CHAPTER 1

INTRODUCTION

1. Introduction

Herbal medicine continue to grow in popularity as consumers adopt more natural approaches for staying healthy, and these have been used since ancient times. In fact, every major culture has used herbalism as the method of healing at some time or the other (Zita, 1997). Since past two decades World Health Organisation is encouraging the use of principal indigenous medicinal plants in developing countries.

World Health Organisation estimates that about 80% of population living in developing countries relies almost exclusively on traditional medicines for their primary health care needs. Since the medicinal plants are the backbone of traditional medicine, this mean that, 3300 million people in the under developed countries utilize medicinal plants on a regular basis. This assumption does not include the developed countries where there has been a great fascination for the herbal medicines and dietary food supplements in the last decade (Dobriyal and Narayana, 1998).

Although world wide growth in usage of traditional systems of health care has been seen in recent years, countries like India, China, Tibet and Brazil, the health care scenario has always been associated with these traditional systems of medicines (TSM). These countries are still having very rich biological as well as cultural diversity and the traditional health care systems have a deep influence on the current healthcare means in these nations. Today these traditional systems are not only flourishing in their respective countries but are also becoming immensely popular among other nations including the western world. In India a number of traditional health care systems have been practiced for many centuries, namely Ayurveda, dating back to more than 4000 years, siddha, Unani and more recently homeopathy. Apart from these systems, there has been a rich heritage of ethno botanical usage of herbs by various tribal communities in the country. All these systems have been codified and documented in oriental languages (Narayana, 1998).

Besides our rich heritage of health care systems, it was only for last 100-150 years that these systems slowly but steadily have been taken up at

the industrial level. Prior to this, these systems were confined to be practiced by individuals like Vaidyas, Hakeems, Traditional Dais, Bone Setters, and Massagers etc. During initial decades of present century, various small and big manufacturing houses of traditional formulations have come up in this country (Narayana, 1998).

Only in the last few decades, a resurgence of interest in plants as sources of medicines and of novel molecules for use in the elucidation of physiological/ biological phenomena seen. There are number of reasons for this. First, there is a genuine expectation in developing countries that their health care problems can be solved through a sensible scientific exploitation of medicinal plants; some of which have been used for generations by local populations. Then there is the world wide 'green' revolution which is reflected in the belief that herbal remedies are safer and less damaging to the human body than synthetic drugs. Further more, underlying this upsurge of interest in plants, it is the fact that many important drugs in use today in modern medicine were derived from plants or from starting molecules of plant origin. Digoxin/digitoxin, the vinca alkaloids, reserpine and tubocurarine are some important examples. Plants have also yielded molecules which are extremely valuable tools in the characterisation of enzymes and the classification of receptor systems: morphine, physostigmine, muscarine, atropine, nicotine and tubocurarine, are the examples (Elizabeth et al., 1978). Some scientists thus expect that the plant kingdom holds the key to the understanding of complex human biochemistry/pathology and the cure of man's perplexing diseases. The initial optimism, engendered by the idea that a sophisticated understanding of receptor systems and of the biochemistry of disease would pave the way to predictable drug development, has not been released., therefore, laboratories around the world are engaged in the screening of plants for biological activity with therapeutic potential. One major criterion for the selection of a plant for such study is to ascertain traditional healer's claims for its therapeutic usefulness. It is thus worth reflecting on the cultural environment in which traditional healers use plant remedies, as well as the materials of plant use, in order to strengthen the research design.

The number of species of higher plants on this planet is estimated to be between 3,10,000 and 5,00,000. All higher plants elaborate chemical secondary metabolites that are of potential medicinal interest. Therefore, the determination of the criteria for selecting plants for phytotherapeutic investigation is perhaps an important exercise as the investigation of itself. The following selection criteria are suggested as a guide (Elizabeth et al., 1978):

1. Selection based on traditional usage.
2. Poisonous plants
3. Selection based on chemical composition.
4. Screening for a specific biological activity
5. Combination of criteria.

1.1 Standardization:

The increasing demand for herbal medicines inevitably led to the issue of obtaining and maintaining their quality. As a result there has been tremendous quality consciousness for the herbs in various countries. Internationally and in our country too, several pharmacopoeias have provided monographs stating quality parameters and standards of many herbs. World Health Organisation (WHO) provided various guidelines on the quality control methods for medicinal plant materials. All these guidelines provided by different organisations take into consideration, macroscopy, microscopy, various physical constants like ash values, extractive values, moisture content etc, chemo profiling through chemical evaluation of the plant drugs. Hence the plant drugs are subjected for standardization process consisting of authentification, macroscopic evaluation, microscopic evaluation, physical, chemical and biological evaluation, to ensure the quality avoiding batch to batch variations starting from the collection of plant materials to preparation of formulations.

