made up the volume to 100 ml in a volumetric flask.

Procedure

This assay was performed according to the method described by Sreejayan and Rao[115]. Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which was measured by Griess reagent. The reaction mixture (3 ml) containing 10 mM sodium nitroprusside in phosphate buffered saline, and the extract or the reference compound (curcumin) at different concentrations (50-800 µg/ml) were incubated at 25°C for 150 min. About 0.5 ml aliquot of the incubated sample was removed at 30 min intervals and 0.5 ml Griess reagent was added. The absorbance of the chromophore formed was measured at 546 nm. Inhibition of the nitric oxide generated was measured by comparing the absorbance values of control, extract and curcumin (25-400 µg/ml).

4.2.5.d. Phosphomolybdate method

The total antioxidant capacity of the extract was determined with phosphomolybdenum using α-tocopherol as the standard[116]. The total antioxidant capacity was determined by phosphomolybdenum method and is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the formation of a green Mo (V) complex which has the maximal absorption at 695 nm.

Procedure

An aliquot of 0.1ml of the extracts (1mg) solution was combined with 1.0 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. After the samples had cooled to room temperature,

the absorbance was measured at 695 nm against the blank using an UV spectrophotometer. The blank solution contained 1.0 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. The total antioxidant capacity was expressed as µg equivalents of α-tocopherol by using the standard tocopherol graph.

4.2.6. *In vivo* hepatoprotective studies

Galactosamine results in acute hepatitis in rats which resembles human viral hepatitis following a single dose. Hence, the present study was undertaken to evaluate the *in vivo* hepatoprotective activity of the various extracts of *Cuscuta reflexa* and *Cassytha filiformis* with d-galactosamine as hepatotoxicant[117].

4.2.6.a. Selection and maintenance of animals

Healthy adult male albino rats of *Wistar* strain weighing between 180-220 g were obtained from the animal house, J.S.S College of Pharmacy, Ootacamund, India for the screening of hepatoprotective activity of the plant extracts. The animals were housed in polypropylene cages in adequately, well ventilated room and maintained under standard environmental conditions (22-28°C, 60-70% relative humidity, 12 h dark/light cycle). The animals were fed with standard rat feed pellets (Amurth Rat Feed, Nav Maharashtra Chakan Oil Mills Ltd., Pune) and water ad *libitum* (Aquaguard filter water). The study was approved by the institutional animal ethics committee; approval no: JSSCP\IAEC\Ph.D\Ph.biotech\01\2008-09.

4.2.6.b. Acute toxicity studies

Acute oral toxicity[118] study was performed as per OECD-423 guidelines (acute toxic class method). *Wistar* rats (n = 3) of either sex selected by random sampling technique were used for the study. The animals were kept fasting for a overnight providing only water, after which the extracts were administered orally at the dose level of 5 mg / kg body weight by intragastric tube and observed for

14 days. If mortality was observed in 2-3 animals, then the dose administered was assigned as toxic dose. If mortality was observed in 1 animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher doses such as 50, 300 and 2000 mg / kg body weight.

Preparation of the drug for the experimental study

The extracts and the standard drugs were administered in the form of suspension in water with 1% sodium carboxymethyl cellulose as suspending agent.

Preparation of the standard

Silymarin, a known hepatoprotective agent was used as the reference for comparison at a dose of 25 mg/kg body weight in 0.3% CMC.

4.2.6.c. Experimental design

Animals were divided into 11groups comprising of 6 animals in each group. Each group received the following treatment.

Group I : Normal control (0.3% CMC)

Group II : Hepatotoxicant (d-galactosamine 600 mg\kg) by i.p route as single dose

Group III : Aqueous extract of *Cuscuta reflexa* at a dose of 200 mg/kg body weight.

Group IV : Aqueous extract of *Cuscuta reflexa* at a dose of 400 mg/kg body weight.

Group V : 50% methanol extract of *Cuscuta reflexa* at a dose of 200 mg/kg body weight.

Group VI : 50% methanol extract of *Cuscuta reflexa* at a dose of 400 mg/kg body weight.

Group VII : Ethanol extract of *Cassytha filiformis* at a dose of 200 mg/kg body weight

Group VIII : Ethanol extract of *Cassytha filiformis* at a dose of 400 mg/kg body weight.

Group IX : 50% methanol extract of *Cassytha filiformis* at a dose of 200 mg/kg body weight.

Group X : 50% methanol extract *Cassytha filiformis* at a dose of 400 mg/kg body weight.

Group XI : Silymarin positive control

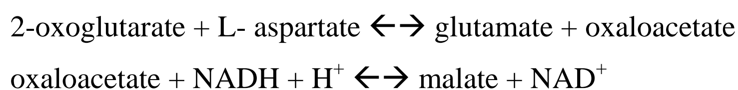
All these treatments were given orally for 14 days. On the 14th day, after 1 h of sample administration, except normal control animals (G-1) all the animal in groups (G-2 to G-11) received single dose of 600 mg/kg intraperitoneal injection of d-galactosamine dissolved in saline. After 48 hours d-galactosamine administration all the animals were sacrificed by cervical decapitation under light ether anaesthesia. The liver and blood were collected from all these animals on the same day for biochemical and histopathological estimations.

4.2 6 d. Isolation of blood serum for biochemical studies

Blood was collected from jugular veins and centrifuged (3000 rpm for 10 min) to obtain serum. The serum was used for marker enzyme estimation.

Assay of aspartate aminotransferase (AST or SGOT)

Asparate amino transferase (AST) catalyzes the following reaction.



2-oxoglutarate + L- aspartate ←→ glutamate + oxaloacetate

oxaloacetate + NADH + H^+ ←→ malate + NAD^+

The rate of NADH consumption was measured photometrically at 540 nm and is directly proportional to the AST activity in the sample, AST level in serum is expressed as U/L.

Estimation of aspartate aminotransferase

Aspartate aminotransferase was estimated by the method of King[119].

Reagents

1. Phosphate buffer 0.1 M, pH 7.5.

2. Substrate: 1.33 g of DL-aspartic acid and 15 mg of 2-oxoglutarate were dissolved in 20.5 ml of 1 N sodium hydroxide and made up to 100 ml with buffer.
3. 0.02% 2,4-dinitrophenyl hydrazine (DNPH): 20 mg of DNPH in 100 ml of 1 N hydrochloric acid.
4. 0.4 N Sodium hydroxide.
5. Standard: 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. This contains 1 µm of pyruvate/ml.

Procedure

1.0 ml of buffered substrate was incubated at 37°C for 10 min. Then 0.2 ml of enzyme was added and mixture was incubated at 37°C for 1 hr. To the control tubes enzyme was added after the reaction and it was arrested by the addition of 1.0 ml of DNPH reagent. The tubes were kept at room temperature for 30 min. A set of standard pyruvate solution was also treated in a similar manner. Then 5.0 ml of sodium hydroxide was added. The colour developed was read at 540 nm.

The enzyme activity is expressed as U/L.

Assay of alanine aminotransferase (ALT or SGPT)

Alanine aminotransferase (ALT) catalyzes the following reactions.

2–oxoglutarate + L – alanine $\leftrightarrow$ glutamate + pyruvate

Pyruvate + NADH + H^+ $\leftrightarrow$ Lactate + NAD^+

The rate of NADH consumption was measured photometrically at 540 nm and is directly proportional to the ALT activity in the sample. Alanine aminotransferase level in serum is expressed as U/L.

Estimation of alanine aminotransferase

Alanine aminotransferase was assayed by the method of King[119].

Reagents

1. Phosphate buffer 0.1 M, pH 7.5.

2. Substrate: 1.78 g of DL-alanine and 30 mg of 2-oxoglutarate were dissolved in 20 ml of buffer, 0.5 ml of 1 N sodium hydroxide was added and made up to 100 ml with phosphate buffer, pH 7.5.
3. 0.02% 2,4-dinitrophenyl hydrazine (DNPH): 20 mg of DNPH in 100 ml of 1 N hydrochloric acid
4. 0.4 N sodium hydroxide.
5. Standard: 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. This contained 1 μm of pyruvate/ml.

Procedure

1.0 ml of substrate was incubated at 37°C for 10 min. Then 0.2 ml of enzyme solution was added. The tubes were incubated at 37°C for 30 min. To the control tubes enzyme was added after arresting the reaction with 1.0 ml of DNPH reagent. The tubes were kept at room temperature for 20 min. Then 5.0 ml of 0.4 N sodium hydroxide was added and then the colour developed was read at 540 nm.

The enzyme activity is expressed as U/L.

Assay of alkaline phosphatase (ALP)

Alkaline phosphatase (ALP) catalyzes the following reaction:

4-nitrophenyl phosphate + H_2O ↔ phosphate + 4-nitrophenolate

The rate of increase in 4-nitrophenolate was determined photometrically at 640 nm and is directly proportional to the ALP activity in the sample. Alkaline phosphate level in serum is expressed as U/L.

Estimation of alkaline phosphatase

Alkaline phosphatase was assayed by the method of King[120].

Reagents

1. Carbonate-bicarbonate buffer 0.1 M, pH 10: 6.36 g of sodium carbonate and 3.36 g of sodium bicarbonate were dissolved in 1000 ml of water.

2. Substrate 0.1 M: 254 mg of disodium phenyl phosphate was dissolved in 100 ml of water.
3. Magnesium chloride 0.1 M: 406 mg of magnesium chloride was dissolved in 20 ml of water.
4. 15% sodium carbonate: 15 g of sodium carbonate was dissolved in 100 ml of water.
5. Folin's phenol reagent: One volume of Folin's reagent was diluted with two volumes of distilled water just before use.
6. TCA 10%
7. Standard: 10 mg of phenol was dissolved in 100 ml of water.

Procedure

The mixture containing 1.5 ml buffer, 1.0 ml substrate and 0.1 ml of magnesium chloride were pre-incubated at 37°C for 10 min. Then 0.1 ml of enzyme was added and incubated at 37°C for 15 min. The reaction was arrested by 1.0 ml of 10% TCA. Control without enzyme was also incubated and the enzyme was added after the addition of TCA solution. Then 1.0 ml of sodium carbonate and 0.5 ml of Folin's phenol reagent were added. After 10 min the blue colour developed was read at 640 nm.

The enzyme activity is expressed as U/L.

Assay of total protein (TP)

Proteins and peptides, in contrast to other nitrogen containing compounds (e.g. creatinine, urea and uric acid) produce a violet coloured complex with copper ions in dilute alkaline solution. The so called biuret reaction is particularly easy to carry out giving reproducible results. The absorbance of the colour complex is directly proportional to the protein concentration in sample materials. The absorbance of the sample and standard was measured against the biuret regent and the absorbance of the blank against distilled water at 640 nm. Total protein level in serum is expressed as mg/dL.

Estimation of protein

Protein was estimated by the method of Lowry[121].

Reagents

1. Alkaline copper reagent

   Solution A: 2% sodium carbonate in 0.1 N sodium hydroxide

   Solution B: 0.5% copper sulphate in 1% sodium potassium tartrate. 50 ml of solution A was mixed with 1 ml of solution B just before use.

2. Folin's phenol reagent: One volume of Folin's reagent was diluted with two volumes of distilled water just before use.

3. Standard bovine serum albumin (BSA): 20 mg of BSA was dissolved in 100 ml of distilled water. Few drops of sodium hydroxide (alkali) were added to aid complete dissolution of BSA and to avoid frothing; it was allowed to stand overnight in a refrigerator.

Procedure

0.1 ml of 10% homogenate was diluted to 1 ml with water. From this diluted samples 0.1 ml was made up to 1 ml with water. Standards were taken and made up to 1 ml with water. 1 ml water was used as blank. To all tubes 4.5 ml of alkaline copper reagent was added and kept at room temperature for 10 min. Then 0.5 ml of Folin's phenol reagent was added and the colour developed was read after 20 min at 640 nm. Protein content is expressed as mg/dl of fresh or wet tissue.

Estimation of total bilirubin

Total bilirubin was estimated by the method of Malloy and Evelyn[122].

Reagents

1. Absolute methanol
2. HCl, 1.5% v/v with water

3. **Diazo reagent:**

Solution A: About 1 gm of sulphanilic acid was dissolved in 15 ml of concentrated HCl and made upto 1 litre with water.

Solution B: About 0.5 gm of sodium nitrate was dissolved in water and made upto 100 ml.

Prepared freshly before use by adding 0.3 ml of solution B to 10 ml of solution A.

4. Standard solution of bilirubin: Prepared a solution containing 10 mg of bilirubin per 100 ml of $CHCl_3$.

Procedure

0.2 ml of serum was taken and 1.8 ml of distilled water was added. To the solution, 0.5 ml of diazo reagent was added. Finally, 2.5 ml of methanol was added and stand for 30 min. Absorbance was read in the colorimeter using a yellow green filter or set upto 540 nm. The amount of total bilirubin was calculated and expressed in terms of mg/dl.

4.2.6.e. Histopathological studies

Immediately after sacrifice, the liver was dissected out, washed in the ice cold saline to remove as much blood as possible. Small pieces of liver tissue were collected and preserved in 10 % formalin solution for histopathological studies.

Tissue preparation for histology [123].

Fixation

Fixative

Picric ac id saturated solution	75 ml
Formaldehyde 40%	25 ml
Glacial acetic acid	5 ml

Procedure

After sacrificing the rats by cervical dislocation, liver tissue were collected, washed in normal saline and fixed in the above said fixative for 24

hours. Then the tissues were washed thoroughly in running tap water followed by a rinse in distilled water.

Dehydration

This is a preliminary but an essential process for removing the water present in tissues and their spaces after fixation to ensure proper penetration of paraffin during embedding. This was performed by graded alcohol.

Procedure

The tissues were placed in specimen jars and passed through ascending grades of ethyl alcohol as follows:

50% alcohol (10 minutes)

70% alcohol (overnight)

80% alcohol (30 minutes)

90% and 95% (2 hours each, 2 changes)

absolute alcohol (1 hour, 2 changes)

Clearing

Xylene is used for the removal of alcohol and it increases the refractive index of the tissue, thereby making them transparent. The dehydrated tissues were transferred to xylene and three changes were given at an interval of 1 hour.

Embedding

It is the process of impregnation of the tissue with the embedding media paraffin.

Material

Embedding oven 55-60°C, paraffin (melting point 58-60°C)

Procedure

The tissues were transferred from xylene to molten paraffin and kept in the paraffin bath for 3–4 hours.

Block preparation

After complete impregnation with paraffin it is necessary to have solid blocks containing the tissues to facilitate proper sectioning of the material. The tissues in molten paraffin were poured into moulds of suitable dimensions and the tissues were set properly within the paraffin. Orientation of tissues is important, which can be done according to the nature of section necessary. To remove any air bubble in the paraffin block the tip of the forceps was gently warmed, inserted and passed around the specimen taking care to avoid any touch with the tissue. This is important as presence of air bubbles in the block hinders proper sectioning. The moulds were then gently immersed in cold water to cool the paraffin rapidly. This prevents crystallization of paraffin. When the blocks became solid, they were removed from water and detached from the mould with a scalpel. The tissues were then ready for sectioning.

Section cutting

The paraffin blocks were sectioned in a microtome to get serial sections, which come out in the form of a paraffin ribbon.

Materials

Properly prepared tissues in paraffin block.

Rotary microtome (Reichert–Jung), Hot plate

Microtome razor

Procedure

The excess paraffin at the sides of the block containing the tissues was trimmed to have a suitable size and fixed on a block holder of the microtome. The block holder was heated on a flame and pressed directly to the base of the block. The surface of the tissue was brought just below the knife-edge by using coarse feed adjustment. The feed mechanism was adjusted to the desired setting of 5 μ. The microtome was operated and section cut. After having a 8″ ribbon, it was detached from the knife by the help of a scalpel and floated in a tray containing warm water. Small portions of this ribbon with serial sections were placed on

clear glass slides and gently stretched using forceps or needles. Water on the slides was drained off and the slides placed on hot plate to allow the paraffin film to dry, set and get attached to the glass slides.

Removal of paraffin wax

Paraffin being poorly permeable to stain, slides were dipped in xylene (two changes each of 2 – 5 minutes) for complete removal of paraffin.

Removal of xylene

To remove xylene, the slides were placed in absolute alcohol for 3 minutes.

Rehydration

The slides were passed through descending grades of alcohol and finally washed in water for 3 minutes.

Staining

Double staining

To differentiate the nucleus and cytoplasm, the basic dye haematoxylin and the acid dye eosin were used.

Staining with haematoxylin

After washing in water, slides were treated with haematoxylin for 2-3 minutes.

Development of colour

To develop the colour of haematoxylin, the slides were washed in tap water for 8 –10 minutes.

Staining with eosin

The eosin was soluble in alcohol medium but not in water medium, the haematoxylin stained slide was again passed through ascending alcohol grades with two final changes of 3 minutes each in absolute alcohol. The slides were placed from absolute alcohol to eosin solution and kept for one to one and half minutes.

Treatment with absolute alcohol

Three changes each of 1 minute were given in absolute alcohol.

Clearing

Sections were treated with xylene 2 – 3 times for clearing.

Mounting

After treatment with xylene very small amount of DPX was placed over the section. Over this a clean cover slip was pressed lightly so far as to avoid formation of bubbles.

4.2.6.f. Statistical Analysis

Statistical analysis was carried out using GraphPad software (GraphPad InStat) by one-way Analysis of Variance (ANOVA) followed by Dunnett's test. Results are expressed as mean ± SEM from six rats in each group. P values < 0.05 were considered significant.

Results & Discussion

RESULTS AND DISCUSSION

Medicinal plants play a key role in the human health care as they have been used as medicines over the centuries. All through human history, there has been a noticeable concern for health care and the cure of diseases, a logical approach to the study of drugs and their activities is the recognition of the basic principles behind the biochemical events leading to drug actions.

The World Health Organization estimates that 65-80% of the world's population use traditional medicine as their primary form of the health care. The use of herbal medicine, the dominant form of the medical treatment in developing countries, has been increasing in developed countries also. Many people believe that because herbal remedies are "natural", they are entirely "safe"[13]. There is a great demand for herbal medicines because of their wide biological activities, higher safety margin and lesser costs than the synthetic drugs [11].

In the dawn of human cultural evolution, the art of curing was essentially magical and was based on logic than on scientific evidence. There was no uniform or standard procedure for maintaining the inventing of these plants and the knowledge about their medicinal properties without any written documentation or regulation. Therefore, it is essential that such uses of natural products be documented and studied for systematic regulation and wide-spread application.

The World Health Organisation has recognized the importance of traditional medicine and has created strategies, guidelines and standards for botanical medicines. Materials of plant origin are prone to contamination, deterioration and variation in composition. Hence it is

necessary to develop methods for rapid, precise and accurate identification and estimation of active constituents in order to bring out consistency of important constituents in the formulations[11].

In this connection, the present study is an attempt to standardize two traditional medicinal plants with respect to their pharmacognostical, phytochemical and biological activity.

5.A. Pharmacognostical studies

Pharmacognostical studies play an important role in the standardization of plant material. In the present study, two plants were selected based on their ethnomedical uses and was authenticated. Their morphological and microscopical characters were determined. A detailed study of microscopic characters of the two plants along with the photographs serves in identifying the special characters of the plants.

5.1.a. *Cuscuta reflexa* Roxb

Macroscopic characters

The genus ***Cuscuta*** Linn is a leafless, twining parasitic ***Vine***. The branchlets are much branched, glabrous, yellowish (achlorophyllous) and slender. According to Gamble there are three species in South India. Henry recorded four species in **Tamil Nadu, South India. *Cuscuta reflexa* *Roxb***. can be differentiated on morphological grounds by the following features.