For the standardization and quality assurance purposes three attributes viz. authenticity, purity and assay are desirable. Authenticity as the name suggests relates to proving that the material is true i.e. it corresponds to the right identity. Authenticity in itself involves many parameters including gross

morphology, microscopy, and chemical analysis. Purity pertains to evaluating that there are no adulterants present in the plant material. It can be evaluated by pharmacognostic evaluations like qualitative and quantitative microscopy, physical constants like, ash values, extractive values etc. Assay part of standardization is chemical and biological profiling by which the chemical and biological effects could be assessed and curative values get established. In biological assays, the drug activity is evaluated through a pharmacological model. For example the efficacy of digitalis a cardiotonic drug can be effectively evaluated by biological assay of its action on cardiac muscles. Similarly the effectiveness of the hepatoprotective drugs can be evaluated by its action on liver. WHO in a number of resolutions has emphasized on the need to ensure the quality control of herbs and herbal formulations by using modern techniques.

The plants are a major constituent of various alternative systems of medicines used world wide since ancient times. To rationalize the use of botanicals, a need-based and novel concept of markers is gaining momentum. In this regard, marker analysis of the plant material as well as their extracts is recommended by various organisations for the evaluation of medicinal plants.

1.1.1 Standardization of botanicals by HPTLC

Quality certification of products of botanical origin has been in the news for several reasons. The Ayurveda, Unani and Siddha systems of medicines have been in practice since thousands of years. The concept of quality in those days was based on physical aspects of the plant material such as identification, colour, odour, size, age, etc. today there are an additional requirement, distinct in nature, for modern routine control of botanical raw materials, in addition to physical tests and identification i.e. chemical composition. There are numerous fractions present in a total extract which add to the efficacy of the product and reduce its toxicity. The total extract can be characterised by its finger print of analytical data. The Govt. of India has adopted the fingerprint approach for botanicals because it supports the traditional concept and is easy to practice at different levels of sophistication.

Unlike in allopathic medicines, the raw materials used to make a traditional medicine may not be found in the finished product. Traditional medicines are not necessarily mixtures of raw materials and excipients.

It is generally realised that for monitoring quality, a chromatographic method that does both quantification of a specified fraction(s) as well as fingerprint is ideal. But the dual purpose analysis is possible for individual crude drugs or some simple mixtures of crude drugs. Formulations containing more than 5 crude drugs are so complex that it is difficult to quantify any particular fraction in a routine manner. Chromatographic techniques, which are used for separating mixtures into individual fractions, are ideal for creating a fingerprint. Gas chromatography however is not suitable for herbal analysis as only substances that can be volatilised at higher temperatures and which are thermolabile can be analysed. HPLC is slow, cannot produce visual images but is useful for quantification.

High Performance Thin Layer Chromatography (HPTLC) is well suited to obtain a detailed fingerprint of herbal extract or product. Such a fingerprint comprises of scanning, in UV; fluorescence; ultraviolet spectra and photographic images in ultraviolet light (254 and 366 nm) and occasionally in visible light after derivatization. HPTLC fingerprint is obtained at low cost and high speed and thus meets the need for a modern quality control method (Charegaonkar, 2005).

1.1.2 Standardization of herbs using marker compound analysis

One of the best methods of the standardising herbs and herbal formulations based on the modern scientific tool is using chromatography. It not only helps in the establishing the current identity but also helps in regulating chemical sanctity of the herbs. One such technique is marker compound testing and fingerprint analysis.

Every herb has a range of chemical constituents, which are produced as a result of metabolic activities in the plant. These compounds either alone or in combination are mainly responsible for the pharmacological activities or therapeutic action in the human body. Hence it would be more practical to test for the presence of these compounds. For example, ashwagandha (*Withania*

somnifera) can be assayed for withanolides, guggul (*Commiphora mukhul*) for guggulusterones, neem (*Azadirachta indica*) for azadirachtine or nimbidine, turmeric (*Curcuma longa*) for curcuminoids. For testing purpose these compounds are referred to as biomarker compounds. On the other hand where the chemical composition of the herbs has been worked out but it is not clearly established whether these chemical entities are responsible for some particular action, any compound which is predominantly present in the herbs can be utilized as marker form the purpose of standardization. This group represents compounds like aegelin in bilva (*Aegle marmelose*), shatavarine in shathavari (*Asparagus racemosus*) etc. hough the activity of these compounds is not linked with the therapeutic purposes the herbs are being recommended, but since the presence of these compounds has been well established they can be used for standardization purposes. These compounds are referred to as chemical marker compounds (Dobriyal and Narayana, 1998).