The plant is a parasitic vine with soft, thick, branched, yellow coloured stem. The plant selected for the present study, is a parasite growing on the host *Morinda Pubescences*. It may also be self-parasitic on it own stem. The *flowers* are subsessile racemes and creamy white in colour. *Calyx* is gamosepalous, five lobed and thick. *Corolla* has five petals, white or cream coloured, gamopetalous, tubular companulate. *Stamens* are five,

epipetalous. *Ovary* has two stout styles which are divergent. *Fruit* is a succulent capsule (Fig. 7).

Fig. 7 Morphological features showing the stem and the flowers of *Cuscuta reflexa*

Microscopical features

Microscopic profile of young stem

The stem varies in thickness from 700 µm to 2 mm. The thin stem is roughly circular in outline with shallow ridges (Fig. 8.1). It consists of a continuous fairly thick epidermal layer of 30 µm thick; the cells are rectangular with prominent cuticle.

The cortex is 150 µm wide comprising of 4-6 layers of wide, angular, thin walled compact parenchyma cells. The pith is wide, parenchymatous, the cells being thin walled, angular and compact. The pith is 400 µm wide.

The stele (vascular tissues) consists of about eight small, discrete collateral vascular bundles which occur in a ring. Each bundle has four to eight xylem elements which are thick walled, lignified and angular in outline, they are 20-40 µm wide; phloem occurs in small nests on the outer part of the xylem mass (Fig. 8.2).

Microscopic profile of thick (old) stem

Thick stem differs from a thin stem in some essential features. The thick stem is also circular in transectional view with irregular ridges and furrows. The epidermis is 30-40 µm thick; the epidermal cells are barrel shaped with thick cuticle (Fig. 9.1, 9.2). The cortex is 240 µm wide with six to eight layers of thin walled, compact, angular parenchyma cells (Fig. 9.1). Three layers inner to the epidermis there is a thin, circular line of crushed cells. The pith is 800 µm wide and the cells are large, thin walled, angular and compact (Fig. 9.2).

The vascular system consists of a thin closed cylinder formed by 10-12 small collateral vascular strands which are interlinked by a thin sclerenchyma cylinder (Fig. 9.2). Each vascular bundle has a small cone of xylem elements and fairly prominent mass of phloem elements.

The xylem elements are narrow, angular, thin walled and compact. The interconnecting sclerenchyma band has tangentially oblong thin walled, wide, lignified cells.

Cell inclusions such as starch grains or calcium oxalate crystals are not evident in the cells.

The stem twines over other plants developing haustoria to derive the nutrition from the host plant. Sometimes the stem may twine with other branch of same ***Cuscuta*** plant forming what is known as **"self-parasitism."** The stem that functions as the parasite develops a broad cup shaped portion with which it clasps over the other branch which acts as the **'host'.** The epidermis of the clasping part of the parasitic branch becomes much wider, the cells elongate considerably into a compact palisade like zone (Fig. 10.1, 10.2). The cortical cells of the parasitic branch develop radially elongated bundle of **haustorium** which penetrates into the cortex of the host branch which later traces into the vascular tissues. The haustorial cells are darkly staining.

The vascular system of the stem that acts as the host is well developed; the vascular bundles are prominent with thick walled larger cluster of xylem and phloem (Fig. 10.2).

When ***Cuscuta*** is parasitic on other host plants of different species, the **haustorium** is well organized comprising of tracheary elements (xylem elements) (Fig. 11.3). The haustorium penetrates the cortex and reaches the xylem cylinder of the host plant (Fig. 11.1). The cortical tissue of the host stem develops thick pad of sclerenchyma around the haustorium (Fig. 11.2). The haustorium and the host tissue develop organic connection so that the parasite gets flow of nutrients from the host tissue.

Fig. 8 Microscopic profile of young stem

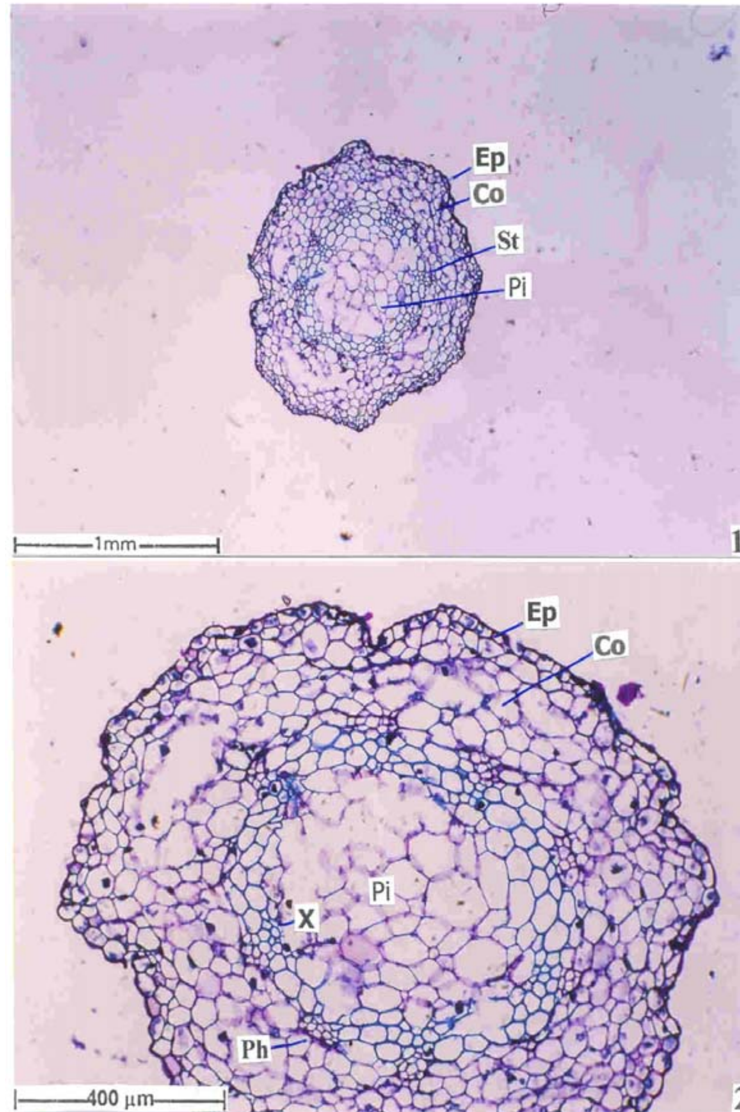


Fig. 8.1 TS of young (thin) stem - entire view

Fig. 8.2 Enlarged section of the stem

(Co-Cortex; Ep-Epidermis; Ph-Phloem; X-Xylem; St-Stele; Pi-Pith)

Fig. 9 Microscopic profile of thick (old) stem

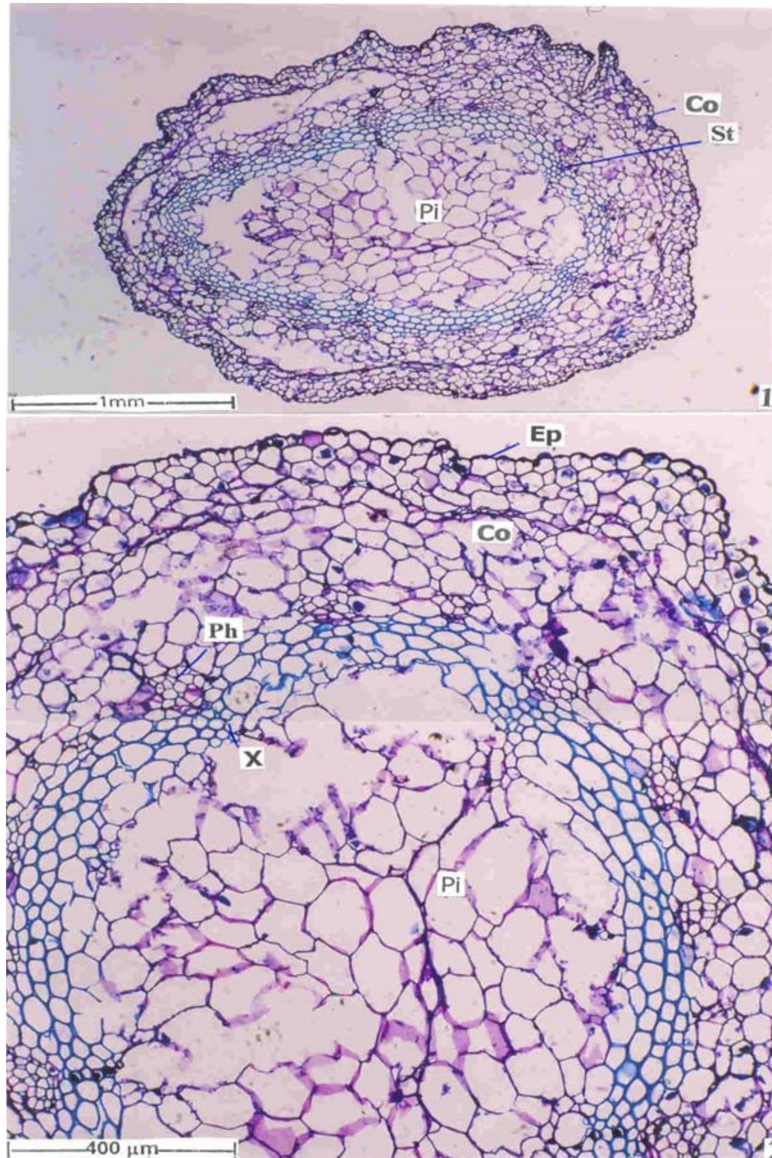


Fig. 9.1 TS of the stem - ground plan

Fig. 9.2 Enlarged section of the stem

(Co-Cortex; Ep-Epidermis; Ph-Phloem; X-Xylem; St-Stele; Pi-Pith)

Fig. 10 Self-parasiting phenomenon

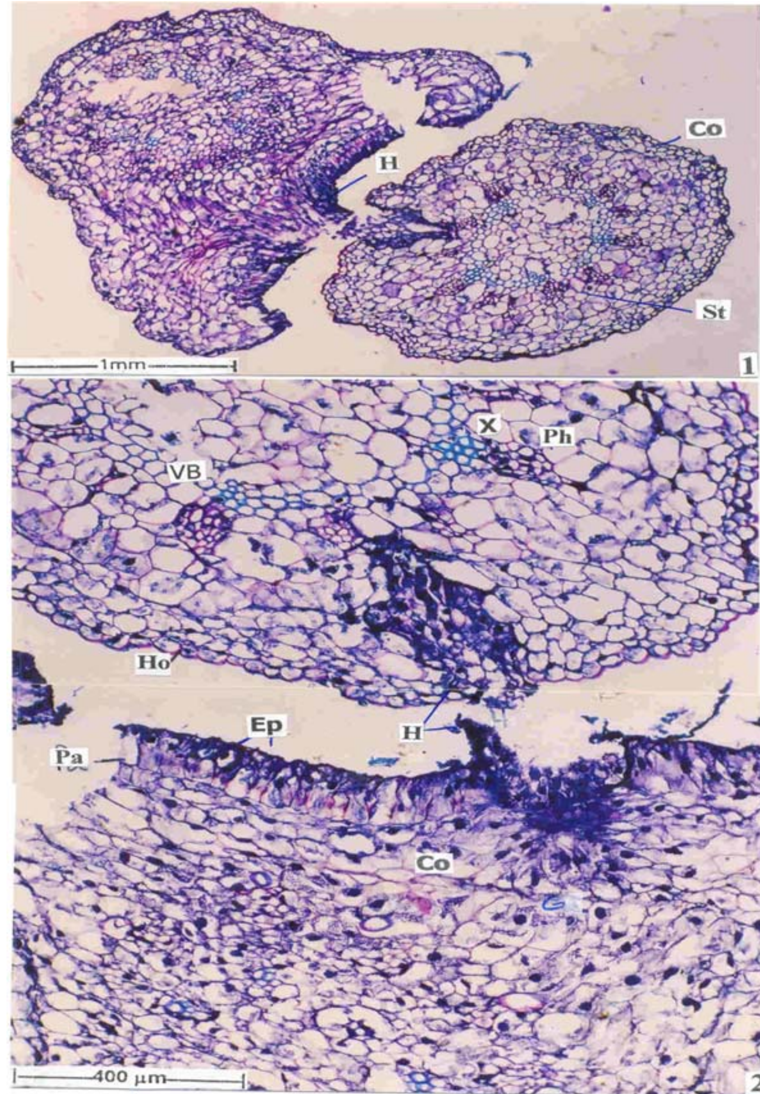


Fig. 10.1 TS of host stem and parasitic stem

Fig. 10.2 Haustorium between host and parasitic stems.

(Co-Cortex; Ep-Epidermis; Ph-Phloem; X-Xylem; St-Stele; Vb-Vascular bundle; Ho-Host stem; Pa-Parasitic stem; H-Haustorium)

Fig. 11 *Cuscuta* parasitic on *Morinda Pubescence*

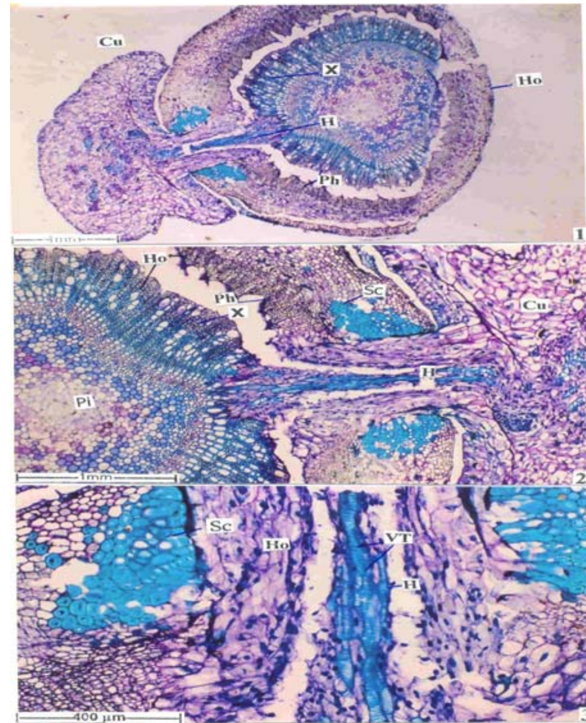


Fig. 11.1 *Cuscuta* parasiting on *Morinda* in TS view

Fig. 11.2 A portion of the host stem, haustorium and the stem of *Cuscuta*; with the presence of sclerenchyma cells on either side of haustorium

Fig. 11.3 Haustorium enlarged to show the xylem elements in the haustorium and sclerenchyma masses on either side of the haustorium.

(Cu-***Cuscuta*** stem; H-Haustorium; Pi-Pith

Ho-Host plant stem; Ph-Phloem; X-Xylem

Vt-Vascular tissue of the haustorium;

Sc-Sclerenchyma mass)

Salient diagnostic features on microscopic characters

- The young stem has a single layer of distinct epidermis, narrow zone of parenchymatous cortex, wide parenchymatous pith and a ring of discrete vascular bundles.
- The xylem elements are not prominent; they are narrow, thin walled and reduced in number.Phloem is comparatively well developed.
- In a thick stem, a thin ring of collapsed cells is seen in the cortex.
- The stele consists of more number of vascular bundles which are fairly prominent, collateral and closed.
- No secondary growth is evident.
- The vascular bundles are interconnected by a thin continuous cylinder of sclerenchyma cells, so that the stele appears as a closed cylinder.
- Cell inclusions such as crystals and starch grains are lacking.

The microscopic characters of *Cuscuta* revealed that the following features are identified as anatomical markers for authentication of this species.

5.1.b. *Cassytha filiformis* Linn

Macroscopic characters

Leafless *twiner. Spikes* terminal or axillary; peduncle 2-4.5 cm; bracteoles 3, ovate, 1.2 mm, ciliate. *Flowers* (sub) sessile, in spikes, 3-merous, *bisexual*, 3 mm across. *Tepals* 6, free, unequal, 3+3, truncate, obtuse; outer lobes ovate-orbicular, 1.2 mm, ciliate; inner lobes obovate, 2.5 mm. Fertile *stamens* 9; filaments 1.5 mm; glands sessile; anthers 2-celled, 1.5 mm; staminodes 3 to 1 mm. *Ovary* 1.5 mm; style 0.5 mm; stigma capitate. *Drupe* globose, enclosed within inflated perianth, crowned by lobes; seed 1, globose, conform to *fruit*: testa thin (Fig. 12).

Fig. 12 Morphological features showing the stem and the flowers of *Cassytha filiformis*

Microscopical features

The stem is circular in cross sectional view with shallow ridges and furrows (Fig. 13.1). The epidermal layer has small squarish cells and thick cuticle. The epidermis is stomatiferous with sunken stomata.

The cortex consists of two or three layers of small, circular air-chambers separated by radially elongated cells of the partitions. The cortex is 200 µm wide. Epidermal layer is 40 µm thick; the cuticle is 10 µm thick (Fig. 13.3).

There is a thin layer of gelatinous fibers along the inner boundary of the cortex. The fibers are either in small discontinuous masses or in thin continuous cylinder (Fig. 13.2).

The stele consists of a continuous thin cylinder of xylem with external phloem. The xylem elements are wide, thin walled and angular in outline. The meta xylem elements are 50-60 µm wide. Phloem elements are in small nests.

Pith is wide and has a central narrow canal and outer intact parenchyma cells. The outer pith cells are wide, thin walled and compact.

Starch grains and calcium oxalate crystals are abundant in outer pith cells (Fig. 14.1, 14.2).

The crystals are prismatic type. The starch grains are circular, simple, concentric and have central hilum. (Fig. 14.2).

The results obtained in pharmacognostical studies reported, herein established the macroscopic and microscopic parameters that characterize the genuine plant drug *Cuscuta reflexa* and *Cassytha filiformis*. These morphological characteristics can be utilized for quick identification of the drug and are particularly useful in the case of powdered materials[124].

The present study on the pharmacognostical characters of the plants may be useful to supplement information in regard to its identification and authentication of the plant and its powdered samples[125].