Different chromatographic methods are used to analyse the marker compounds in herbs with the help of modern sophisticated tools. High performance thin layer chromatography is being used frequently where only fingerprinting of the herbs is required without quantifying the compounds though the same can also be quantified with the help of densitometer. Marker testing is no way a substitute for other tests like, physico-chemical parameters, microscopic characterisation etc. but is an efficient method to ensure the identity and purity of herbal raw materials.

Biomarker profiling is being highlighted in quality control and standardization of herbal medicine to establish the quality control approaches with chemoprofiling techniques with the lead from some therapeutically potent medicinal plants (Mukherjee et al, 2006).

1.2 Hepatoprotective activity

The modern system of medicine still lack in providing suitable medicament for a large number of adverse conditions, in spite of tremendous advances made in discovery of new compounds. A few of these diseases can be mentioned like, hepatic disorders, viral infections, AIDS, rheumatic

diseases etc (Mohammed Ali, 1994) The available therapeutic agents only bring about symptomatic relief without any influence on the curative process, thus, causing the risks of relapses and danger of untoward effects. A large number of populations suffer, due to various reasons, from hepatic diseases and also inflammatory conditions of known and unknown origin. The development of antihepatotoxic drugs being a major thrust area has drawn attention of majority of workers in the field of natural product research.

1.2.1 Liver Disorders

Liver, the largest and the most versatile by organ for metabolism and excretion, plays an important role in the maintenance of body's internal environment through its multiple and diverse functions. It is continually exposed to a variety of xenobiotics and therapeutic agents due to inadequately controlled environmental pollution and expanding therapeutic uses of potent drugs. Thus, the disorders associated with this organ are numerous and varied. Although a strict delineation of various hepatic disorders is not yet possible, from didactic point of view, these may be classified as acute or chronic hepatitis (inflammatory diseases), hepatosis (non-inflammatory disorders) and liver cirrhosis (degenerative disorder resulting in fibrosis).

The following are some of the Liver diseases that are commonly observed.

a) Necrosis
b) Cirrhosis
c) Hepatitis- may be of viral, toxic or deficiency type.
d) Hepatic failure-Acute or Chronic.
e) Chemical/Drug induced Hepatotoxicity: Generally may be hepatitis, jaundice and carcinogenesis.
f) Liver disorders due to impaired metabolic function. Generally the disorders associated with fat (liposis) and bilirubin (jaundice) metabolisms are very commonly seen.

1. Disorders associated with fat metabolism: Fatty Liver

2. Disorders associated with bilirubin metabolism- Jaundice or which may be of different types based upon mechanisms of action and etiology.

I. Haemolytic / pre-hepatic jaundice.

II. Obstructive (post-hepatic / cholestatic jaundice).

III. Hepatogenous/hepatic jaundice/cholestasis.

In these three conditions there occurs unconjugated hyperbilirubinaemia.

IV. Hereditary jaundice or pure cholestasis: Gilbert's syndrome, Dubin-Johnson syndrome and Crigler-Najjar syndrome etc, Rotor's syndrome are some of the hereditary Jaundice types usually observed.

Gilbert's syndrome and Crigler-Najjar syndrome are examples of hereditary non-haemolytic unconjugated hyperbilirubinaemia, where as Dubin-Johnson syndrome and Rotor's syndrome are conditions with hereditary conjugated hyperbilirubinaemia.

1.2.2 Investigations of liver functions

When the liver is diseased, one or more but not necessarily all of its functions are impaired. There can be no test for liver functions as a whole. The various 'liver function tests' (LFTS) are tests of derangements of individual functions of the liver. Since many tests give similar abnormal results in a particular liver disease, it may be possible to extend a conclusion drawn from a single test. The liver biopsy result may not be comparable with the LFTS since many functional changes are not mirrored by obvious structural changes in the liver cells (Praful 1996).

Thus a battery of liver function tests is employed for accurate diagnosis, to assess the severity of the damage, to judge the prognosis and to evaluate therapy (Harsh Mohan, 2005). These tests are described below in relation to major liver functions.

I. Tests for manufacture and excretion of bile.

Bile is produced by liver, stored in the gallbladder and is secreted via biliary ducts into the duodenum. Bile consists of biliary phospholipids and primary and secondary bile acids. Jaundice will develop if bilirubin is

excessively produced, or there is impaired hepatic uptake and conjugation of bilirubin or it is insufficiently excreted into the duodenum. Tests employed to assess the synthesis and elimination of bilirubin pigment, urobilinogen and bile acids are as follows.

1. Bilirubin: Bilirubin pigment can be detected in serum, faeces and urine. The bile pigment bilirubin is forme from the breakdown of haemoglobin molecule. Haemoglobin consists of four protein chains, each of which contains porphyrin ring and an iron atom. A portion of the porphyrin ring breaks and the cyclic structure becomes the open chain tetrapyrrole derivative biliverdin. Further reduction by enzyme biliverdin reductase leads to the formation of unconjugated bilirubin which is insoluble in water. After the molecules of unconjugated bilirubin enter the hepatocytes, they attach to ligandin and Z protein, two soluble transport proteins. One or two glucuronic acid molecules attach to each bilirubin molecule in a reaction mediated by UDP-glucuronyltransferase. Formation of glucuronyl conjugate now makes the bilirubin complex soluble in water, now termed conjugated bilirubin.