Fig. 13 TS of stem - *Cassytha filiformis*

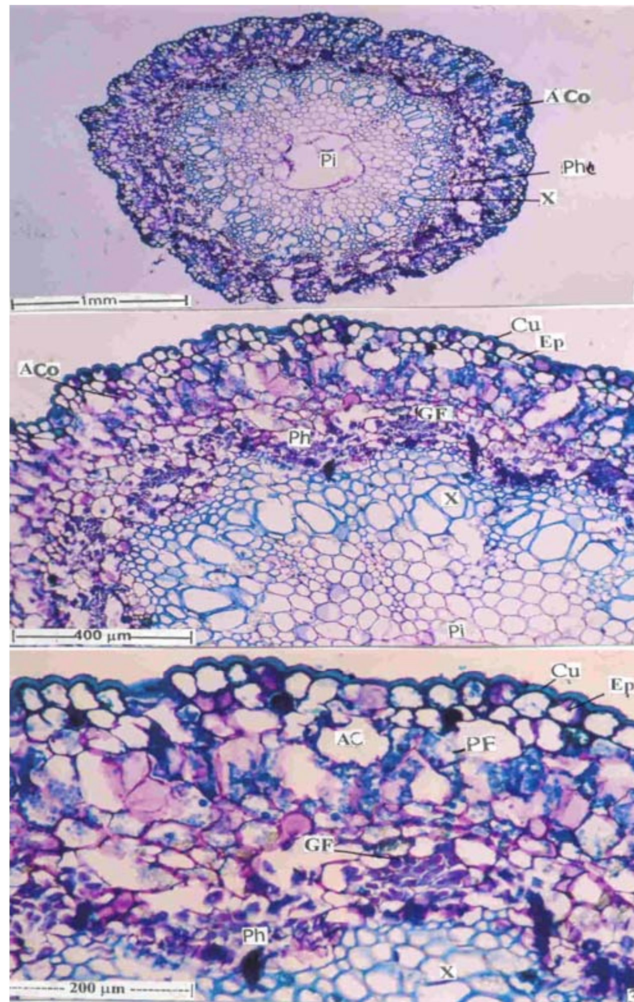


Fig. 13.1 TS of stem - entire view

Fig. 13.2 TS of stem-A section enlarged showing cortex and the stele

Fig. 13.3 TS of stem showing aerenchymatous cortex and thick cuticle of the epidermis

(Ac-Air chamber; Aco-Aerenchymatous cortex;

Cu-Cuticle; Ep-Epidermis; Gf-Gelatinous fibers;
Ph-Phloem; Pi-Pith; X-Xylem)

Fig. 14 Crystals and starch grains in the stem tissue as seen under polarized light microscope

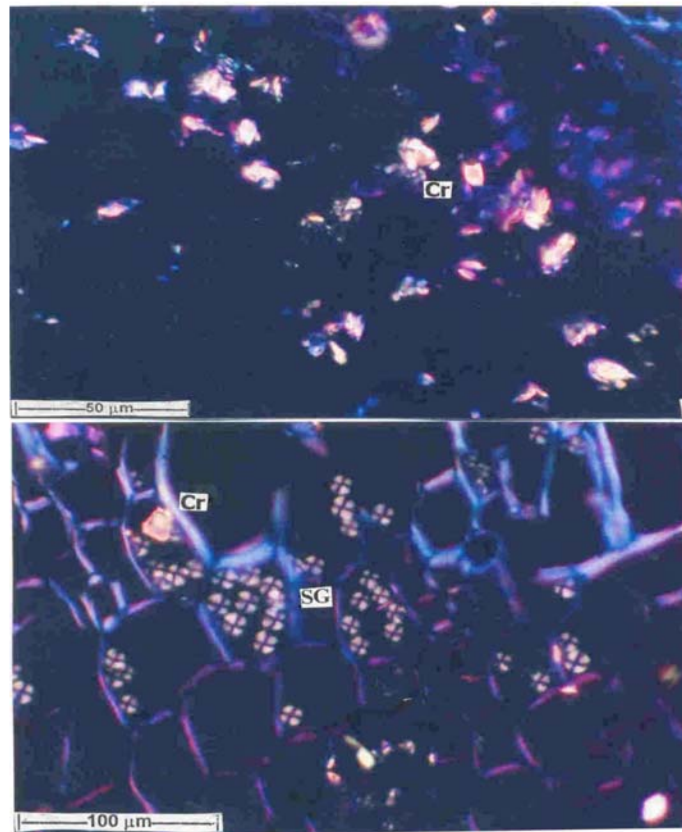


Fig. 14.1 Calcium oxalate prismatic crystals in the cortical cells
Fig. 14.2 Concentric starch grains in the ground parenchyma cells
(Cr-Crystals of prismatic type; SG-Starch grains with “+”mark due to double refringent nature of the starch grains).

5.2. Physiochemical studies

Determination of physiochemical constants is important for the purpose of evaluation of crude drugs. The quality parameters of the crude drugs as raw materials were established with the help of several official determinations based on physical and physiochemical studies. These studies were aimed at ensuring standardisation of herbal drugs under investigations[126]. Several physiochemical parameters were established for the two plants.

5.2.a. Ash values

The total ash and acid insoluble ash values for *Cuscuta reflexa* were found to be the higher than *Cassytha filiformis* and were 7.298 and 4.397 w/w respectively. The total ash values and acid insoluble ash values for *Cassytha* were 5.2970 and 3.3711 w/w. The sulphated ash value is high in the case of *Cassytha filiformis* and the value was 11.4908 w/w. The sulphated ash value of *Cuscuta reflexa* was found to be 4.8712 w/w. The water soluble ash values were 3.911 and 3.970 w/w for *Cuscuta* and *Cassytha* respectively. The ash values of the two plant materials are shown in Table 5.

Table 5

Ash values of the plant materials

S. No.	Plant	Total ash (% w/w)	Acid insoluble ash (%w/w)	Water soluble ash (% w/w)	Sulphated ash (%w/w)
1.	*Cuscuta reflexa*	7.298	4.397	3.911	4.8712
2.	*Cassytha filiformis*	5.270	3.3711	3.970	11.4908

All the values are average of four determinations

Ash values are helpful in determining the quality and purity of crude drugs in powdered form according to the standard procedure[126]. Ash value of a drug gives an idea of the earthy matter or the inorganic composition and other impurities present along with the drug[97].

5.2.b. Extractive values

The plant powders of *Cuscuta* and *Cassytha* showed alcohol and water soluble extractive values as 20.18, 18.26 w/w and 20.84, 18.75 w/w respectively (Table 6).

Table 6

Extractive values of the plant materials

S. No.	Plant	Extractive values	
		Alcohol soluble extractives	Water soluble extractives
1.	*Cuscuta reflexa*	20.18	18.26
2.	*Cassytha filiformis*	20.84	18.75

All the values are average of four determinations

Extractive values are useful for evaluation of crude drugs and gives idea about the nature of chemical constituents present in them. The amount of extractive, a drug yield to a given solvent, is often an approximate measure of a certain constituent or group of related constituents the drug contains. In some cases the amount of drug soluble in a given solvent is an index of its purity[126]. Extractive values are primarily useful, for the determination of exhausted or adulterated drugs[97].

5.2.c. Fluorescence analysis

The fluorescence analysis of the raw plant powders on treatment with various reagents showed the presence of various constituents and is shown in Table 7 & 8. The powders showing blue colour with iodine and brown colour with ferric chloride indicates the presence of starch (carbohydrates) and phenolic compounds like tannins flavanoids, etc. The formation of green colour with nitric acid and yellow colour in alcohol indicates the presence of flavanoids and phenolic compounds. Starch was found to be absent in *Cuscuta reflexa* but present in *Cassytha filiformis.*

Table 7

Fluoresence analysis of raw powder of *Cuscuta reflexa*

S. No.	Sample + Reagents	UV Light		Visible Light
		Short 254 nm	Long 366 nm	
1	Drug powder	Pale green	No visible colour	Pale brown
2	Powder + aqueous sodium hydroxide	Pale green	No visible colour	Pale yellow
3	Powder + alkaline sodium hydroxide	Pale green	No visible colour	Pale yellow
4	Powder + IN hydrochloric acid	Pale green	No visible colour	Pale yellow
5	Powder + 50% sulphuric acid	Pale green	Pale brown	Reddish brown
6	Powder + 50% nitric acid	Pale green	Pale green	Orange
7	Powder + picric acid	Pale yellow	Pale green	Yellow
8	Powder + acetic acid	No visible colour	No visible colour	Pale brown
9	Powder + ferricchloride	Pale green	Pale green	Bluish green
10	Powder + nitric acid + ammonia	Pale green	Pale green	Orange with orange fames

Table 8

Fluoresence analysis of raw powder of *Cassytha filiformis*

S. No.	Sample + Reagents	UV Light		Visible Light
		Short 254 nm	Long 366 nm	
1	Drug powder	Light yellow	Pale yellow	Greenish brown
2	Powder + aqueous sodium hydroxide	Green	Cream	Pale green
3	Powder + alkaline sodium hydroxide	Green	Cream	Pale green
4	Powder + IN hydrochloric acid	Light yellow	Light green	Reddish brown
5	Powder + 50% sulphuric acid	Green	Dark yellow	Greenish yellow
6	Powder + 50% nitric acid	Green	Dark red	Green
7	Powder + picric acid	Brownish yellow	Brownish yellow	Yellowish green
8	Powder + acetic acid	Dark brown	No visible colour	Pale brown
9	Powder + ferric chloride	Brownish green	Brownish green	Pale brown
10	Powder + nitric acid + ammonia	Pale green	Dark green	Pale green

The fluorescence behaviour of the powdered drug in different solutions towards ordinary light and ultraviolet light (both long and short wavelengths) gives an idea about various phytoconstituents present in the plant drugs. These results indicate the presence of some particular phytoconstituents in the respective plant powders and extracts which was later, confirmed by the phytochemical tests.

5.2.d. Phytochemical studies

5.2.d.i. Organoleptic characters of *Cuscuta reflexa*

The whole plant powder of *Cuscuta reflexa* is pale brown in colour with no characteristic odour or taste. The powder was coarse in appearance and when triturated with water it was non sticky in nature. The powder on shaking with water gives foam like froth and no oil stain was found when the powder was pressed between filter papers for 24 hours.

5.2.d.ii. Organoleptic characters of *Cassytha filiformis*

The whole plant powder of *Cassytha filiformis* was pale greenish brown in colour with no characteristic odour or taste. The powder was coarse in appearance and when triturated with water it was found to be sticky in nature. The powder on shaking with water gives foam like froth and no oil stain was found when the powder was pressed between filter papers for 24 hours.

A preliminary organoleptic characters of both the powdered plant material was studied and the results are shown in Table 9 &10.

Table 9

Organoleptic characters of *Cuscuta reflexa* raw powdered material whole plant

Powder Character	
Colour	Pale brown
Appearance	Coarse powder
Odour	No characteristic smell
Taste	No characteristic taste

Treatment	Observation
Powder triturated with water	Non sticky
Powder shaken with water	Foam like froth
Powder pressed between filter paper for 24 hrs	No oil stain

Table 10

Organoleptic characters of *Cassytha filiformis* raw powdered material - whole plant

Powder Character	
Colour	Pale greenish brown
Appearance	Coarse powder
Odour	No characteristic smell
Taste	No characteristic taste

Treatment	Observation
Powder triturated with water	sticky
Powder shaken with water	Foam like froth
Powder pressed between filter paper for 24 hrs	No oil stain

5.2.d.iii. Qualitative phytochemical analysis

In the qualitative phytochemical analysis of *Cuscuta reflexa*, the raw powder in methanol was found to show positive results for the presence of alkaloid, tannin, flavanoid, phenols, carbohydrates, and mucilage wherein the raw powder in water showed the presence of alkaloid, tannin, saponin, flavanoid, phenols, carbohydrates, glycoside and mucilage. The 50% methanol extract and aqueous extract of *Cuscuta reflexa*, showed the presence of alkaloids, terpenoid, tannins, flavanoid, phenol, carbohydrates, glycoside and mucilage. In the case of *Cassytha filiformis* the raw powder in methanol showed the presence of alkaloid, terpenoid, steroids, tannin, flavanoid, phenols and carbohydrates whereas the raw powder in water showed positive results for the presence of alkaloid, steroids, tannin, saponin, flavanoid, phenols, carbohydrates and mucilage. The ethanol extract showed the presence of alkaloid, tannin, flavanoid, phenols, carbohydrates and glycoside wherein 50% methanol extract showed positive results for the presence of alkaloids, terpenoid, tannins, flavanoid, phenols, carbohydrates, glycosides and gum.

Both the plant extracts showed the presence of alkaloid, tannins, flavanoid, phenols, carbohydrates and glycosides. These details of the results are summarized in Table 11.

Table 11

Qualitative phytochemical analysis of the raw powder and extracts of the plants

S. No.	Constituents	Raw powder				50% methanol extracts of *Cuscuta reflexa*	Aqueous extract of *Cuscuta reflexa*	Ethanol extract of *Cassytha filiformis*	50% methanol extracts of *Cassytha filiformis*
		Cuscuta reflexa		*Cassytha filiformis*					
		Alc	Aq	Alc	Aq				
1	Alkaloid	+	+	+	+	+	+	+	+
2	Terpenoid	–	–	+	–	+	+	–	+
3	Steroids	–	–	+	+	–	–	–	–
4	Tannin	+	+	+	+	+	+	+	+
5	Saponin	–	+	–	+	–	–	–	–
6	Flavonoid	+	+	+	+	+	+	+	+
7	Phenols	+	+	+	+	+	+	+	+
8	Protein	–	–	–	–	–	–	–	–
9	Carbohydrate	+	+	+	+	+	+	+	+
10	Glycoside	–	+	–	–	+	+	+	+
11	Gum	–	–	–	–	–	–	–	+
12	Fixed oil	–	–	–	–	–	–	–	–
13	Mucilage	+	+	–	+	+	+	–	–

+ Present; – Absent

Phytochemical evaluation helps in laying down the pharmacopoeial standards[98].

5.2.e. Estimation of total phenolic content

Total phenolic content was estimated by using Folin-Ciocalteu reagent. Total phenolic content of the extracts were solvent dependent and expressed as µg pyrocatechol equivalent. The content of the total phenolics in the extracts decreased in the following order as ethanol extract of *Cassytha filiformis,* aqueous extract of *Cuscuta reflexa,* 50% methanol extracts of *Cassytha filiformis* and 50% methanol extracts of *Cuscuta reflexa* (Table 12).

Table 12

Estimation of total phenolic content

Plant extract	Total phenolic content (µg pyrocatechol equivalent / mg)
50% methanol extract of *Cuscuta reflexa*	36
Aqueous extract of *Cuscuta reflexa*	60
Ethanol extract of *Cassytha filiformis*	94
50% methanol extract of *Cassytha filiformis*	58

Phenolics are ubiquitous secondary metabolites in plants and possess a wide range of therapeutic uses such as antioxidant, antimutagenic, anticarcinogenic, free radical scavenging activities and also decreases cardiovascular complications[127]. The scavenging ability of the phenolics is mainly due to the presence of hydroxyl groups. Total phenolic assay by using Folin-Ciocalteu reagent is a simple, convenient and reproducible method. It is employed routinely in studying phenolic antioxidants[128].

5.2.f. Estimation of flavanoid content

The flavanoid content in the extracts was expressed as µg quercetin equivalent. The 50% methanol extracts of *Cassytha filiformis* showed highest amount of flavanoids among the extracts tested. The content of total flavanoids in the extracts decreased in the following order, 50% methanol extracts of *Cassytha filiformis*, 50% methanol extracts of *Cuscuta reflexa,* aqueous extract of C*uscuta reflexa* and ethanol extract of *Cassytha filiformis* (Table 13).

Table 13

Estimation of flavonoid content

Plant extract	Flavonoid content (µg quercetin equivalent / mg)
50% methanol extract of *Cuscuta reflexa*	138
Aqueous extract of C*uscuta reflexa*	110
Ethanol extract of *Cassytha filiformis*	68
50% methanol extract of *Cassytha filiformis*	145

Flavanoids are a group of polyphenolic compounds, which exhibit several biological effects such as antiinflammatory, antihepatotoxic, antiulcer, antiallergic, antiviral, anticancer activities. They also inhibit enzymes such as aldose reductase and xanthine oxidase. They are capable of effectively scavenging the reactive oxygen species because of their phenolic hydroxyl groups and are potent antioxidants[129]. In view of their wide pharmacological and biological actions, they have a greater therapeutic potential. The presence of high phenolic and flavanoid content

in the fractions has contributed directly to the antioxidant activity by neutralising the free radicals.

5.3. *In vitro* antioxidant activity

The *in vitro* antioxidant activity of the plant extract exhibited potent antioxidant activity with low IC_{50} values in DPPH, reducing power ability, nitric oxide and phosphomolybdate (total antioxidant capacity) scavenging methods (Fig. 15).

Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms[130].

5.3.a. DPPH method

All the extracts demonstrated H-donor activity. The highest DPPH radical scavenging activity was detected in 50% methanol extracts of *Cuscuta reflexa* (IC_{50} 44.5 μg/ml), followed by the 50% methanol extracts of *Cassytha filiformis*, aqueous extract of *Cuscuta reflexa* and ethanol extract of *Cassytha filiformis* (IC_{50} 54.2, 56.9 and 63 μg/ml respectively; Table 14). These activities are less than that of ascorbic acid.

Table 14

Hydrogen donating ability (DPPH assay)

Extract	Concentration (µg/ml)	Absorbance at 517 nm	% inhibition	IC_{50} µg/ml
Control		0.104		
50% methanol extract of *Cuscuta reflexa*	10 20 40 80 160	0.086 ± 0.005 0.075 ± 0.003 0.054 ± 0.002 0.038 ± 0.001 0.013 ± 0.003	17.31 ± 0.04 27.88± 0.06 48.08 ± 0.06 63.46 ± 0.02 87.50 ± 0.01	44.5
Aqueous extract of *Cuscuta reflexa*	10 20 40 80 160	0.084 ± 0.006 0.065 ± 0.005 0.059 ± 0.003 0.048 ± 0.006 0.026 ± 0.003	19.23 ± 0.41 37.50 ± 0.68 43.27± 0.07 53.84 ± 0.06 75.00 ± 0.06	56.9
Ethanol extract of *Cassytha filiformis*	10 20 40 80 160	0.089 ± 0.003 0.073 ± 0.005 0.065 ± 0.002 0.051 ± 0.001 0.036 ± 0.002	14.42 ± 0.02 29.81 ± 0.30 37.50 ± 0.01 50.96 ± 0.32 65.38 ± 0.03	63.0
50% methanol extract of *Cassytha filiformis*	10 20 40 80 160	0.082 ± 0.005 0.068 ± 0.004 0.040 ± 0.002 0.037 ± 0.001 0.024 ± 0.002	21.15 ± 0.06 34.62 ± 0.06 61.54 ± 0.03 64.42 ± 0.09 76.92 ± 0.03	54.2
Ascorbic acid (standard)	1 2 4 8 16	0.078 ± 0.001 0.057 ± 0.002 0.036 ± 0.002 0.021 ± 0.002 0.007 ± 0.001	25.01 ± 0.30 45.19 ± 0.16 65.38 ± 0.22 79.81 ± 0.13 93.27 ± 0.05	39.6

Values are mean ± S.E.M. (n=3).

DPPH assay is one of the most widely used methods for screening of antioxidant activity of plant extracts[131]. DPPH is a stable, nitrogen-centered free radical which produces violet colour in ethanol solution. It was reduced to a yellow coloured product, diphenylpicryl hydrazine, with the addition of the fractions in a concentration-dependent manner. The reduction in the number of DPPH molecules can be correlated with the number of available hydroxyl groups. All the extracts showed significantly higher inhibition percentage (stronger hydrogen-donating ability) and positively correlated with total phenolic content.

5.3.b. Measurement of reducing power ability activity

Table 15 shows the reductive capabilities of the extracts in comparison to the standard, BHT. In this the antioxidant activity, the reducing power increased with increasing amount of the extracts. The ethanol extract of *Cassytha filiformis* showed the highest reducing ability (absorbance 0.799) than all the other extracts tested. However, the activity was less than the standard, BHT (absorbance 1.534). The aqueous extract of *Cuscuta reflexa*, 50% methanol extracts of *Cassytha filiformis* and 50% methanol extracts of *Cuscuta reflexa* also showed reductive ability.

Table 15

Reducing power ability

Extract	Concentration (µg/ml)	Absorbance at 700 nm
50% methanol extract of *Cuscuta reflexa*	50 100 200 400 800	0.132 ± 0.003 0.149 ± 0.005 0.240 ± 0.003 0.368 ± 0.006 0.601 ± 0.003
Aqueous extract of *Cuscuta reflexa*	50 100 200 400 800	0.035 ± 0.003 0.068 ± 0.002 0.141 ± 0.005 0.304 ± 0.006 0.599 ± 0.008
Ethanol extract of *Cassytha filiformis*	50 100 200 400 800	0.056 ± 0.003 0.107 ± 0.005 0.224 ± 0.006 0.411 ± 0.003 0.799 ± 0.002
50% methanol extract of *Cassytha filiformis*	50 100 200 400 800	0.034 ± 0.003 0.071 ± 0.004 0.149 ± 0.005 0.308 ± 0.003 0.582 ± 0.011
BHT (standard)	50 100 200 400 800	0.244 ± 0.003 0.328 ± 0.005 0.489 ± 0.002 0.976 ± 0.008 1.534 ± 0.011

Values are mean ± S.E.M. (n=3)

The transformation of Fe^{3+} into Fe^{2+} in the presence of various extracts was measured to determine the reducing power ability. The reducing ability of a compound generally depends on the presence of

reductones (antioxidants), which exert the antioxidant activity by breaking the free radical chain by donating a hydrogen atom[112].

The antioxidant principles present in the extracts caused the reduction of Fe^{3+}/ ferricyanide complex to the ferrous form, and thus proved the reducing power ablity.

5.3.c. Nitric oxide radical inhibition activity

All the extracts effectively reduced the generation of nitric oxide from sodium nitroprusside. The 50% methanol extracts of *Cassytha filiformis* showed strong nitric oxide scavenging activity (IC_{50} 83.53 µg/ml) and that of standard curcumin was 75.6 µg/ml. The 50% methanol extracts of *Cuscuta reflexa* (104.5 µg/ml), aqueous extract of C*uscuta reflexa* (125.7 µg/ml) and ethanol extract of *Cassytha filiformis* (133.9 µg/ml) also showed good scavenging activities (Table 16).

Table 16

Scavenging of nitric oxide radical

Extract	Concentration (µg/ml)	Absorbance at 546 nm	% inhibition	IC_{50} µg/ml
Control		1.224		
50% methanol extract of *Cuscuta reflexa*	25 50 100 200 400	0.956 ± 0.015 0.886 ± 0.005 0.647± 0.003 0.420± 0.014 0.299 ± 0.003	23.15 ± 0.46 28.78 ± 0.58 47.14 ± 0.57 65.69 ± 0.33 75.57 ± 0.46	104.5
Aqueous extract of *Cuscuta reflexa*	25 50 100 200 400	0.996 ± 0.012 0.935 ± 0.005 0.750 ± 0.005 0.524 ± 0.014 0.410 ± 0.061	18.63 ± 0.66 23.61 ± 0.46 38.73 ± 0.46 57.19 ± 0.49 66.50 ± 0.52	125.7
Ethanol extract of *Cassytha filiformis*	25 50 100 200 400	0.942 ± 0.008 0.825 ± 0.005 0.755 ± 0.012 0.620 ± 0.005 0.508 ± 0.003	23.04 ± 0.65 32.59 ± 0.12 38.32 ± 0.33 49.35 ± 0.44 58.49 ± 0.37	133.9
50% methanol extract of *Cassytha filiformis*	25 50 100 200 400	0.860 ± 0.017 0.715 ± 0.005 0.599 ± 0.004 0.314 ± 0.005 0.214 ± 0.003	29.74 ± 0.15 41.58 ± 0.22 51.06 ± 0.46 74.35 ± 0.54 82.52 ± 0.49	83.53
Curcumin (standard)	25 50 100 200 400	0.813 ± 0.023 0.615± 0.005 0.421 ± 0.012 0.222 ± 0.012 0.109 ± 0.005	33.60 ± 0.39 49.78 ± 0.26 65.63 ± 0.52 81.86 ± 0.75 91.40 ± 0.14	75.6

Values are mean ± S.E.M. (n=3)

In vitro inhibition of nitric oxide radical is a measure of antioxidant activity of plant drugs. Nitric oxide is a free radical which plays an important role in the pathogenesis of pain, inflammation, etc. Scavenging of nitric oxide radical is based on the generation of nitric oxide from sodium nitroprusside in buffered saline, which reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent[132]. The absorbance of the chromophore is measured at 546 nm in the presence of the fractions. All the extracts decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*. This may be due to the antioxidant principles in the extracts which compete with oxygen to react with NO thereby inhibiting the generation of nitrite.

5.3.d. Phosphomolybdate method

The phosphomolybdate method is quantitative, since the total antioxidant capacity is expressed as α-tocopherol equivalents. Among the extracts tested, the aqueous extract of *Cuscuta reflexa* contains 134 µg vitamin E equivalent/ mg. The antioxidant activity increased in the order of aqueous extract of *Cuscuta reflexa* >50% methanol extracts of *Cuscuta reflexa* >50% methanol extracts of *Cassytha filiformis* > ethanol extract of *Cassytha filiformis* (Table 17).

Table 17

Total antioxidant activity

Extract	**Total antioxidant activity (phosphomolybdenum method) (µg α-tocopherol equivalent/mg)**
50% methanol extract of *Cuscuta reflexa*	124
Aqueous extract of *Cuscuta reflexa*	134
Ethanol extract of *Cassytha filiformis*	70
50% methanol extract of *Cassytha filiformis*	96

The phosphomolybdate method has been routinely used to evaluate the total antioxidant capacity of the extracts[133]. In the presence of the fractions, the Mo (VI) is reduced to Mo (V) and forms a green coloured phosphomolybdenum V complex which shows maximum absorbance at 695 nm. All the extracts possessed antioxidant activity.

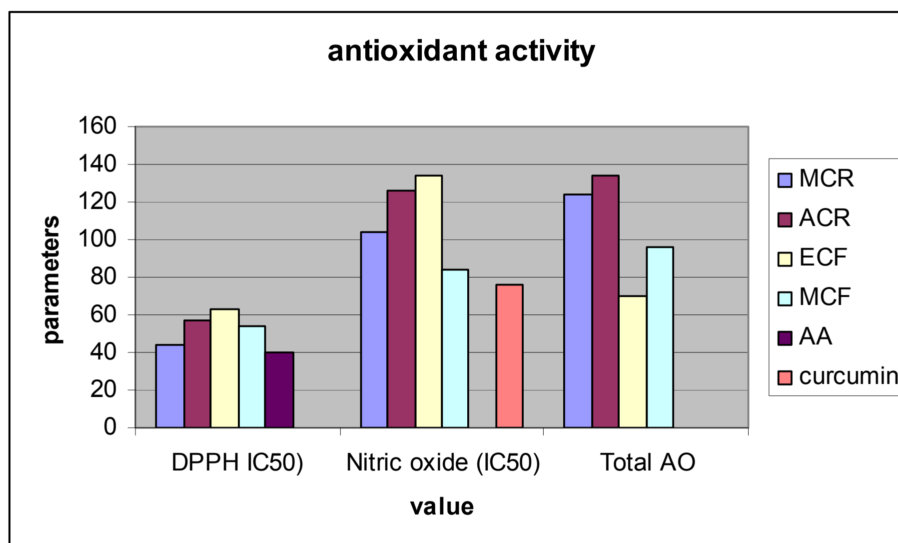


Fig. 15 *In vitro* antioxidant activity

5.B. Chemical studies

5.1.a. Characterization of compound-I

The aqueous extract of *Cuscuta reflexa* was subjected to column chromatography on silica gel. Elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 5 ml portions and monitored on normal TLC with appropriate solvent system. The chemically similar fractions that showed similar spots were pooled together. The fractions 260-300 yielded a yellowish coloured solid mass and the TLC profile showed two spots. This yellowish solid was designated as fraction-I. The fraction-I was further purified by solvent extraction and recrystallization technique and yielded a colourless solid. It was designated as compound-I. The isolated compound-I was subjected to TLC physical, chemical and spectroscopic studies for its characterization.

The homogeneity of compound-I was proved by melting point and a single spot in TLC and HPTLC using pre-coated plates of silica gel GF_{254} as adsorbent and solvent mixtures of different polarities as developers. The solvent systems used and their corresponding R_f values are shown below

S. No.	Mobile phase	No. of spots	R_f value
1	Ethyl acetate:formic acid:glacial acetic acid:water (100:11:11:27)	Single	0.48
2	n-Butanol:glacial acetic acid:water(40:10:50)	Single	0.49
3	n-Butanol:ethyl acetate:water (4:1:2.2)	Single	0.32

The compound-I exhibited a positive test for phenols. It gave a green colour with ferric chloride.

The melting point of the isolated compound-I was found to be in between 207-209°C (uncorrected). The isolated compound-I was subjected to UV, IR, ^{1}H NMR, ^{13}C NMR, and mass spectroscopic analysis to find out the structure.

Spectral studies of compound-I

UV λ_{max} MeOH (nm): 255, 354 (Fig. 16).

IRν_{max}cm: 671.25, 790.84, 1207.48, 1222.91, 1602.90, 2357.09, 2854.74, 3016.77, 3607.01, 3695.73 (Fig. 17).

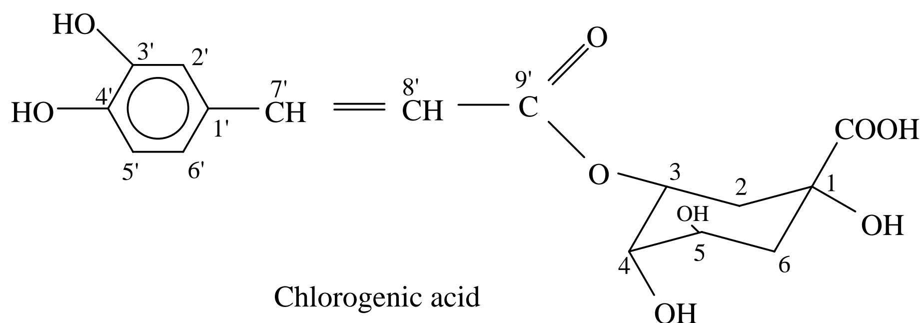
^{1}HNMR: δ 7.43 (d, J = 15.8 Hz, C-7'H), 7.07 (d, J = 1.5 Hz, C-2'H), 6.91 (dd, J = 8.2 Hz, C-6'H), 6.85 (d, J = 8.2 Hz, C-5'H), 6.27 (d, J = 15.8 Hz, C-

8'H), 5.11 (dd, J = 8.5 Hz, C-3H), 4.21 (dd, C-5H), 3.67 (dd, C-4H), 2.04 (dd, J = 11.9, J = 2.4 Hz, C-6Hα), 1.98 (2H, d, J = 8.5 Hz, C-$2H_2$), 1.42 (dd, J = 11.9, J = 1.5 Hz, C-6Hß) (Fig. 18)

^{13}CNMR: δ 168.4 (s, C-9'), 149.2 (s, C-4'), 146.6 (s, C-3'), 146.4 (d, C-7'), 127.8 (s, C-1'), 122.9 (s, C-6'), 116.6 (d, C-5'), 115.4 (d, C-8'), 115.1 (d, C-2'), 76.7 (s, C-1), 74.4 (d, C-4), 71.7 (d, C-3), 70.8 (d, C-5), 49.0 (t, C-2), 38.2 (t, C-6) (Fig. 19).

LC-MS *m/z* (re lint): 355.2 $[M]^+$. (Fig. 20).

The spectral data matched with that of the chlorogenic acid[134], thus confirming the structure of chlorogenic acid.



Chlorogenic acid

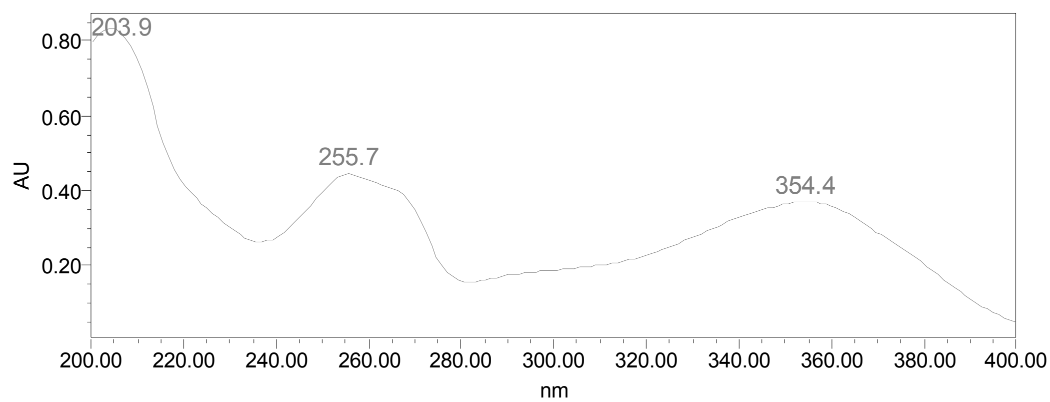
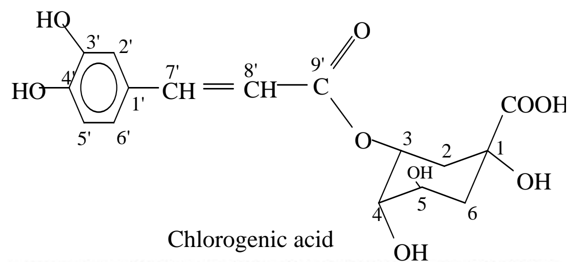


Fig. 16 UV data of compound - I



Chlorogenic acid

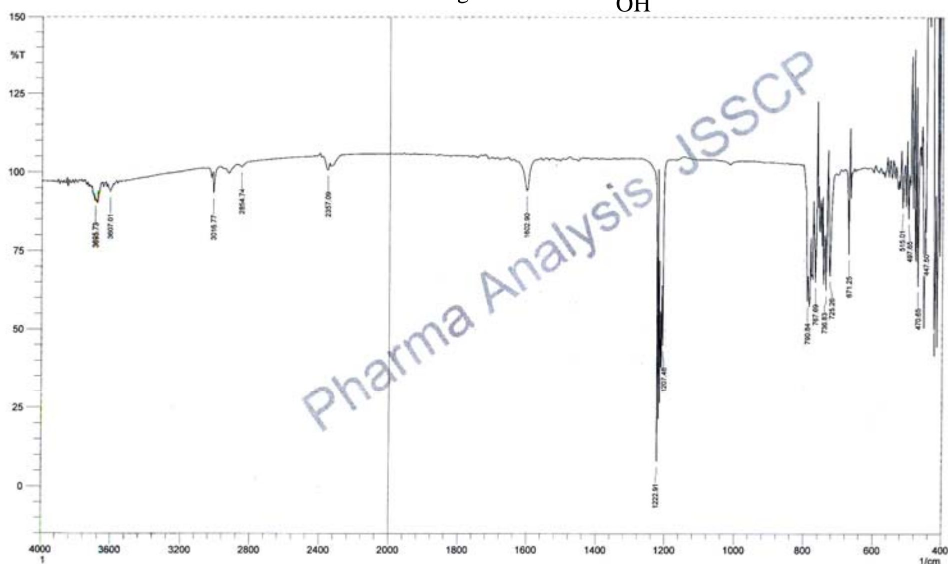


Fig. 17 IR of compound - I

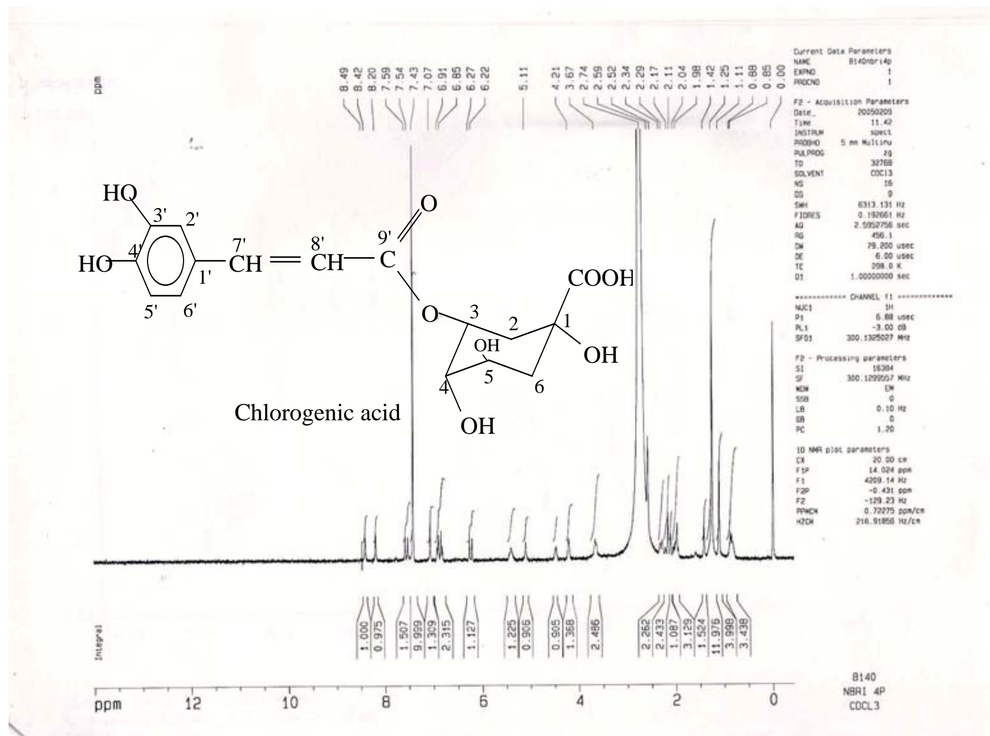


Fig. 18 ^{1}H NMR of compound - I

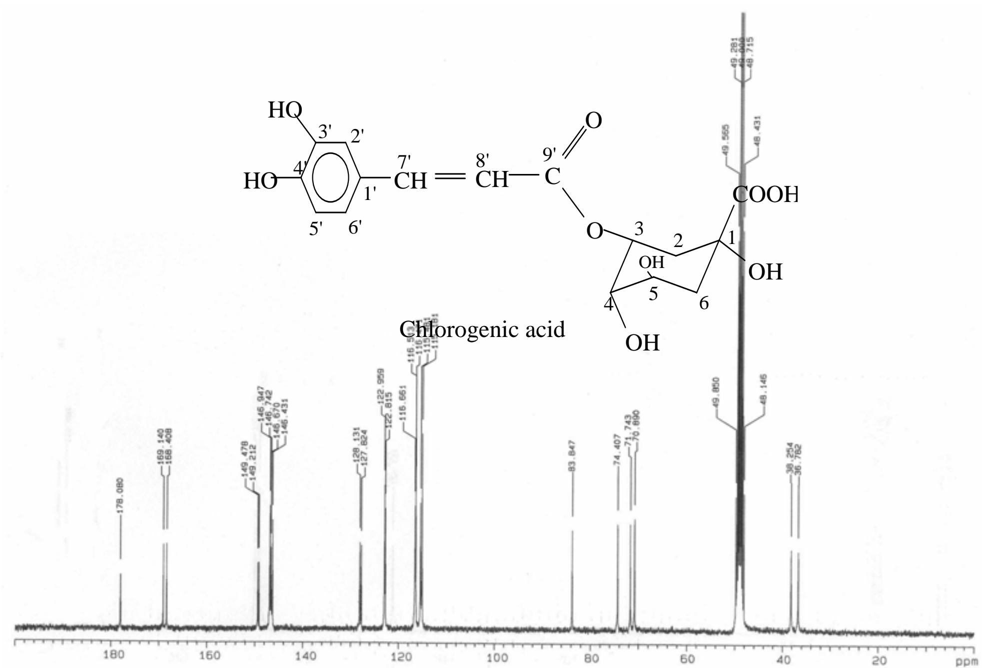
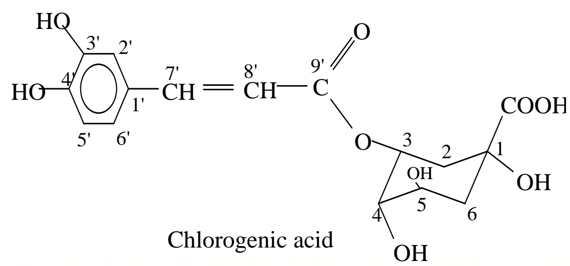


Fig. 19 ^{13}C NMR of compound - I



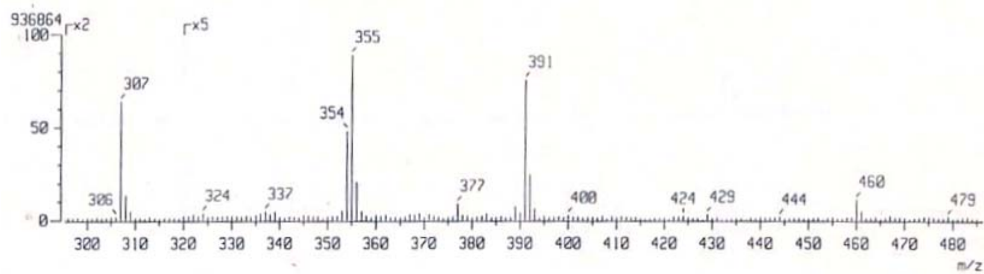


Fig. 20 Mass of compound - I

5.1.b. Characterization of compound-II

The fraction 400-480 yielded dark greenish coloured solid and its TLC profile showed three spots which was designated as fraction-II. The fraction-II was purified further by solvent extraction and recrystallization technique and yielded a light yellow colour solid. It was designated as compound-II. The isolated compound-II was subjected to TLC physical, chemical and spectroscopic studies for its characterization.