Bilirubin determination in serum is based on the reaction with diazo reagent. Bilirubin level rises in diseases of hepatocytes, obstruction to biliary excretion into duodenum, in haemolysis, and defects of hepatic uptake and conjugation of bilirubin pigment such as Gilbert's disease.

2. Urobilinogen: In small intestine the bilirubin reduction occurs producing a group of molecules known as urobilinogens. It is normally excreted in urine. Its semi quantitative estimation is based on the reaction with Ehrlich's aldehyde reagent. An increase in urobilinogen in the urine is found in hepatocellular dysfunctions such as alcoholic liver disease, cirrhosis, and malignancy of the liver.

3. Bile acid (Bile salts): The primary bile acids cholic acid and chenodeoxycholic acid are formed from cholesterol in the hepatocytes. These bile acids on secretion into the gut come in contact with colonic bacteria and undergo deconjugation with the production of secondary metabolites (deoxycholic acid and lithocholic acid). Most of these bile acids are

reabsorbed through enterohepatic circulation and reach the liver. Only about 10% of the total bile acids are excreted in the faeces.

Hepatobiliary diseases with cholestasis are associated with raised levels of serum bile acids which are responsible for producing pruritus.

II. Serum enzyme assays.

Determination of certain serum enzymes is considered to be useful in various types of liver injury, whether hepatocellular or cholestatic, as well as in quantifying liver damage. A combination of serum transaminases and alkaline phosphatase estimation is adequate to diagnose liver injury.

1. Alkaline phosphatase: Serum alkaline phosphatase is produced by many tissues, especially bone, liver, intestine, and placenta and is excreted in bile. Elevation in activity of the enzyme can thus be found in diseases of bone, liver and in a pregnancy. In absence of bone disease and pregnancy, elevated serum alkaline phosphatase levels generally reflect hepatobiliary disease. The great elevation occurs in biliary tract obstruction. Slight to moderate increase is seen in parenchymal liver diseases such as in hepatitis and cirrhosis and in metastatic liver disease.

2. Transaminases:

GOT (AST) and GPT (ALT): Aspartate transaminase or AST (Glutamic oxaloacetic transaminase or GOT) is a mitochondrial enzyme released form heart, liver, skeletal muscle and kidney. Alanine transaminase or ALT (Glutamic pyruvic transaminase or GPT) is a cytosolic enzyme primarily present in liver. Serum levels of GOT and GPT are increased on damage to the tissues producing them. Thus serum estimation of GPT which is fairly specific for liver tissue of greater value in liver cell injury, whereas GOT level may raise in acute necrosis or ischaemia of other organs such as the myocardium, besides liver cell injury.

3. γ-Glutamyl transpeptidase (γ-GT): The primary source of the enzyme, γ-GT in serum is the liver. Its serum level parallels serum alkaline phosphatase and is used to confirm that the elevated serum alkaline phosphatase is of hepatobiliary origin.

4. Other enzymes:

i) 5^1-Nucleotidase: it is another phosphatase derived from the liver. Its determination is useful to distinguish alkaline phosphatase of hepatic origin from that of bony tissue.

ii) Lactic dehydrogenase: It is widely distributed in the tissues (Heart, liver, kidney, skeletal muscles) of the body. High concentrations are found in heart and liver. Even small amounts of tissue damage are reflected in measurable elevations of the enzyme activity. The activity is elevated in number of situations associate with liver damage.

This enzyme may provide an important assessment of liver function, particularly in monitoring the progress of patients treated surgically for cirrhosis or those who undergone liver transplant.

iii) Cholinesterase: It is located in heart, liver, pancreas and brain. Enzyme synthesized by liver is released in high concentrations into the serum. Serum cholinesterase values decrease noticeably in liver disease.

III. Tests for metabolic functions.

The liver is the principal site of metabolism and synthesis of plasma proteins and amino acids, lipid and lipoproteins, carbohydrates, and vitamins, besides detoxification of drugs and alcohol.

1. Protein and amino acid metabolism: Amino acids derived form the diet and from tissue break down are metabolised in liver to ammonia and urea. A number of plasma proteins and immunoglobulins are synthesised on polyribosomes bound to the rough endoplasmic reticulum within the hepatocytes and discharged into plasma. Based on these metabolic functions of the liver, serum estimation of proteins, immunoglobulins and ammonia are employed to assess the liver cell damage.

i) Serum proteins: Liver cells synthesise albumin, fibrinogen, prothrombin, haptoglobin, ceruloplasmin, transferrin, alpha fetoproteins and acute phase reactant proteins. The blood levels of these plasma proteins are decreased in extensive liver damage. Routinely estimated are total concentration of serum proteins, serum albumin, serum globulin, and albumin/globulin ratio. Due to the availability of protein electrophoresis, thymol turbidity and flocculation test based on altered plasma protein components have been discontinued.