The homogeneity of compound-II was proved by melting point and a single spot in TLC and HPTLC using pre-coated plates of silica gel GF_{254} as adsorbent and solvent mixtures of different polarities as developers. The solvent systems used and their corresponding R_f values are shown below

S. No.	Mobile phase	No. of spots	R_f value
1	Ethyl acetate:formic acid:glacial acetic acid:water (100:11:11:27)	Single	0.63
2	n-Butanol:glacial acetic acid:water(40:10:50)	Single	0.62
3	n-Butanol:ethyl acetate:water (4:1:2.2)	Single	0.48

The compound-II exhibited a positive test for flavanoid and phenols. It gave an olive green colour with ferric chloride, a reddish pink colour in Shinoda’s test and a yellow colour with ammonia. It gave positive colour reaction in Molish’s test indicating the presence of a glycoside.

The melting point of isolated compound-II was found to be in between 216-218ºC (uncorrected). The isolated compound-II was subjected to UV, IR, Proton NMR, ^{13}C NMR, and Mass Spectroscopic analysis to find out the structure.

Spectral studies of compound-II

UV λ_{max} MeOH (nm): 253, 368 (Fig. 21).

IR ν_{max} cm^{-1}: 3392, 3369, 1654, 1609, 1558, 1508, 1458, 1429 (Fig. 22).

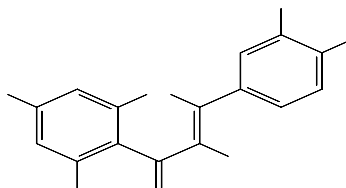
^{1}HNMR: δ 7.15 (CH), 6.93(CH), 6.72(CH), 6.25(CH), 5.94(CH), 11.85(OH), 10.68(OH), 10.29(OH), 9.48(OH) (Fig. 23).

^{13}CNMR: δ 136.5 (C), 146.9 (C), 146.5(C), 145.9(C), 158.8 (C), 161.8(C), 166.4(C), 122.8(C), 115.3 (CH), 104.5 (C), 117.2 (CH), 176.1 (C), 98.3(CH), 94.0(CH), 121.8(CH) (Fig. 24).

FAB-MS: pso. Ions 303 [M - H] (Fig. 25).

The spectral data matched with that of the quercetin, thus confirming the structure of quercetin.

Quercetin (3,3',4',5,7- pentahydroxy flavone)



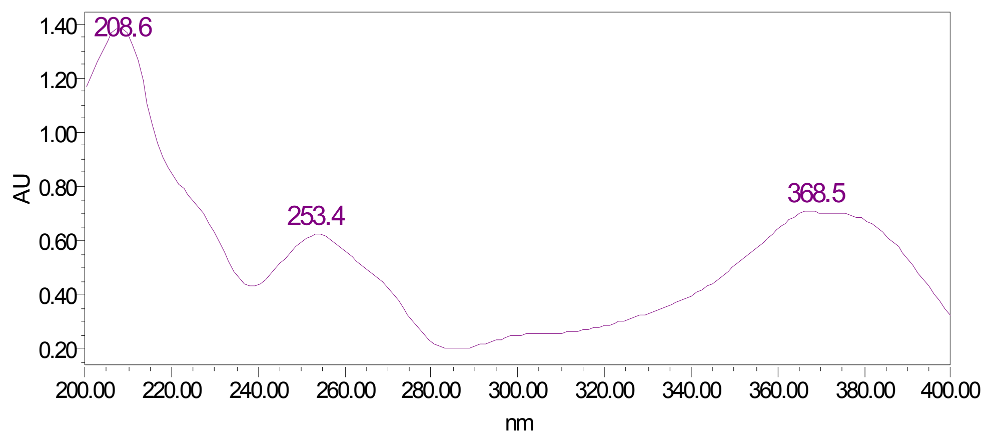
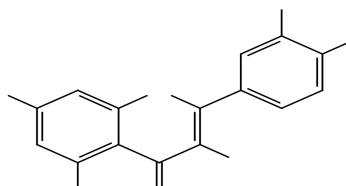


Fig. 21 UV data of compound - II

Fig. 22 IR of compound - II



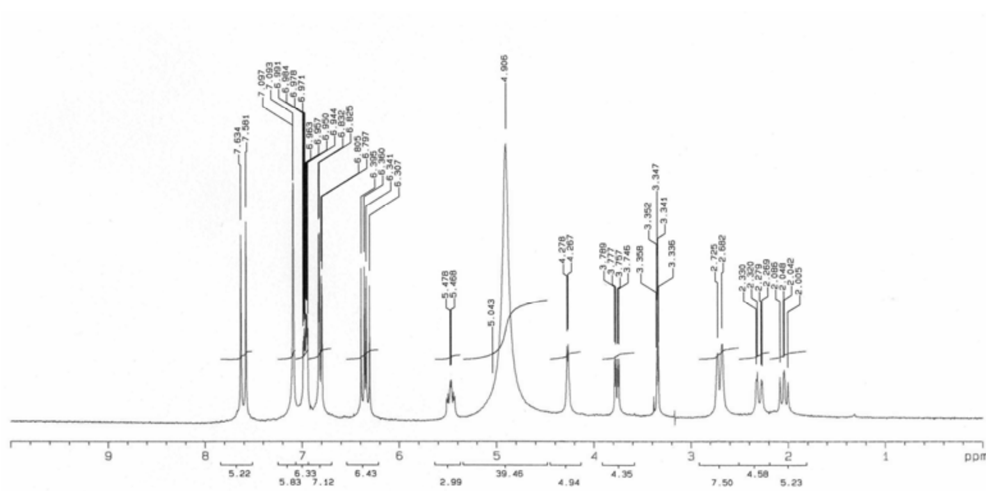


Fig. 23 ^{1}H NMR of compound - II

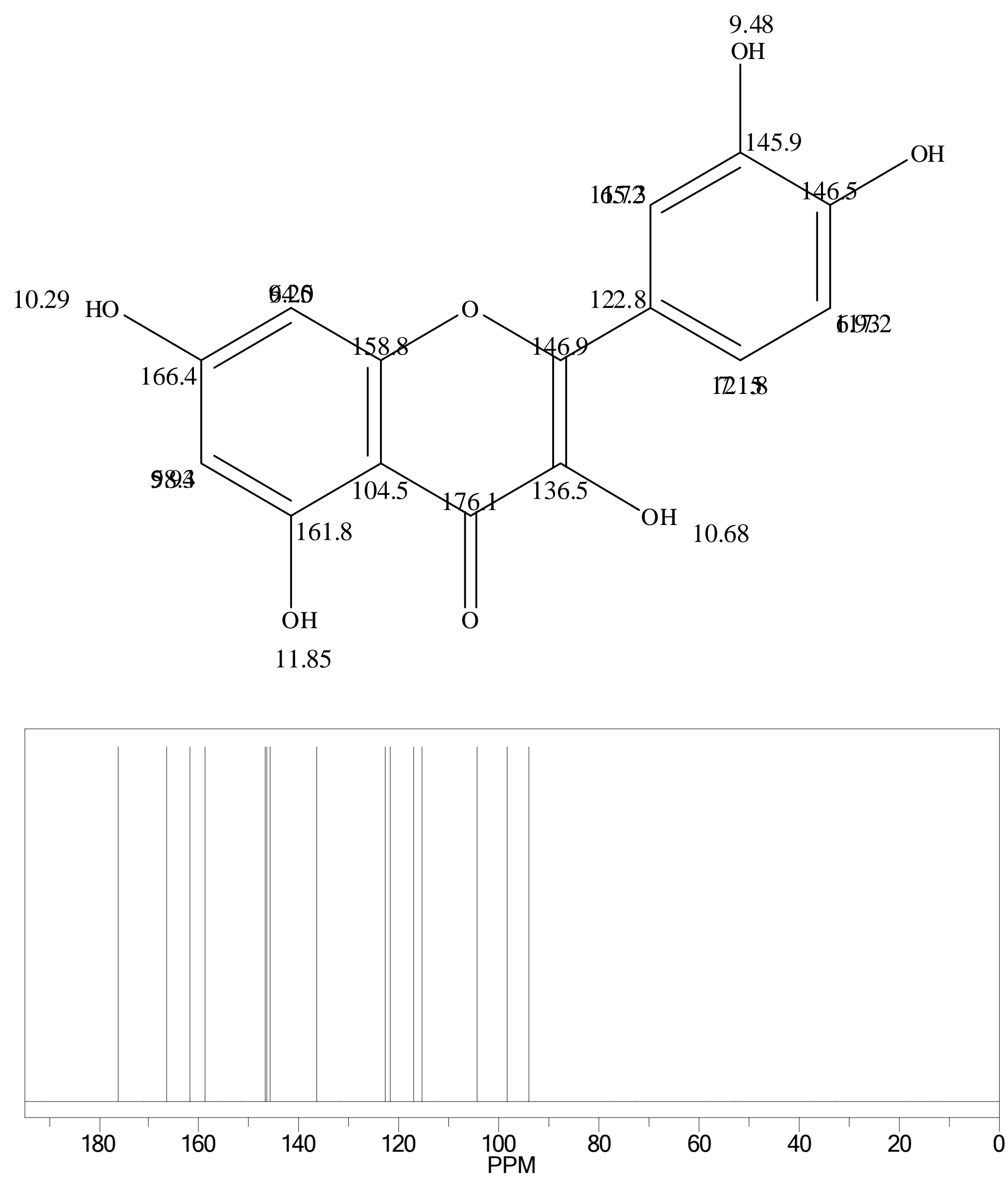


Fig. 24 ^{13}C NMR of compound - II

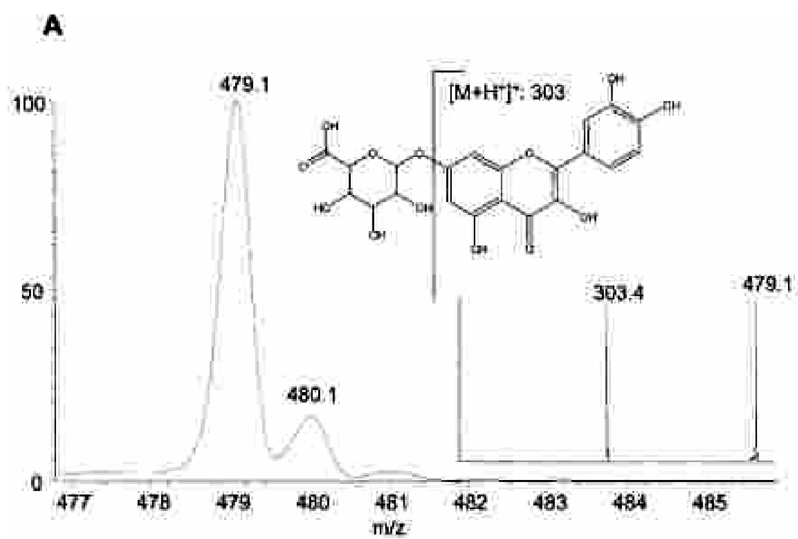


Fig. 25 Mass of compound - II

5.1.c. Characterization of compound-III

The ethanol extract of *Cassytha filiformis* was suspended in distilled water (100 ml) and was fractionated successively in a separating funnel with n-hexane petroleum ether (60°-80° C), chloroform, ethyl acetate and methanol in that order. The fraction was washed with distilled water, dried over anhydrous sodium sulphate and the solvent was removed by distillation under reduced pressure.

The dark greenish residue of ethyl acetate fraction was dissolved in methanol and was then loaded on a silica gel column. The column was eluted with 100% ethyl acetate followed by graded mixtures of 1%, 2%, 3%, 5% methanol in ethyl acetate. Elution of the different fraction was monitored by TLC silica-gel (ethyl acetate : methanol : water; 100 : 16.5 : 13.5) and visualized with UV/NH_3.

The eluate obtained with 100% ethyl acetate and 1% methanol in ethyl acetate showed the presence of a single spot on the TLC. The eluate on concentration deposited a yellowish brown colour compound. The compound was designated as compound-III, which was further purified and recrystallized with acetone. The solid precipitate was subjected to spectroscopic studies for its characterization.

The homogeneity of compound-III was proved by melting point and a single spot in TLC and HPTLC using pre-coated plates of silica gel GF_{254} as adsorbent and solvent mixtures of different polarities as developers. The solvent systems used and their corresponding R_f values are shown below.

S. No.	Mobile Phase	No. of spots	R_f value
1	Ethyl acetate:formic acid:glacial acetic acid:water (100:11:11:27)	Single	0.65
2	n-Butanol:glacial acetic acid:water(40:10:50)	Single	0.62
3	n-Butanol:ethyl acetate:water (4:1:2.2)	Single	0.59

The melting point of isolated compound-III was found to be in between 217-219°C (uncorrected). The isolated compound-III was subjected to UV, IR, ^{1}H NMR, ^{13}C NMR, and mass spectroscopic analysis to find out the structure. From the spectral studies of compound-III it was confirmed as quercetin as that of compound-II of *Cuscuta reflexa.*

The aqueous extract of *Cuscuta reflexa* and ethanol extract of *Cassytha filiformis* were subjected to column chromatography and fractionation for isolation of its active constituents. The aqueous extract of *Cuscuta reflexa* was chromatographed on silica gel column. Elution was carried out with solvents and solvent mixtures of increasing polarity. Fractions were collected in 5 ml portions and monitored by TLC and the fractions showing similar spots were combined. Three compounds were isolated and their purity was confirmed as single spot by TLC using three different solvent systems. The melting points of compound-I was 207-209ºC and its R_f was 0.48. The melting points of compound-II and compound-III was 216-218ºC and. 217-219ºC and its R_f value was 0.61 and 0.63 respectively. These compounds were subjected to spectral analysis viz., UV, IR, NMR and mass spectrum. From the spectral data compound-I was characterized as chlorogenic acid. The compound-II and compound-III was characterized as quercetin.

5.C. *In vivo* hepatoprotective studies

5.1.a. Biochemical studies

The ethanol, 50% methanol, and aqueous extracts of *Cuscuta reflexa* and *Cassytha filiformis* were evaluated for its hepatoprotective activity against d-galactosamine induced hepatic damage. Healthy adult male albino rats of *Wistar* strain weighing 180-220 g were used for the study. Silymarin was used as positive control. The plant extracts at two different dose levels (200, 400 mg/kg, po), showed significant hepatoprotective activity as evidenced by an alteration in the serum enzyme levels.

The effect of *Cuscuta reflexa* and *Cassytha filiformis* at both the dose levels on marker enzymes in serum against d-galactosamine induced hepatotoxicity were shown in Table-18. Liver damage induced by d-galactosamine significantly increased the marker enzymes like AST, ALT and ALP in serum ($P<0.05$). Oral administration of the plant extracts of *Cuscuta reflexa* and *Cassytha filiformis* significantly decreased the level of marker enzymes AST, ALT and ALP ($P<0.01$) in serum. The total bilirubin level was significantly increased ($P<0.05$) in d-galactosamine treated animals. The plant extracts (*Cuscuta reflexa* and *Cassytha filiformis)* treated animals showed a significantly lower bilirubin level in serum. The total protein level in serum was considerably reduced in d-galactosamine toxicity. The plant extracts (*Cuscuta reflexa* and *Cassytha filiformis)* treated animals significantly increased ($P<0.05$) the total protein level in serum (Fig. 27, 28).

In the assessment of liver damage by d-galactosamine, the determination of enzyme levels is largely used. The changes observed with viral hepatitis are also seen in d-galactosamine administration[135] Hence d-galactosamine induced liver toxicity was chosen as the experimental model. The ability of the liver protective drugs to reduce the

injurious effects or to preserve the normal hepatic physiological mechanisms, which had been disturbed by the hepatotoxin, is the index of its protective effect[136]. The enzymes like AST and ALT are cytoplasmic in origin and necrosis or membrane damage releases the enzymes into circulation and therefore can be measured in the serum [137]. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver[138]. In addition, destruction of hepatic cells causes an elevation in the serum levels of acid phosphatase (ACP) and bilirubin. ACP is localized almost exclusively in the particles and its release parallels that of lysosomal hydrolases. Increase in the serum level of ALP is due to increased synthesis, in the presence of increasing biliary pressure.

The present study revealed a significant increase in the marker enzymes like AST, ALT, ALP and serum bilirubin levels, on exposure to d-galactosamine, indicating considerable hepatocellular injury. Oral administration of C*uscuta reflexa* and *Cassytha filiformis* at two different dose levels attenuated the increased levels of the marker enzymes produced by d-galactosamine and caused a subsequent recovery towards normalization almost like that of standard silymarin treatment. The decreased total protein level observed in the rats treated with d-galactosamine may be due to the decrease in the number of hepatocytes which in turn may result in decreased hepatic capacity to synthesis protein[139]. On administration of extracts C*uscuta reflexa* and *Cassytha filiformis* showed significant increase in total protein level, which indicates the increase in hepatocyte levels, accounting for its hepatoprotective effect. The subsequent recovery towards normalization of these enzymes strongly suggest the possibility of the extracts being capable of conditioning the hepatocytes so as to cause accelerated regeneration of

parenchymal cells. The results showed that the extracts of the plant drugs *Cuscuta reflexa* and *Cassytha filiformis* at different dose levels offer hepatoprotection.

Table 18

Effect of the extracts on serum transaminases, alkaline phosphatase, total protein and bilirubin on control and experimental animals

Group	Drug and dose (mg\kg)	AST (U/L)	ALT (U/L)	ALP (U/L)	TP (mg/dl)	TB (mg/dl)
1.	Normal control (1% SCMC 10 ml/ kg)	45.12±0.86	31.6±2.39	124.40±19.15	6.46±0.15	0.49±0.01
2.	Hepatotoxicant d-galactosamine (600 mg/kg)	98.32±8.62[a]	79.85±3.62[a]	236.80±12.65[a]	0.98±0.24[a]	1.85±0.12[a]
3.	Aqueous extract of *Cuscuta reflexa* (200 mg/kg)	67.54±2.80[b]	62.81±2.30[d]	184.55±15.63[d]	4.86±1.25[c]	1.04±0.02[b]
4.	Aqueous extract of *Cuscuta reflexa* (400 mg/kg)	54.36±2.56[b]	56.32±8.65[b]	167.40±10.96[b]	5.12±0.86[c]	0.76±0.01[b]
5.	50% methanol extract of *Cuscuta reflexa* (200 mg/kg)	69.32±1.36[b]	65.60±6.35[d]	202.50±9.86[d]	3.64±1.21[d]	0.98±0.02[b]
6.	50% methanol extract of *Cuscuta reflexa* (400 mg/kg)	52.15±3.86[b]	57.30±4.65[b]	175.40±9.42[c]	4.85±0.92[c]	0.64±0.02[b]
7.	50% methanol of *Cassytha filiformis* (200 mg/kg)	59.86±1.32[b]	68.71±3.60[d]	182.40±13.56[c]	4.97±0.64[c]	1.12±0.12[b]
8.	50% methanol of *Cassytha filiformis* (400 mg/kg)	53.96±2.68[b]	59.82±4.30[b]	162.30±19.50[b]	4.84±1.39[c]	0.77±0.03[b]
9.	Ethanol extract of *Cassytha filiformis* (200 mg/kg)	64.35±1.45[b]	67.90±3.25[d]	173.50±11.50[c]	3.12±0.84[d]	1.02±0.12[b]
10.	Ethanol extract of *Cassytha filiformis* (400 mg/kg)	51.36±2.68[b]	49.30±2.80[b]	159.63±12.84[b]	4.84±1.29[c]	0.64±0.11[b]
11.	Positive control (silymarin) (25 mg/kg)	49.45±2.86[b]	42.95±2.38[b]	149.56±5.26[b]	5.12±0.82[c]	0.54±0.01[b]

Values are expressed as mean ± SEM; n = 6 in each group. [a]P <0.01 when compared to control; [b]P <0.01, [c]P<0.05 and [d]P>0.05 when compared to d-galactosamine control (one way ANOVA followed by Dunnett’s test).