Hypoalbuminemia may occur in liver diseases having destruction of hepatocytes. Hyperglobulinemia may be present in chronic inflammatory disorders such as in cirrhosis and chronic hepatitis.

ii) Immunoglobulins: The levels of serum immunoglobulins produced by lymphocytes and plasma cells (IgG, IgM and IgA) show non-specific abnormalities in liver diseases and represent inflammatory or immune response rather than liver cell dysfunction. IgA is the predominant immunoglobulin in bile and its level is raised in cirrhosis, IgG is markedly raised in chronic active hepatitis and IgM is markedly increased in primary biliary cirrhosis.

iii) Clotting factors: Hepatic synthetic function of several clotting factors can be assessed by few simple coagulation tests. Prothrombin time and partial thromboplastin time, both of which reflect the activities of various clotting factors, are prolonged in patients with hepatocellular disease. Prothrombin time is dependant upon both hepatic synthesis of clotting factors and intestinal uptake of vitamin K. Thus obstruction of bile duct and intrahepatic cholestasis which results in vitamin K deficiency due to impaired lipid absorption, are associated with prolonged prothrombin time.

iv) Serum ammonia: High blood levels of ammonia are found in acute fulminant hepatitis, cirrhosis and hepatic encephalopathy. The rise in serum ammonia is due to inability of severely damaged liver to convert ammonia to urea. Thus, urea synthesis is reduced in chronic liver disease.

2. Lipid and lipoprotein metabolism.

Lipids synthesised in the liver include cholesterol and cholesterol esters, phospholipids and triglycerides. These are insoluble in water and are carried in circulation with three major types of lipoproteins which contain apoproteins. These are: high density lipoproteins (HDL), low density lipoproteins (LDL) and very low density lipoproteins (VLDL).

i) Blood lipids (total serum cholesterol, triglycerides and lipoprotein fractions): Estimation of total serum cholesterol, triglycerides and lipoprotein fractions are frequently done in patients with liver disorders.

There is raise in total serum cholesterol in cholestasis, probably due to retention of cholesterol which is normally excreted in bile. Serum triglyceride is also elevated in cholestasis. Values are lowered in acute or chronic diffuse liver diseases.

3. Carbohydrate metabolism:

The liver plays a central role in carbohydrate metabolism. Blood glucose level is lowered in fulminant acute hepatic necrosis. In chronic liver disease, there is impaired glucose tolerance and relative insulin resistance.

IV. Immunologic tests.

Liver diseases are associated with various immunologic abnormalities which may be non-specific immunologic reactions or may be antibodies against specific etiologic agents.

1. Non-specific immunologic reactions: These include the following:

i) Smooth muscle antibody: Smooth muscle antibody to actin component of muscle is formed in certain hepatic disorders with hepatic necrosis. It appears that hepatocytes have a protein which is immunologically similar to actin.

ii) Mitochondrial antibody: Mitochondrial antibody develops in patients with primary biliary cirrhosis.

iii) Antinuclear antibody: Antinuclear antibody is present in some patients of chronic hepatitis.

2. Antibodies to specific etiologic agents:

i) Antibodies to hepatitis B surface antigen (HBsAg) can be demonstrated in cases of serum hepatitis. A confirmed positive test for HBsAg is definite proof of hepatitis B infection.

ii) Hepatitis B core antibody (HBc) can be detected in all patients with hepatitis B.

iii) Hepatitis B e antigen (HBeAg) can be found in chronic varieties of hepatitis B.

ii) Amoeba antibodies to Entamoeba histolytica develop in patients with amoebic or liver abscess.

V. Ancillary diagnostic tests.

In addition to laboratory tests described above, two ancillary tests which are invariably done are ultrasonography and percutaneous liver biopsy.

1. Ultrasound examination: It is indicated in following situations.

i) Cholestasis of various etiologies to see the dilated intra and extrahepatic canalicular tree.

ii) Space-occupying lesions within liver to determine whether they are neoplasms or non neoplastic cysts.

2. Percutaneous liver biopsy: It is employed to examine the microscopic changes of hepatic morphology in various diseases.

1.2.3 Evaluation of Hepatoprotective Activity

Until recently it had been accepted almost as dogma that there was not and could not be any screening method for standardization and evaluation of hepatoprotective drugs since most of the available methods do not simulate the clinical hepatic diseased conditions. Therefore evaluation of any compound with hepatoprotective claims in a single model does not suffice the purpose and needs to be based on multi models, which are in great demand today. A review of literature reveals that several chemical substances and drugs having specific actions on liver are used as hepatotoxins in experimental animals to simulate ideal diseased conditions.

Hepatotoxins may be grouped into direct or indirect types depending upon their intrinsic capability, host susceptibility and circumstances of exposure. Generally direct toxins injure many tissues including liver (eg.CCl_4), an indirect affects particular metabolic pathway of the liver (eg. galactosamine). Thus the hepatotoxins affect the liver in a number of ways as:

1. Interference with hepatic bilirubin uptake, conjugation and excretion eg. Rifampicin.

2. Dose and time dependant reactions.

   a) Acute toxic hepatitis eg. Paracetamol

   b) Fatty liver eg. Tetracycline

3. Dose independent reactions.

   a) Diffuse hepatocellular damage eg. Isoniazid

   b) Cholestatic hepatitis eg. Chlorpromazine.

c) Granulomatous infiltration eg. Phenytoin, Chlorpropamide

Thus hepatoprotective activity can be most easily evaluated/screened with the aid of several model systems of liver damage in experimental animals.

In all test model systems, conditions for liver damage are implemented and an attempt is made to counteract this toxicosis with the substance/preparation under test. The magnitude of the protective effect can be measured by estimating the enzyme activities and the rate of survival and can be verified histologically. The available methods are *in vivo*, *ex vivo* & *in vitro* methods (Visen, 1993). All these methods are used to study the protective or curative effects of any compound under test. In order to test for hepatoprotective activity the test substance and the hepatotoxin are administered simultaneously whereas in case of antihepatotoxic or curative activity the test substance is generally administered after induction of hepatotoxicity.

a) In vitro methods: In these methods hepatocytes are generally isolated by using in-situ, two step recirculating collagenase perfusion technique. These are then seeded in small containers and exposed to test samples and toxins. After a specified time period the degree of toxicity or protection is assessed by viability tests and enzyme levels such as GOT and GPT.

Invitro methods employing primary culture hepatocytes using carbon tetrachloride, galactosamine, thioacetamide, ethanol and paracetamol etc. as hepatotoxins have been devised. These have a number of advantages over in vivo methods such as their ability to dispose numerous samples at a time, low cost with a small size, little variation and reproducibility of results. The major disadvantage is that sometimes it may not reflect the events which occur in animals.

b) Ex vivo models: In this after completion of preselected in vivo test protocol hepatocytes are isolated and the percentage of viable cells and biochemical parameters are determined as liver function tests. These methods are somewhat better correlated to clinical models than in vitro or in vivo methods.

c) In vivo methods: These are of two types.

1) Based on serum parameters: In vivo methods are used not only to study the nature of the given compound but also to study the mechanism of the toxicant. Hepatotoxicity is produced in experimental animals by the administration of known dose of hepatotoxin like carbon tetrachloride, paracetamol, D-galactosamine, thioacetamide, and ethyl alcohol etc., which produce marked measurable effects, the magnitude of which can be measured by carrying out various liver function tests viz. morphological, metabolic or functional, biochemical and histopathological determinations. Although it is a very convenient laboratory method, reproducibility of results is rather poor.

2) Based on bile parameters: The compounds having hepatoprotective claims are also evaluated in general for their choleretic or anticholestatic activity in order to know whether the liver disorder is due to an abnormality of bilirubin metabolism or not. Choleretics are those agents which increase the outputs of bile by stimulating the liver where as anticholestatics are those which correct the retention and accumulation of bile due to intrinsic and extrinsic factors in the liver. These activities are evaluated by studying bile flow content in conscious and anaesthetised animals for 5 hours.

1.2.4 Experimental models for hepatoprotective screening

Several chemical reagents and drugs which induce liposis, necrosis, cirrhosis, carcinogenesis and hepatobiliary dysfunctions in experimental animals are classified as hepatotoxins. The most important ones used are carbon tetrachloride, D-galactosamine, paracetamol, chloroform, ethyl alcohol and thioacetamide. The following are some of the experimental rat models employing these hepatotoxins.

1. CCl_4 model:

A number of CCl_4 models are devised depending upon its dosage through different routes of administration.

a) Acute hepatic damage: Acute liver damage, characterised by ischemia, hydropic degeneration and central necrosis is caused by oral or subcutaneous administration of CCl_4 (1.25ml / kg). The maximum elevation of biochemical parameters are found to be 24 hours after the CCl_4 administration normally

administered as 50 % v/v solution in liquid paraffin or olive oil (Rage et al., 1989).

b) Chronic reversible hepatic damage: Administration of CCl_4 (1ml/ kg s.c) twice weekly for 8 weeks produces chronic, reversible liver damage (Saraf, et al., 1991).

c) Chronic, irreversible hepatic damage: Administration of CCl_4 (1 ml/kg s.c) twice weekly for 12 weeks simulates chronic, irreversible liver damage (Pathak et al., 1991).