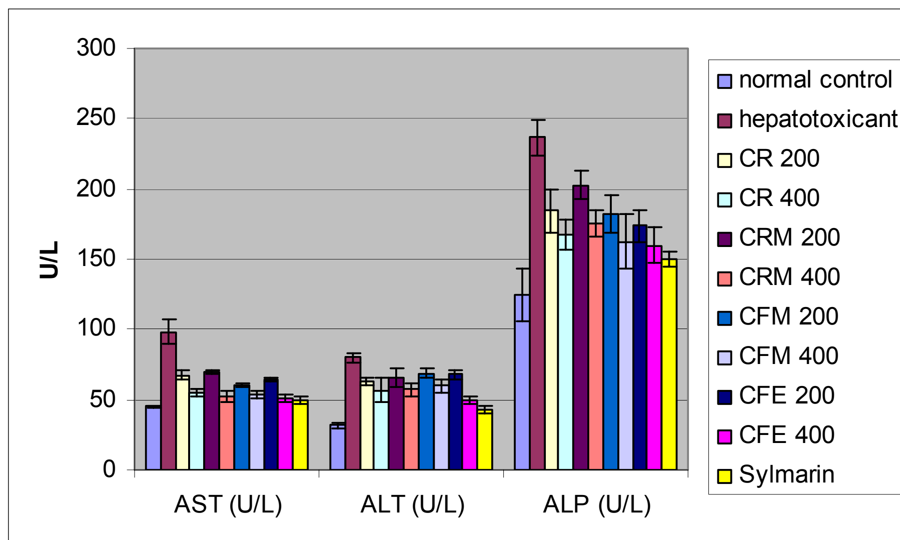


Fig. 26 Biochemical parameters of AST, ALT, ALP

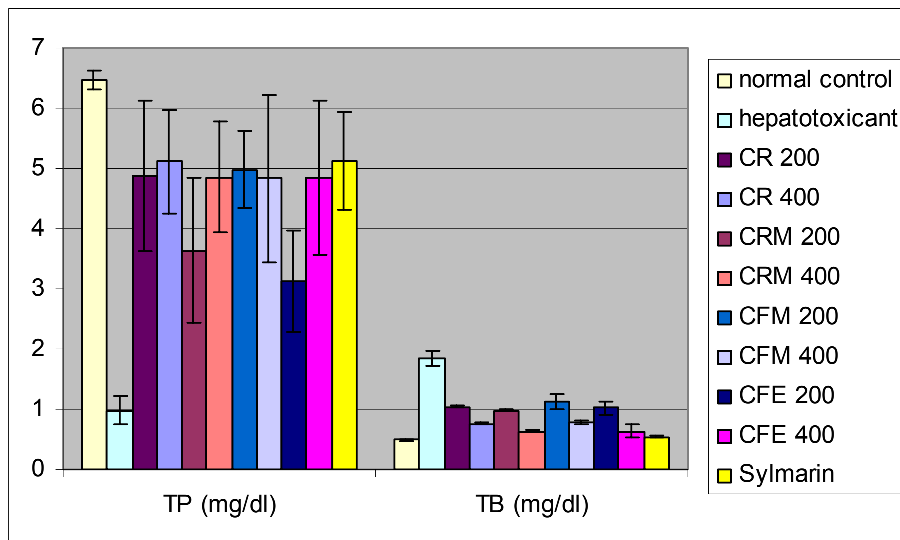


Fig. 27 Biochemical parameters of TP, TB

5.1.b. Histopathological studies

The histopathological profile of the rat liver of the normal control group showed normal liver with central vein and cords of hepatocytes.

Liver of the rat treated with hepatotoxicant d-galactosamine showed severe hepatocellular degeneration with hepatocytes showing fatty changes. Hepatocytes showed early degenerative changes. The rat group treated with hepatotoxicant and aqueous extract of *Cuscuta reflexa* at a dose level of 200 mg/kg body weight showed mild hepatocellular degeneration and that of the dose level 400 mg/kg body weight showed mild perilobular hepatocellular fatty changes.

The liver sections of the group treated with hepatotoxicant and 50% methanol extract of *Cuscuta reflexa* at a lower dose of 200 mg/kg body weight showed dilated central vein and mild perilobular hepatocellular fatty changes and at a higher dose of 400 mg/kg body weight showed mild hepatocellular fatty changes.

The rat group treated with hepatotoxicant and ethanol extract of *Cassytha filiformis* at a dose level of 200 mg/kg body weight showed less fatty changes and vacuolated hepatocytes and that of the dose level 400 mg/kg bodyweight showed normal central vein with mild hepatocytic fatty changes. The liver sections of the group treated with hepatotoxicant and 50% methanol extract of *Cassytha filiformis* at a lower dose of 200 mg/kg body weight showed dilated central vein with less hepatocytic fatty changes and at a higher dose of 400 mg/kg body weight showed mild hepatocytes with fatty changes. The treatment of 25 mg/kg body weight of silymarin as positive control showed normal central vein with mild hepatocytic fatty changes. The histopathological effect of *Cuscuta reflexa* and *Cassytha filiformis* on d-galactosamine induced liver injury in rats are shown in Table 19 (Fig. 28-38).

Exogenous administration of d-galactosamine has been found to induce liver damage, which closely resembles human viral hepatitis[140]. After a single injection with d-galactosamine, the uracil nucleotides are

considerably decreased in the liver[141]. The toxicity of d-galactosamine causes cell injury by depleting uridine nucleotides resulting in inhibition of RNA and protein synthesis in the liver leading to necrosis[142].

The metabolism of d-galactosamine may deplete several uracil nucleotides including UDP-glucose, UDP-galactose and UTP which are trapped in the formation of uridinediphosphogalactosamine[143]. Accumulation of UDP-sugar nucleotides[144] may contribute to the changes in the rough endoplasmic reticulum and to the disturbance of protein metabolism. Further, intense galactosamination of membrane structures is thought to be responsible for loss in the activity of ionic pump. The impairment in the calcium pump, with consequent increase in the intracellular calcium is considered to be responsible for cell death. An evidence of hepatic injury is leakage of cellular enzymes into the plasma. When liver cell plasma membrane is damaged, a variety of enzymes normally located in the cytosol are released into the blood stream. Their estimation in the serum is useful quantitative marker of the extent and type of hepatocellular damage.

In present study d-galactosamine in larger dose produced liver necrosis. This may be due to depletion of several uracil nucleotides including UDP-glucose, UDP-galactose and UTP. This resulted in elevation in levels of quantitative markers in serum (i.e.) AST, ALT, ALP and total bilirubin[143]. Pretreatment with the extracts of the plants offered hepatoprotection as evidenced by an inhibition in the elevated AST, ALT, ALP and total bilirubin levels. The histopathological observations supported the evidence.

On the basis of results obtained in the present investigation, it can be concluded that the histopathological study of the liver of the extract treated rats showed normal structure which also confirms the

hepatoprotective nature of the extracts. The hepatoprotective property of the extracts may be attributed to the presence of flavanoids, which are present in the plant. Several phenols and flavanoids are reported from the plants taken for study[59,60,66,83]. A large number of these compounds are known to possess strong antioxidant and hepatoprotective properties[71,72]. Hence, the observed antioxidant and hepatoprotective activity of the various extracts of *Cuscuta reflexa* and *Cassytha filiformis* may be due to the presence of any of these compounds.

Table 19

Histopathological effect of *Cuscuta reflexa* and *Cassytha filiformis* against d-galactosamine induced liver injury in rats.

Group	Dose	Observation
1	Normal control	Normal liver with central vein and cords of hepatocytes.
2	D-Galactosamine (600 mg/kg)	Hepatocellular degeneration with fatty changes.
3	Aqueous extract of *Cuscuta reflexa* (200 mg/kg)	Mild hepatocellular degeneration
4	Aqueous extract of *Cuscuta reflexa* (400 mg/kg)	Mild perilobular hepatocellular fatty changes.
5	50% methanol extract of *Cuscuta reflexa* (200 mg/kg)	Dilated central vein mild perilobular hepatocellular fatty changes.
6	50% methanol extract of *Cuscuta reflexa* (400 mg/kg)	Mild hepatocellular fatty changes.
7	50% methanol extract of *Cassytha filiformis* (200 mg/kg)	Less fatty changes, vacuolated hepatocytes.
8	50% methanolic extract of *Cassytha filiformis* (400 mg/kg)	Normal central vein with mild hepatocytic fatty changes.
9	Ethanol extract of *Cassytha filiformis* (200 mg/kg)	Dilated central vein with less hepatocytic fatty changes.
10	Ethanol extract of *Cassytha filiformis* (400 mg/kg)	Mild hepatocytes with fatty changes.
11	Silymarin (25 mg/kg)	Normal central vein with mild hepatocytic fatty changes.

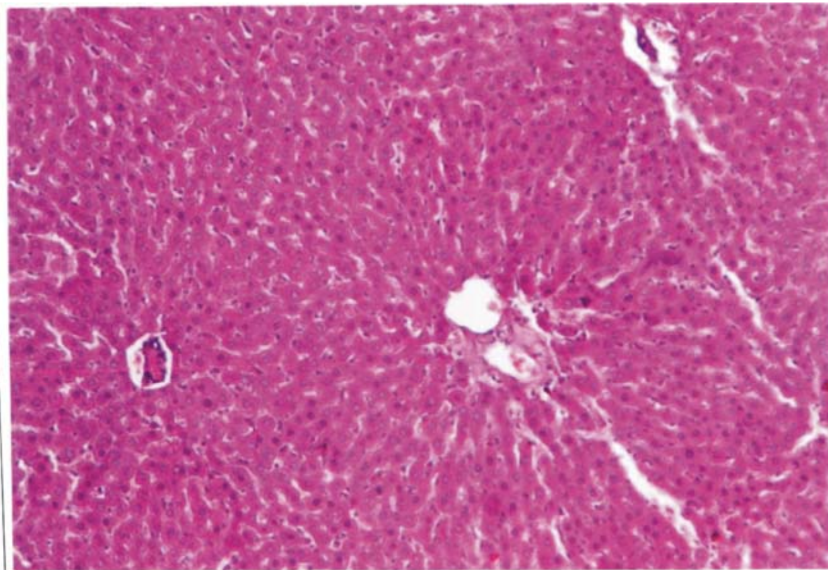


Fig. 28 Liver section of group 1 rat (normal), showing normal liver with central vein and cords of hepatocytes. H & E ×100

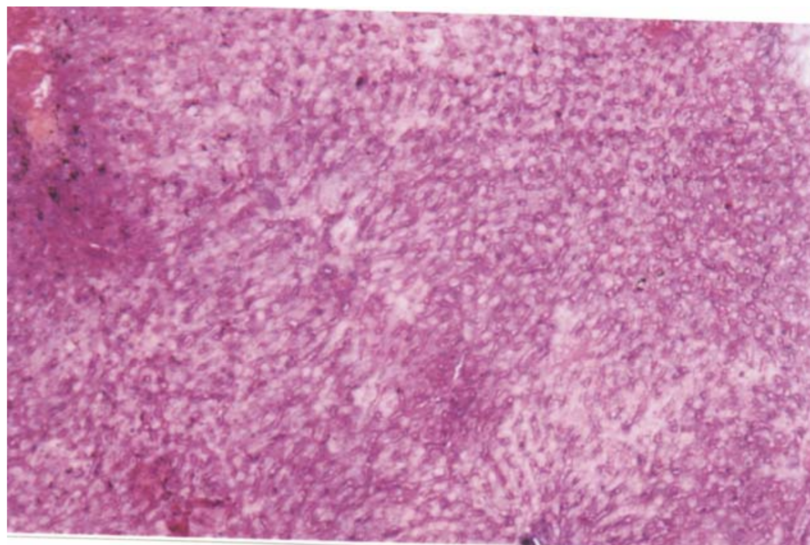


Fig. 29 Liver section of group 2 rat (hepatotoxicant d-galactosamine), showing hepatocellular degeneration with fatty changes. H & E ×100

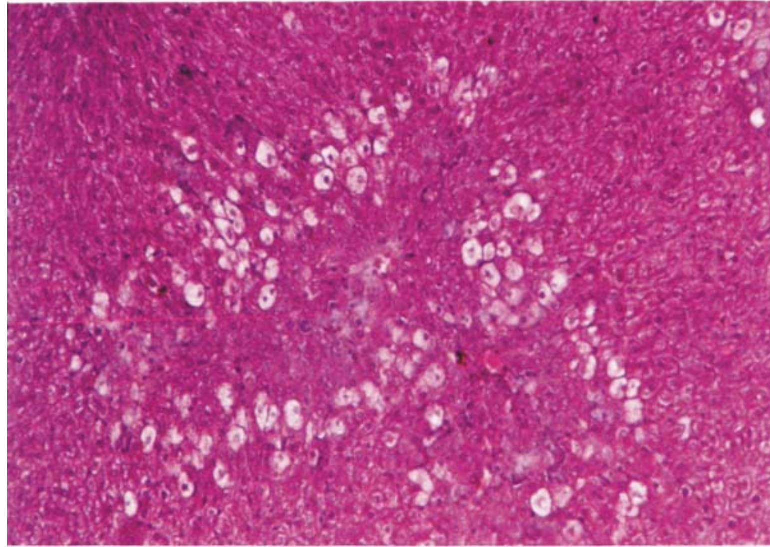


Fig. 30 Liver section from group 3 rat treated with aqueous extract of *Cuscuta reflexa* (200 mg/kg), showing mild hepatocellular degeneration. H & E ×100

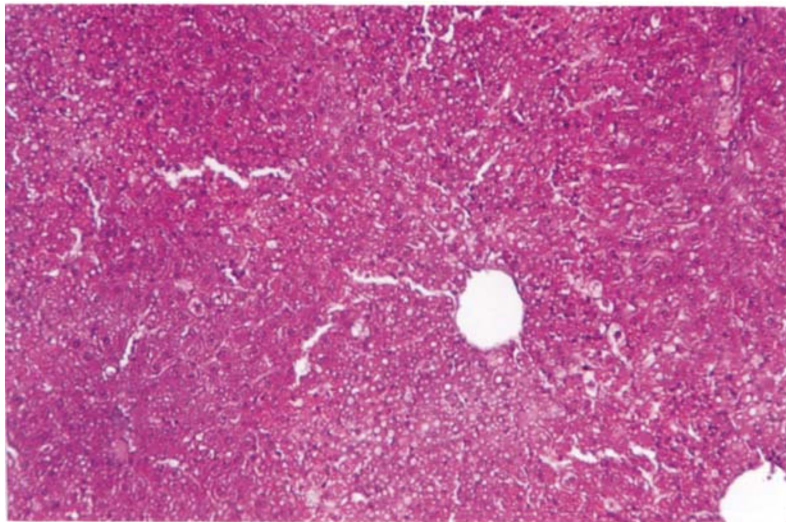


Fig. 31 Liver section from group 4 rat treated with aqueous extract of *Cuscuta reflexa* (400 mg/kg), showing mild perilobular hepatocellular fatty changes degeneration. H & E × 100

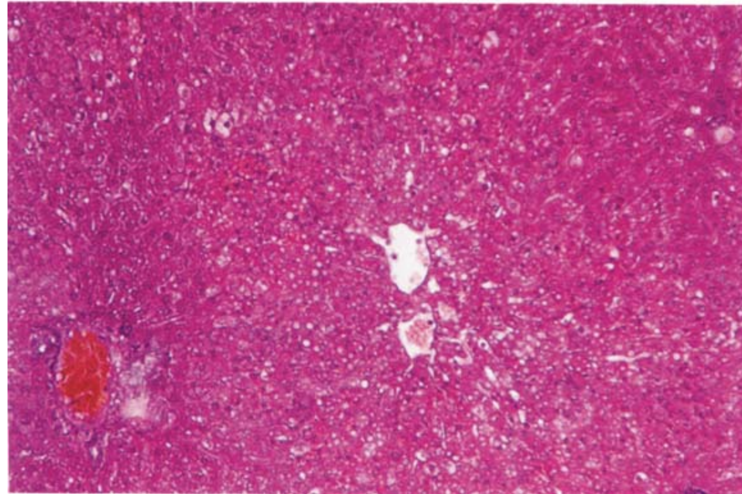


Fig. 32 Liver section from group 5 rat treated with 50% methanol extract of *Cuscuta reflexa* (200 mg/kg), showing dilated central vein with perilobular hepatocellular fatty changes. H & E × 100

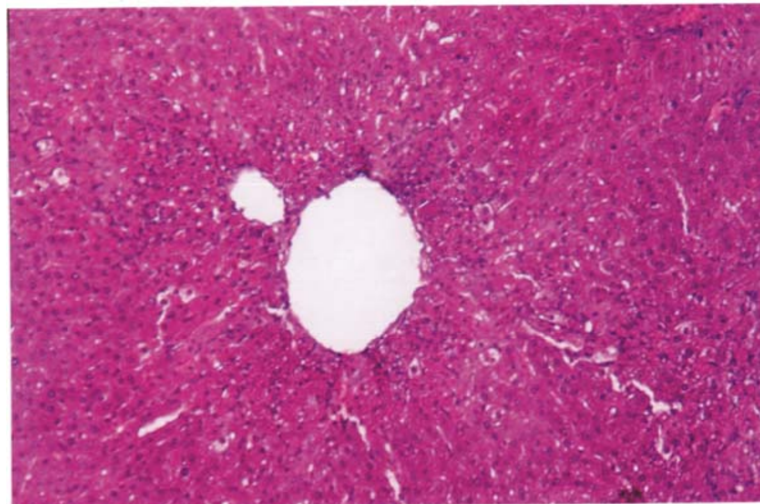


Fig. 33 Liver section from group 6 rat treated with 50% methanol extract of *Cuscuta reflexa* (400 mg/kg), showing mild hepatocellular fatty changes. H & E × 100

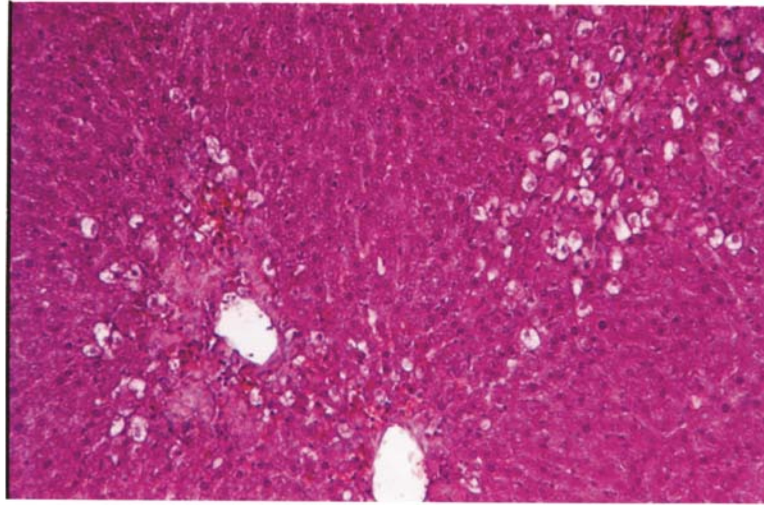


Fig. 34 Liver section from group 7 rat treated with 50% methanol extract of *Cassytha filiformis* (200 mg/kg), showing Less fatty changes vacuolated hepatocytes. H & E ×100