2. Thioacetamide model:

Thioacetamide (100mg/kg s.c) induces acute hepatic damage after 48 hrs of administration by causing sinusoidal congestion and hydropic swelling with increased mitosis (Saraf et al., 1992).

3. D-Galactosamine model:

D- Galactosamine 800 mg/kg i.p induces acute hepatotoxicity after 48 hrs of administration with diffused necrosis and steatosis (Kiso et al., 1983).

4. Paracetamol model:

Paracetamol induces acute hepatotoxicity depending upon its dosage through different routes of administration, such as-

a. Paracetamol 800 mg/kg i.p. induces centrilobular necrosis without steatosis (Rachmilewitz et al., 1950).

b. Paracetamol at a single dose of 3 g/ kg p.o stimulates acute hepatic damage. It takes 48 hours to induce the toxicity (Handa and Anupama, 1990).

5. Chloroform model:

It produces hepatotoxicity with extensive central necrosis, fatty metamorphosis, hepatic cell degeneration and necrosis either by inhalation (for 1 hr in atmosphere) or by subcutaneous administration. (0.4-1.5ml/ kg) (Goldschmidt et al., 1939).

6. Ethanol model:

Ethanol induces liposis to a different degree depending upon its dose, route and period of administration as follows-

a. A single dose of ethanol 1ml/kg induces fatty degeneration (Goodell et al., 1944).

b. Administration of 40% (v/v) ethanol 2 ml/ 100g/day p.o for 21 days produces fatty liver (Thripati et al., 1991).

c. Administration of country made liquor 3ml/100 gm/day p.o for 21 days produces liposis (Gulati et al., 1991).

1.2.5 Mechanism of action of some selected hepatotoxins

In this section, a general idea about the possible ways of induction of toxicity by some hepatotoxins is described.

1. Carbon tetrachloride (CCl_4):

The hepatotoxicity of CCl_4 is due to the metabolic formation of the highly reactive trichloromethyl free radical which attacks the polyunsaturated fatty acids of the membrane of the endoplasmic reticulum and initiates a chain reaction. It is enhanced by induction of hepatic microsomal enzyme systems and vice versa by antioxidants which mop up the free radicals. The first cells to be damaged are those in the centrilobular region where microsomal enzyme activity is the greatest. The initial damage produced is highly localised in the endoplasmic reticulum which results in loss of cytochrome P_{450} leading to its functional failure with a decrease in protein synthesis and accumulation of triglycerides leading to fatty liver, a characteristic of CCl_4 poisoning. If the damage is severe, it leads to disturbances in the water and electrolyte balance of hepatocytes leading to an abnormal increase in liver enzymes in plasma, there by impairing mitochondrial functions, followed by hepatocellular necrosis (Recknagel, 1967; Slater, 1966).

2. Paracetamol:

Paracetamol an analgesic and antipyretic is assumed to be safe in recommended doses, overdoses however taken with suicidal intent, produce hepatic necrosis. Small doses are eliminated by conjugation followed by excretion, but when the conjugation enzymes are saturated the drug is diverted to an alternative metabolic pathway, resulting in the formation of a hydroxylamine derivative by cytochrome P_{450} enzyme. The hydroxylamine derivative, a reactive electrophillic agent, reacts non-enzymatically with glutathione and detoxifies. When the hepatic reserves of glutathione depletes, the hydroxylamine reacts with macromolecules and disrupts their structure

and function. Extensive liver damage by paracetamol it self decreases its rate of metabolism and other substrates for hepatic microsomal enzymes (Savides et al., 1983).

3. Thioacetamide:

Thioacetamide, a substitute for H_2S with less toxicity and obnoxious smell, on repeated exposure produces cirrhosis by inhibiting the respiratory metabolism of the liver due to the uncontrolled entry of Ca^{+2} ions into hepatocytes, resulting in inhibition of oxidative phosphorylation. Early metabolic disturbances increase the RNA and protein content of the nuclear fraction of hepatocytes leading to varying graded liver damage including nodular cirrhosis, liver cell proliferation, production of pseudo lobules and parenchymal cell necrosis. The serum levels of glutamic dehydrogenase are also found to increase, indicative of mitochondrial injury, which plays an important role in thioacetamide induced hepatotoxicity (Gallagher et al., 1956).

4. Rifampicin:

All the antitubercular drugs are liable to produce liver damage with a broad spectrum of liver dysfunction, ranging from slight elevations of transaminases to severe hepatocellular necrosis.