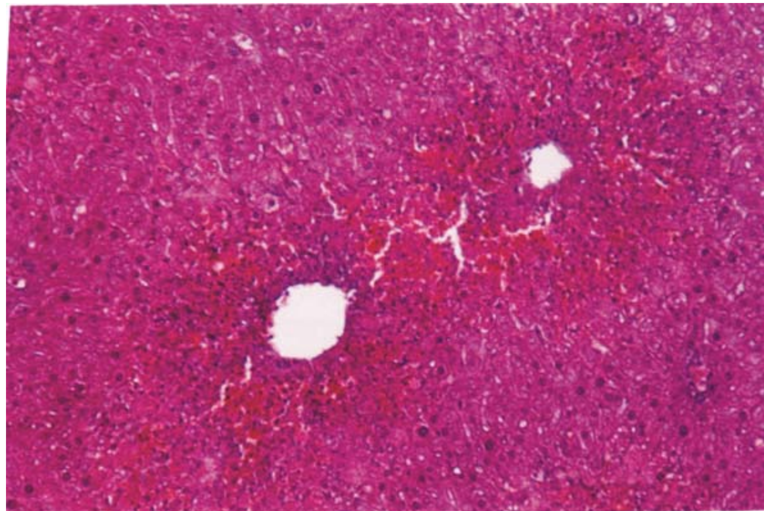


Fig. 35 Liver section from group 8 rat treated with 50% methanol extract of *Cassytha filiformis* (400 mg/kg), showing Normal central vein with mild hepatocytic fatty changes. H & E ×100

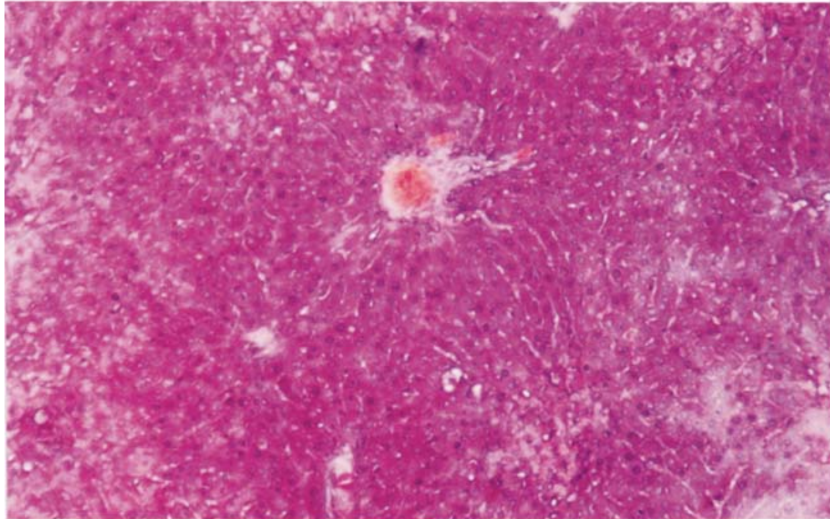


Fig. 36 Liver section from group 9 rat treated with ethanol extract of *Cassytha filiformis* (200 mg/kg), showing diliated central vein with less hepatocytic fatty changes. H & E ×100

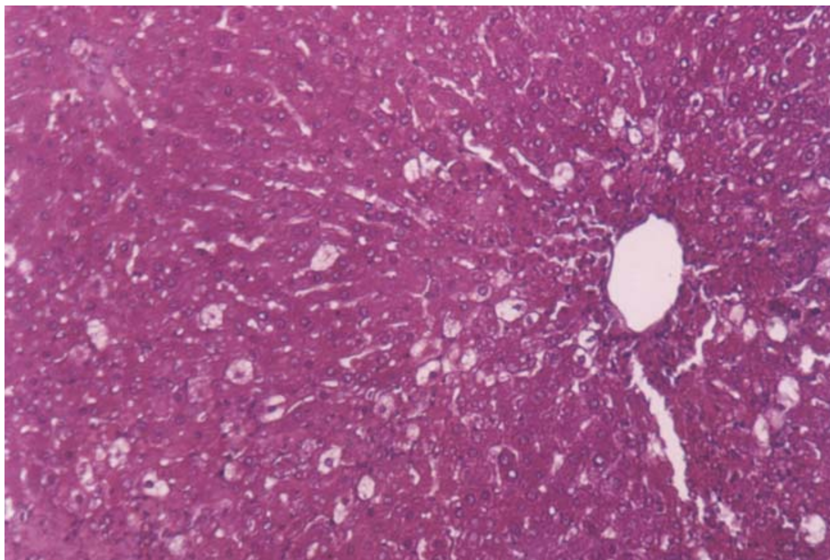


Fig. 37 Liver section from group 10 rat treated with ethanol extract of *Cassytha filiformis* (400 mg/kg), showing mild hepatocytes with fatty changes. H & E ×100

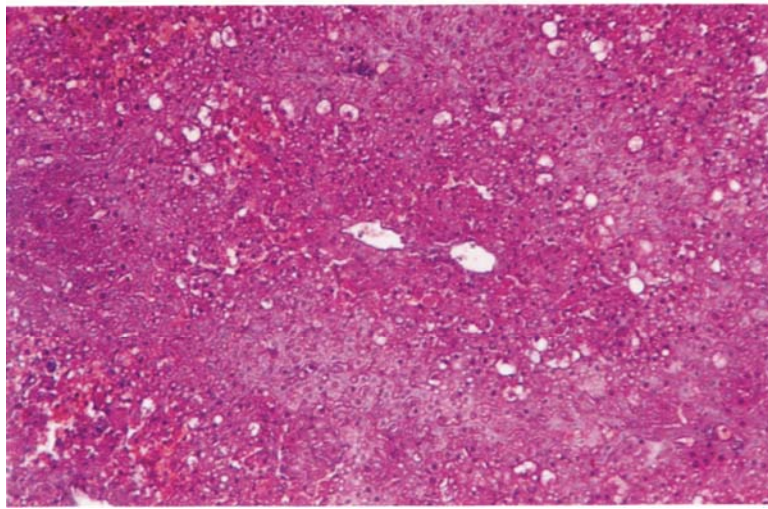


Fig. 38 Liver section from group 11 rat (positive control) treated with Silymarin (25 mg/kg), showing normal central vein with mild hepatocytic fatty changes. H & E ×100

Summary & Conclusion

SUMMARY AND CONCLUSION

The first chapter has been studied under different parts. The first part deals with the usefulness of plants to mankind, a brief introduction to herbal drugs where plants and phytoconstituents have been suggested as good alternatives to synthetic drugs. The next part deals with liver and its functions, liver disorders, reasons for liver disorders and finally the experimental models for the evaluation of antihepatotoxic activity. The last part is about reviews on plants used in general as hepatoprotectants to cure liver disorders and antioxidant activity of plants.

The second chapter of the thesis explains the scope and objective of the present investigation in detail with the name of the plants selected to evaluate for their hepatoprotective activity. The plan of work in brief has also been explained in this chapter.

The next chapter deals with the plant profile, ethnomedical/tribal literature information, chemical and pharmacological reviews of the selected two plants of my study.

The subsequent experimental or materials and methods chapter of the thesis has been broadly divided into three sections. a) Pharmacognostical studies b) Chemical studies c) Pharmacological studies.

The first section deals with collection, authentication, drying and powdering of the plant *Cuscuta reflexa* and *Cassytha filiformis*. The two plant materials were collected from different parts of Chennai, Tamil Nadu and authenticated. The dried and powdered plant materials were extracted with suitable solvents. The extraction procedures like cold maceration done for the powdered plant materials and standardisation of plant extracts had

also been explained. Fresh plant materials were used for morphological and microscopical evaluation.

The next part of the work was carried out in various phases, which includes pharmacognostical evaluation in order to establish the identity and to standardize the plant materials. Morphological and microscopical characters were also studied.

Physicochemical parameters like total ash, acid insoluble ash, water soluble ash, sulphated ash values, alcohol soluble extractive and water soluble extractive values were determined.

Fluorescence analysis was carried out for the plant powder and their extracts by treating with several reagents. The colour changes were observed under UV and visible lights. In the micro chemical analysis, the plant powders were treated with several reagents, and the colour changes were observed under microscope.

Estimation of total phenolic and flavanoid content was done for all the extracts of both the plant materials.

The preliminary phytochemical analysis of the plant powders and extracts were carried out.

The section b of materials and methods deals with the isolation of phytoconstituents from the plant extracts using column chromatography by gradient elution method with solvents like n-hexane, petroleum ether, chloroform, ethyl acetate and methanol. The plant extracts were subjected to TLC and HPTLC analysis using several solvent systems.

An *in vitro* antioxidant activity studies were also done for various extracts by different methods.

The final section c of the chapter deals with the pharmacological investigation such as selection of animals and dose, acute toxicity studies, experimental model to damage liver with hepatotoxicant d-galactosamine,

experimental design for screening of the plant extracts for *in vivo* hepatoprotective activity, their biochemical, histopathological parameters and finally statistical analysis for the biochemical parameters.

The following chapter of the thesis deals with the results and discussion pertaining to the present study have been elaborated separately under corresponding sections of the previous chapter such as 5a, 5b and 5c supported by tables and figures.

Some of the important findings of the present study based on the results are:

- The phytochemical studies of the plant extracts showed the presence of alkaloids, glycosides, triterpenoids, flavanoids, saponins, carbohydrates, tannins, gums and mucilage. These results gave clues regarding the presence of some particular phytoconstituents in the respective plant extracts.
- Isolated compounds were subjected to spectral studies to identify the structures. The structure confirms the presence of flavanoids like quercetin.
- The *in vitro* antioxidant activity of the plant extracts exhibited potent antioxidant activity with low IC_{50} values in DPPH, reducing power ability, Nitric oxide and phosphomolybdate scavenging methods.
- The *in vivo* screening for hepatoprotective activity of both the plant extracts exhibited significant activity against d-galactosamine as hepatotoxicant.

Hence in our study the significant hepatoprotective activity and *in vitro* antioxidant activity of the various extracts may be attributed to the presence of flavanoid like quercetin. From the study carried out, it is evident that both the plants are endowed with significant hepatoprotective

activity against d-galactosamine induced hepatic injury, thereby justifying its use for their hepatoprotective activity in the traditional system of medicine.

Finally I would like to conclude that my present study throws immense light on two angiosperm-parasitic taxa with reference to pharmacognostical, phytochemical and biological aspects enriching with new vistas.

Bibliography

BIBLIOGRAPHY

1. Naranjo P. The urgent need for the study of medicinal plants. In: Schultes RE, Reis SV, editors. Ethnobotany: Evolution of a discipline. London: Chapman & Hall; 1995. p. 362-68.

2. Gottlieb OR. Micromolecular evolution, systematics and ecology. Berlin: Springer-Verlag; 1982. p. 789.

3. Rao KC, Sangeetaa W. Alternate sweeteners. Vatika. 1993;4:193.

4. Schmeltz I. Naturally occurring insecticides. In: Jacobson M, Crosby DG, editors. Naturally occurring insecticides. New York: Dekker; 1971. p. 99-136.

5. Cassady JM, Chang CJ, McLaughlin JE. Recent advances in the isolation and structural elucidation of antineoplastic agents in higher plants. In: Beal JL, Reinhard E, editors. Natural products as medicinal agents. Suppl. Planta Medica. Stuttgart: Hippokrates Verlag; 1981. p. 93-124.

6. Naranjo P. Farmacologia y medicina tradicional. In: Samaniego E, Escaleras R, editors. Fundamentos de Farmacologia Medica. Quito: Univ. Central; 1981.

7. WHO (World Health Organisation). Promocion y desarrolla de la medicina tradicional. Ginebra; 1978.

8. Pushpangadan P. Role of traditional medicine in primary health care. In: Iyengar PK, Damodaran VK, Pushpangandan P, editors. Science for health. Thiruvanathapuram: State Committee on Science, Technology and Environment. Govt of Kerala; 1995. p. 24-8.

9. Mills S, Bone K. Principles and practice of phytotherapy. New York: Churchill LivingStone; 2000. p. 17 – 20.
10. Padh H. A report of the seminar on herbal drugs present status and future prospects. Curr Sci. 2001 Jul 10;81(1):15.
11. Shrikumar S, Ravi TK. Approaches towards development and promotion of herbal drugs. Phcog Rev. 2007 Jan-May;1(1):180-83.
12. Muttikkal JJ, Parambi DGT. Research guidelines for evaluating the safety of herbal medicines. Indian J Nat Prod. 2006;23(1):3-7.
13. Thakkar NH, Bhatt SP. Herbal medicine: Impression for moment. Adv Pharmacol Toxicol. 2008;9(1):9,10.
14. Handa SS. Future trends of plants as drugs. Pharma Times. 1991;4:13-23.
15. Subramoniam A, Pushpangadan P. Development of phytomedicines for liver diseases. Indian J Pharmacol. 1999;3(3):166-175.
16. Singh D, Gupta RS. Hepatoprotective activity of medicinal plants. In: Prof Trivedi PC, editor. Indian folk medicine. Jaipur: Pointer Publishers; 2007. p. 21-57.
17. Veeresham C, Asres K. Antioxidants of plant origin. Indian J Nat Prod. 2000 Dec;21(4):3-18.
18. Thibodeau GA, Patton TP. Anthony`s Textbook of anatomy and physiology.17th ed. New Delhi: Elsevier; 2004. p. 757-59.
19. Tortora GJ, Grabowski SR. Principles of anatomy and physiology. 10th ed. Hoboken(NJ): John Wiley & Sons Inc; 2003. p. 880.

20. Podolsky DL, Isselbacher KJ. Diagnostic tests in liver. In: Isselbacher KJ, Braunwald E, Wilson JD, Martin JB, Fauci AS, Kasper DL, editors. Harrison`s Principles of internal medicine. Vol 2. 13th ed. New Delhi: Mc Graw-Hill Inc, Health Professions Division; 1994. p. 1444-46.

21. Ward FM, Daly MJ. Liver disease. In: Walker R, Edwards C, editors. Clinical pharmacy and therapeutics. 3rd ed. Edinburgh: Churchill Living Stone; 2003. p. 209-13.

22. Waugh A, Grant A. Ross and Wilson Anatomy and physiology in health and illness. 9th ed. London: Churchill Living Stone; 2001. p. 333-35.

23. Zimmerman HJ. Hepatotoxicity. New York: Appleton Century Crofts; 1978. p. 32,210,213,10,169-179.

24. Mcintyre N, Benhamou JP, Bircher J, Rizzetto M, Rodes J. Oxford text book of clinical hepatology. Vol 2. 2nd ed. New York: Oxford University Press; 1991. p. 907-09.

25. Mitchell JR, Jollow DJ, Potter WZ, Davis DC, Gillette JR, Brodie BB. Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. J Pharmacol Exp Ther.1973 Oct;187(1):185-94.

26. Keppler D, Lesch R, Reutter W, Decker K. Experimental hepatitis induced by d-galactosamine. Exp Mol Pathol. 1968 Oct;9(2):279-90.

27. Feo C, Garcon E. Study of steatosis induced in the rat by orotic acid. Biomedicine. 1973 Feb 10;19(2):61-4.

28. Faber E. Ethionine fatty liver. Adv Lipid Res. 1967;5:119-83.

29. Sharma A, Singh RT, Sehgal V, Handa SS. Antihepatotoxic activity of some plants used in herbal formulations. Fitoterapia. 1991;62:131.

30. Jain SP, Takade AR, Joshi UM, Kale RH, Prohit R. N. Protective effect of *Gingko biloba* on antitubercular drugs induced hepatotoxicity in rats. Indian Drugs. 2005;42(3):167-71.

31. Wealth of India. A Dictionary of Indian raw materials and industrial products. Vol 3. Ci-Cy. New Delhi: Publication and Information Directorate CSIR; 1992. p. 296,297.

32. Kirtikar KR, Basu BD. Indian Medicinal Plants. 2nd ed. Vol 3. Dehradun: International Book Distributors; 1999. p. 1741,1742,2163,2164.

33. Warrier PK, Nambiar VPK, Ramankurtty C. Indian Medicinal Plants. Vol 2. Chennai: Orient Longman; 1994. p. 262-64.

34. Nadkarni's KM. Indian Materia Medica. Vol 1. Bombay: Popular Prakashan; 1976. p. 419-20,292.

35. The Useful Plants of India. New Delhi: Publication and Information Directorate CSIR; 1986. p. 152,110.

36. Chopra RN, Nayar SL, Chopra IC. Glossary of Indian Medicinal Plants. New Delhi: Publication and Information Directorate CSIR; 1956. p. 85,55.

37. Matthew KM. The Flora of the Tamilnadu Carnatic. Vol 3. Tiruchirapalli: The Rapinat Herbarium St. Joseph's College; 1983. p. 1013,1014,1358.

38. Wealth of India. A dictionary of Indian raw materials and industrial products. Vol 3. Ca-Ci. New Delhi: Publication and Information Directorate CSIR; 1992. p. 373,374.

39. Borah PK, Gogoi P, Phukan AC, Mahanta J. Traditional medicine in the treatment of gastrointestinal diseases in upper Assam. Indian Journal of Traditional Knowledge Oct 2006;5(4):510-12.

40. Gogi R, Borthakur SK. Notes on herbal recipes of Bodo tribe in Kamrup district, Assam. Ethno Botany. 2001;13:15-23.

41. Patil DA. Origin of medicines vis-à-vis doctrine of signatures. Ethno Botany 2004;16:52-58.

42. Mahato RB, Chaudhary.Ram P. Ethnomedicial plants of palpa district, Nepal. Ethno Botany. 2005;17:152-163.

43. Singh VK, Ali ZA, Siddiqui.MK. Folk herbal remedies of the kheri district forests (Uttar Pradesh) India. In: Singh VK, Govil.JN, Singh G, editors. Recent progress in medicinal plants, Ethnomedicine and Pharmacognosy. Vol 1. USA: Sci Tech Publishing LLC; 2002. p. 87-118.

44. Kumar S, Chauhan AKS. Medicinal plants used by local inhabitants in Bharatpur district, Rajasthan. Ethno Botany. 2005;17:179-183.

45. Girach RD, Aminuddin. Ethnomedicinal uses of plants among the tribals of Singbhum district, Bihar, India. Ethno Botany. 1995;7:103-7.

46. Maliya SD. Uses of some Indian plants in folk medicines of Nepal. Ethno Botany. 2005;17:202-4.

47. Kumar S, Chavhan AKS. Less known medicinal uses of plant species in Keoladeo national park, Bharatpur, Rajasthan. Ethno Botany. 2006;18:153-6.

48. Verma S, Chauhan NS. Studies on ethno-medico-botany of Kunihar forest division, district, Solan (HP). Ethno Botany. 2006;18:160-5.

49. Jain AK, Patole SN. Less- known medicinal uses of plants among some tribal and rural communities of Pachmarhi forest (MP). Ethno Botany. 2001;12:96-100.

50. Arjariya A, Rawat A. Birth control practice among rural and tribal women of Chhatarpur district (MP). In: Prabhuji SK, Rao GP, Patil S. K, editors. Recent advances in medicinal plant research: vision 21st century. New Delhi: Satish Publication House; 2005. p. 319-22.

51. Sahoo AK, Mudgal V. Less known ethnobotanical uses of plants of Phulbani district, Orissa, India. Ethno Botany. 1995;7:63-7.

52. Prusti AB. Plants used as ethnomedicine by Bondotribe of Malkan giri district, Orissa. Ethno Botany. 2007;19:105-10.

53. Deokota R, Chhetri RB. Ethno botanical study in Sunsari district of eastern Nepal. Ethno Botany. 2007;19:67-72.

54. Jadhav D. Ethnomedicinal plants used by Bhil tribe of Bibdod, Madhya Pradesh. Indian Journal of Traditional Knowledge. 2006 April;5(2):263-67.

55. Behera SK, Panda A, Behera SK, Misra MK. Medicinal plants used by the Kandhas of Kandhamal district of Orissa. Indian Journal of Traditional Knowledge. 2006 Oct;5(4):519-28.

56. Panda T, Padhy RN. Ethnomedicinal plants used by tribes of Kalahandi district Orissa. Indian Journal of Traditional Knowledge. 2008 April;7(2):242-49.

57. Katewa SS, Galav PK, Nag A, Jain A. Poisonous plants of the southern Aravalli hills of Rajasthan. Indian Journal of Traditional Knowledge. 2008 April;7(2):269-72.

58. Uddin SJ, Shilpi AJ, Middleton M, Byres M, Shoeb M, Nahar L, et al. Swarnalin and Cis-Swarnalin, two new tetrahydrofuran derivatives with free radical scavenging activity, from the aerial parts of *Cuscuta reflexa*. Nat Prod Res. 2007 Jun;21(7):663-68.

59. Tripathi VJ, Yadav SB, Upadhyay AK. A new flavanone reflexin, from *Cuscuta reflexa* and it selective sensing of nitric oxide. Appl Biochem Biotechnol. 2005 Oct;127(1):63-67.

60. Loffler C, Sahm AJ, Wray V, Czygan FC, Proksch P. Soluble phenolic constituents from *Cuscuta reflexa* and *Cuscuta platyloba*. Biochem Syst Ecol.1995;23(2):121-28.

61. Loffler C, Czygan CF, Proksch P. Phenolic constituents as taxonomic markers in the genus *Cuscuta* (Cuscutaceae). Biochem Syst Ecol. 1997;25 (4):297-303.

62. Tewari KK, Singh M. Acid Soluble and acid insoluble inorganic polyphosphates in *Cuscuta reflexa*. Phytochemistry. 1964;3:341-47.

63. Bhavsar GC, Pundarikakshudu K, Momin MM, Patel PJ. Cardiac glycosides from *Cuscuta reflexa* Roxb. grown on *Nerium indicum* Linn. Indian Drugs. 2003 May; 40(5):267-70.

64. Bhavsar GC, Pundarikakshudu K, Patel RK, Patel PJ. Accumulation of vasicine and vasicinone in *Cuscuta reflexa* Roxb. grown on *Adhatoda vasica* Ness. Indian Drugs. 2005 Jun;42(6):387,388.

65. Anis E, Anis I, Ahmed S, Mustafa G, Malik A, Afza N, et al. α-Glucosidase inhibitory constituents from *Cuscuta reflexa*. Chem Pharm Bull. 2002;50(1): 112-14.

66. Chatterjee U, Chauhan HOS, Sanwal GG. Physico-chemical and functional characterization of a high molecular weight carboxymethyl cellulase from *Cuscuta reflexa*. Indian J Biochem Biophys. 1997 Aug;34:354-364.

67. Anis E, Mustafa G, Ahmed S, Ullah N, Malik A, Atza N, et al. Sterols and sterol glycosides from *Cuscuta reflexa*. Nat Prod Sci. 1999;5(3):124-26.

68. Anis E, Ahmed Z, Mustafa G, Ullah N, Nadeem RZ, Malik A, et al. Long chain esters of oleane series from *Cuscuta reflexa*. Fitoterapia. 1998;69(5):474,475.

69. Mattoo RL, Mattoo PR. Effect of detergents on the alkaline phosphatase of *Cuscuta reflexa*. Phytochemistry. 1974;13:1727-29.

70. Srivastava S, Nighojkar A, Kumar A. Purification and characterization of starch phosphorylase from *Cuscuta reflexa* filaments. Phytochemistry. 1995;39(5):1001-05.

71. Srivastava S, Nighojkar A, Kumar A. Muitiple forms of pectin methylesterase from *Cuscuta reflexa* filaments. Phytochemistry. 1994;37(5):1233-36.

72. Yadav SB, Tripath V, Singh RK, Pandey HP. Flavanoid glycosides from *Cuscuta reflexa* stems and their antioxidant activity. Indian Drugs. 2001;38(2):95,96.

73. Yadav SB, Tripathi V, Singh RK, Pandey HP. Antioxidant activity of *Cuscuta reflexa* stems. Indian J Pharm Sci. 2000 Nov-Dec;62(6): 477,478.

74. Pal DK, Mandal M, Senthilkumar GP, Padhiari A. Antibacterial activity of *Cuscuta reflexa* stem and *Corchorus olitorius* seed. Fitoterapia. 2006;77:589-91.

75. Mazumder UK, Gupta M, Pal DK, Bhattacharya S. Induction of carbonic anhydrase by *Cuscuta reflexa* stem and *Corchorus olitorius* seed in mice. Indian J Pharm Sci. 2003 Jul-Aug;65(4):401-03.

76. Gupta M, Mazumder UK, Pal DK, Bhattacharya S. Onset of puberty and ovarian steroidogenesis following administration of methanolic extract of *Cuscuta reflexa* Roxb stem and *Corchorus olitorius* Linn seed in mice. J Ethnopharmacol. 2003;89:55–59.

77. Gupta M, Mazumder UK, Pal DK, Bhattacharya S. Anti-steroidogenic activity of methanolic extract of *Cuscuta reflexa* Roxb. Stem and *Corchorus olitorius* Linn. seed in mouse ovary. Ind J Exp Biol. 2003 Jun; 41: 641-44.

78. Pandit S, Chauhan NS, Dixit VK. Effect of *Cuscuta reflexa* Roxb. on androgen-induced alopecia. J Cosmet Dermatol. 2008 Sep;7(3):199-204.

79. Babu BH, Bindu AC, Jisha KG, Padikkala J. Cytotoxic and tumour reducing activity of *Cuscuta reflexa* a total stem parasite. Amala Res Bull. 2001;21: 92-8.

80. Poudel RC, Shrestha KK, Tiwari NN. Anti-hepatitis herbal formulations of traditional healers in mild hills of central Nepal. Proceedings of the 2nd world congress on biotechnological developments of herbal medicine;2003 Feb20th-22nd; Lucknow(UP): NBRI; 2003. p. 16.

81. Reddy ShS, Raju VS. Folklore biomedicine for common veterinary diseases in Nalgonda district, Andra Pradesh, India. Ethno Botany. 2000;12:113-17.

82. Rao DM, Pullaiah T. Ethno-medico-botanical studies in Guntur district of Andra Pradesh, India. Ethno Botany. 2001;13:40-44.

83. Wu YC, Chao YC, Chang FR, Chen YY. Alkaloids from *Cassytha filiformis*. Phytochemistry. 1997;46(1):181-84.

84. Chang FR, Chao YC, Teng CM, Wu YC. Chemical constituents from *Cassytha filiformis* II. J. Nat. Prod. 1998Jul;61(7):863-66.

85. Tsai TH, Wang GJ, Lin LC. Vasorelaxing alkaloids and flavanoids from *Cassytha filiformis*. J Nat Prod. 2008;71:289-91.

86. Babayi HM, Udeme JJI, Abalaka JA, Okogun JI, Salawu OA, Akumka DD, et al. Effect of oral administration of aqueous whole extract of *Cassytha filiformis* on haematograms and plasma biochemical Parameters in rats. J Med Toxicol. 2007 Dec;3(4):146-51.

87. Chang CW, Ko FN, Su MJ, Wu JC, Teng CM. Pharmacological evaluation of ocoteine, isolated from *Cassytha filiformis*, as an α_1-adrenoceptor antagonist in rat thoracic aorta. J Pharmacol. 1997;73:207-14.

88. Khan MR. Antibacterial activity of some Tanzanian medicinal plants. Pharm Biol. 2001;39(3):206-12.

89. Akah PA. Abortifacient activity of some Nigerian Medicinal Plants. Phytother Res. 1994;8:106-08.

90. Hoet S, Stevigny C, Block S, Opperdoes F, Colson P, Baldeyrou B, et al. Alkaloids from *Cassytha filiformis* and related aporphines: Antitrypanosomal activity, cytotoxicity, and Interaction with DNA and Topoisomerases. Planta Med. 2004;70:407-13.

91. Mukherjee P K. Quality control of herbal drugs. New Delhi: Business Horizons Pharmaceutical Publishers; 2002. p. 184-86.

92. Sass JE. Elements of botanical micro technique. New York: McGraw Hill Book Co; 1940. p. 222.

93. Johnson DA. Plant micro technique. New York: McGraw Hill Book Co; 1940. p. 523.

94. O'Brien TP, Feder N, Mc Cull ME. Polychromatic staining of plant cell walls by toluidine blue-o. Protoplasma. 1964;59:364-373.

95. Easu K. Plant anatomy. New York: John Wiley & Sons; 1964. p. 767.

96. Indian Pharmacopeia. Ministry of health and family welfare, Govt of India. Vol 2. 4th ed. New Delhi: The Controller of Publication; 1996. p. A-53,A-54.

97. Vijaya Bharathi R. Vamsadhara C.Pharmacognostical evaluation of *Andrographis stenophylla* C. B. Clarke. Nat Prod Sci. 2007;13(3):241-46.

98. Shantha TR, Vasanth Kumar KG, Gopa Kumar K. Pharmacognosy of tala fruits (*Borassus flabellier* L.). Arya Vaidyan. 2007 May-Jul;20(4):199-205.

99. Chemical standardization of herbal drugs. In: Rastogi S, Govindarajan R. editors. Post harvest technology of medicinal and aromatic plants. Lucknow: National Botanical Research Institute; 2003.

100. Raaman N. Phytochemical Techniques. New Delhi: New India Publishing Agency; 2006. p. 18-24.

101. Pourmorad F, Hosseinimehr SJ, Shahabimajd N. Antioxidant activity, phenol and flavanoid contents of some selected Iranian medicinal plants. African Journal of Biotechnology. 2006 Jun;5(11):1142-45.

102. Harborne JB. Phytochemical methods: A guide to modern techniques of plant analysis. 2nd ed. London: Chapman & Hall; 1984. p. 37-99.

103. Wagner H, Bladt S. Plant drug analysis-A thin layer chromatography atlas. 2nd ed. Berlin: Springer-Verlag; 1996. p. 195-245.

104. Tiwari AK. Imbalance in antioxidant defence and human disease: Multiple approach of natural antioxidants therapy. Curr Sci. 2001;81(9):1179-87.

105. Alho H, Leinonen J. Total antioxidant activity measured by chemiluminescence method. Methods Enzymol. 1999;299:3-15.

106. Tanizawa H, Ohkawa Y, Takino Y, Miyase T, Ueno A, Kageyama T, et al. Studies on natural antioxidants in citrus species. I. Determination of antioxidative activities of citrus fruits. Chem Pharm Bull. 1992;40:1940-42.

107. Rao AL, Bharani M, Pallavi V. Role of antioxidants and free radicals in health and disease. Adv Pharmacol Toxicol.2006;7(1):29-38.

108. Cheeseman KH, Slater TF. An introduction to free radical chemistry. Br Med Bull. 1993;49(3):481-93.

109. Mondal SK, Chakraborty G, Gupta M, Mazumder UK. *In vitro* antioxidant activity of *Diospyros malabarica* Kostel. bark. Indian J Exp Biol. 2006 Jan;44:39-44.

110. Mensor LL, Menezes FS, Leitao GG, Reis AS, Dos Santos TC, Coube CS.. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother. Res.2001;15:127-30.

111. Fejes S, Blazovics A, Lugasi A, Lemberkovics E, Petri G, Kery, A. *In vitro* antioxidant activity of *Anthriscus cerefolium* L. (Hoffm.) extracts. J. Ethnopharmacol. 2000;69(3):259-65.

112. Meir S, Kanner J, Akiri B, Hadar SP. Determination and involvement of aqueous reducing compounds in oxidative systems of various senescing leaves. J. Agric. Food. Chem. 1995;43:1813-17.

113. Bahu BH, Shylesh BS, Padikkala J. Antioxidant and hepatoprotective effect of *Acanthus ilicifolius*. Fitoterapia. 2001;72(3):272-77.

114. Garrat DC. The quantitative analysis of drugs. 3rd ed. London: Chapman & Hall Ltd; 1964. p. 456-58.

115. Sreejayan N, Roa MNA. Nitric oxide scavenging by curcuminoids. J. Pharm. Pharmacol. 1997;49:105-07.

116. Jayaprakasha GK, Jena BS, Negi PS, Sakariah KK. Evaluation of antioxidant activities and antimutagenicity of turmeric oil: A byproduct from curcumin production. Z. Naturforsch. 2002;57c:828-35.

117. Oh WY, Pyo S, Lee KR, Lee BK, Shin DH, Cho SI, et al. Effect of *Holotrichia diomphalia* larvae on liver fibrosis and hepatotoxicity in rats. J Ethnopharmacol. 2003;87:175-80.

118. Ecobichon DJ. The basis of toxicology testing. New York: CRC press; 1997. p. 43-86.

119. King J. The transferases-alainine and aspartate transaminases. In: Van D, editor. Practical clinical enzymology. London: Nostrand Company Ltd; 1965. p. 191-208.

120. King J. The hydrolases-acid and alkaline phosphatases. In: Van D, editor. Practical clinical enzymology. London: Nostrand Company Ltd; 1965. p. 191-208.

121. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin Phenol reagent. J Biol Chem. 1951;193:265-75.

122. Malloy HT, Evelyn KA. Determination of bilirubin. J Biol Chem. 1937;119:481-90.

123. Pearse AGE. In: Histochemistry. Vol 1. 3rd ed. London; Churchill Livingstone: 1968. p. 20-100.

124. Arce ER, Gattuso M, Alvarado R, Zarate E, Aguero J, Feria I, Lozoya X. Pharmacognostical studies of the plant drug *Mimosae tenuiflorae* cortex. J. Ethnopharmacol. 2007;133:400-08.

125. Latha S, Selvamani P, Pal TK, Gupta JK, Ghosh. Pharmacognostical studies on leaves of *Commiphora caudate* (Wight & ARN) Engl. Ancient Science of Life. 2006 Jul-Dec;26(1&2):19-25.

126. Suresh J, Elango K, Dhanabal SP, Paramakrishnan N, Suresh B. Pharmacognostical evaluation of *Artemisia pallens* Wall ex DC. Aryavaidyan. 2007 May-Jul;20(4):242-48.

127. Yen GC, Duh PD, Tsai CL. Relationship between antioxidant activity and maturity of peanut hulls. Journal. Agric. Food. Chem. 1993;41:67-70.

128. Huang D, Ou B, Proir RL. The chemistry behind the antioxidant capacity assays. J. Agric. Food. Chem. 2005;53:1841-56.

129. Cao G, Sofic E, Prior RL. Antioxidant and pro-oxidant behaviour of flavanoids: structure activity relationships. Free Radic. Biol. Med. 1997;22:749-60.

130. Bandyopadhyay U, Das A, Bannerjee RK. Reactive oxygen species: Oxygen damage and pathogenesis. Curr. Sci. 1999;77(5):658-66.

131. Nanjo F, Goto K, Seto R, Suzuki M, Sakai M, Hara YO. Scavenging effects of tea catechins and their derivatives on 1,1-diphenyl-2-picryl hydrazyl radical. Free. Radic. Biol. Med. 1996;21:895-902.

132. Marcocci PL, Sckaki A, Albert GM. Antioxidant action of *Ginkgo biloba* extracts EGP761. Methods. Enzymol. 1994;234:462-75.

133. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal. Biochem. 1999;269:337-41.

134. Cetto AA, Wiedenfeld H. Hypoglycemic effect of *Cecropia btusifolia* on Streptozotocin diabetic rats. J. Ethnopharmacol. 2001;78:145-49.

135. Suja SR, Latha PG, Pushpangadan P, Rajasekaran S. Evaluation of hepatoprotective effects of *Helminthost achys Zeylanica* (L) Hook against carbon tetra chloride induced liver damage in *wistar* rats. J. Ethnopharmacol. 2004;92:61-66.

136. Yadav NP, Dixit VK. Hepatoprotective activity of leaves of *Kalanchoe pinnata pers*. J. Ethnopharmacol. 2003;86:197-202.

137. Achliya GS, Wadodkar SG and Dorle AK. Evaluation of hepatoprotective effect of *Amalkadi Ghrita* against carbon tetra chloride induced hepatic damage in rats. J. Ethnopharmacol. 2004;90:229-32.

138. Shenoy AK, Somayaji SN, Bairy KL. Evaluation of hepatoprotective activity of *Gingo biloba* in rats. Indian. J. Pharmacol. 2002;46(2):167-74.

139. Bhandarkar MR, Khan A. Antihepatotoxic effect of *Nymphaea stellata* Willd against carbon tetrachloride induced hepatic damage in albino rats. J. Ethnopharmacol. 2004;91:61-64.

140. Decker K, Keppler D. Galactosamine-induced liver injury. In: Popper H, Schaffner F, editors. Progress in liver disease. New York: Grune,Stratton; 1972. p. 183-99.

141. Dhanabal SP, Syamala G, Elango K, Suresh B. Protective effect of *Pterocarpus santalinus* on galactosamine induced liver damage. Nat. Prod. Sci. 2006;12(1):8-13.

142. Karan M, Vasisht K, Handa SS. Antihepato toxic activity of *Swertia chirata* on paracetomol and galactosamine induced hepatotoxicity in rats. Phytother. Res. 1999;13:95-101.

143. Deshmukh PT, Bhatt JK. Preclinical evaluation of hepatoprotective activity of *Calotropis procera* root bark in experimental liver damage induced by d-galactosamine in rats. Indian Drugs. 2008 Jun;45(6):499-504.

144. Mitra SK, Venkataranganna MV, Sundaram R, Gopumadhavan S. Protective effect of HD-03, a herbal formulation, against various hepatotoxic agents in rats. J. Ethnopharmacol. 1998;63:181-86.

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AUTHENTICATION CERTIFICATE

Based upon the organoleptic/ macroscopic/ microscopic examination of fresh/ market sample, it is certified that the specimen given by...Mrs. R. Padma..................

....

is identified as below:

Binomial: Cuscuta reflexa Roxb.

Synonym(s): Nil

Family: Convolvulaceae

Regional names: Hindi – Akasbel; Sanskrit – Amarvela;

References: Henry. A.N.; et al.; 1987; Flora of Tamil Nadu; Vol - II, BSI, Coimbatore-03

Nature of the specimen: Fresh Plant

Voucher No of the specimen: PARC/2007/108

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AUTHENTICATION CERTIFICATE

Based upon the organoleptic/ macroscopic/ microscopic examination of fresh/ market sample, it is certified that the specimen given by Mrs. R. Padma ....

is identified as below:

Binomial: Cassytha filiformis L.

Synonym(s): Nil

Family: Lauraceae

Regional names: Hindi - Amarbeli; Sanskrit - Akashavalli; Tamil - Erumaikkottan.

References: Henry. A.N., et al., 1987. Flora of Tamil Nadu; Vol - II, BSI, Coimbatore-03.

Nature of the specimen: Fresh Plant

Voucher No of the specimen: PARC/2007/109

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Committee for the Purpose of control and Supervision of Experiments on Animals (CPCSEA)
Institutional Animal Ethics committee (IAEC).

CERTIFICATE

Title of the Project: Screening of selected Medicinal plants for their hepatoprotective activity.

Proposal Number: JSSCP/IAEC/PH.D/ PH. BIOTECH/02/2008-09

Date received after modification (if any):

Date received after second modification: 21S-12-2007.

Approval date: 22/10/08 **Animals:** Wistar rats/ Albino mice
Rabbits / Guinea pigs

No. of animals sanctioned: 60 **Male/Female**

Expiry date (Termination of the Project): One month.

Name of IAEC/CPCSEA chairperson: Prof. K. Elango

Date: 22/10/08

Signature of Chairperson

(Prof. K. Elango)
(Vice Principal)