Rifampicin, a broad spectrum antibiotic is the most commonly and widely used antitubercular drug particularly in combination with isonicotinic acid hydrazide (INH) or other similar agents. Both Rifampicin and INH are known to be hepatotoxic, when administered separately and continued particularly in combination with INH. Although Rifampicin does not appear to accumulate in hepatic dysfunction, it should be used cautiously because of its hepatotoxic nature. It is largely metabolised to desacetyl rifampicin which undergoes entero-hepatic circulation. It is distributed throughout the body and about 85% of it gets bound to serum proteins. Since it is largely metabolised in the liver, its serum concentration rises in liver disease with fatal liver damage and acute hepatic failure. It reduces drug metabolising enzymes in liver and thereby impairs other drugs effectiveness. Since it actively and specifically binds to RNA polymerases, it inhibits the synthesis of all forms of

RNA, thus by inhibiting nucleic acid and protein synthesis; it induces fatty liver and finally cirrhosis (Sherlock, 1972).

5. D-Galactosamine:

Generally it produces reproducible cell injuries. D-Galactosamine induced hepatotoxicity is due to conversion of the toxicant to UDP-Hexosamine which functions as a trap for Uridine nucleotides and leads to fall in the concentration of uridine triphosphate (UTP), Uridine diphosphate (UDP) and Uridine monophosphate (UMP). D-Galactosamine-1-phosphate and UDP-Galactosamine are found to be main metabolites of D-Galactosamine. UDP-Glucose (UDPG) catalyses the conversion of Galactosamine-1-phosphate to UDP-Galactosamine which shows lesser affinity to D-Galactose-1-phosphate and Uridyl transferase. This gets accumulated in the liver due to reduced levels of UDPG and high levels of Galactosamine. Reduced levels of UDPG affect the UDPG-linked synthesis of glycogen, hetero polysaccharides and glucuronides, as well as trapping of uridine phosphates by formation of UDP-hexosamines which plays an important role in induction of galactosamine induced hepatitis (Keppler et al., 1969; Keppler et al., 1968). Thus the depletion of uracil nucleotides (UTP, UDP-glucose and UDP-galactose) results in the inhibition of RNA synthesis and disturbance of biosynthesis of glycoproteins, leading to deterioration of the cell membranes. Morphologically it produces both diffused necrosis and steatosis (Farber et al., 1973).

1.3 Hepatoactive Medicaments

The literature survey reveals a large number of drugs of plant origin are endowed with hepatoactive claims either directly or indirectly. These are generally classified into 3 categories without any strict delineation among them viz.

1. Anti hepatotoxic agents:

These generally antagonise the effects of any hepatotoxin causing hepatitis or any liver disorder or disease.

2. Hepatotropic agents:

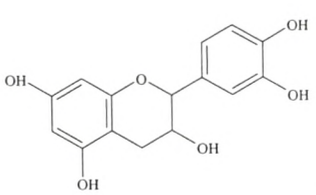
These generally support or promote the healing process of the liver. In practice these two activities cannot be easily distinguished from each other.

3. Hepatoprotective agents:

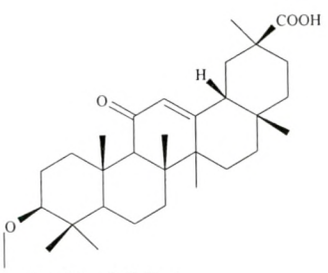
These generally prevent various types of liver affections prophylactically.

In general any hepatoprotective agent can act as an antihepatotoxic toxic or hepatotropic agent but the vice versa is always not true.

There are number of phytoconstituents from plants which have exhibited antihepatotoxic activity. Some of the reported constituents with pharmacologically/therapeutically proved claims may be enlisted as Silymarin, Glycyrrhizin, (+)-Catechin, Saikosaponins, Curcumin, Picroside I and II and Gomisin etc (Wagner et al., 1998). Acetylbergenin (Lim et al., 2000), Kolaviron (flavonone) (Oluwatosin and Edward, 2006) was also reported for its hepatoprotective properties.

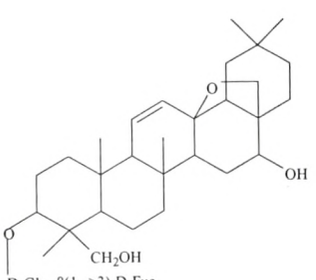


(+)-Catechin



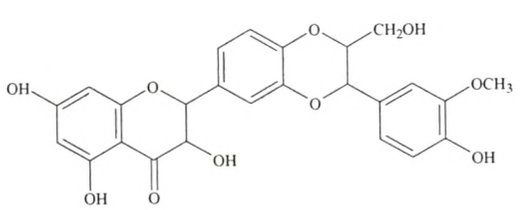
D-Glc A-ß (1-->2)-D-Glc A

Glycyrrhizin

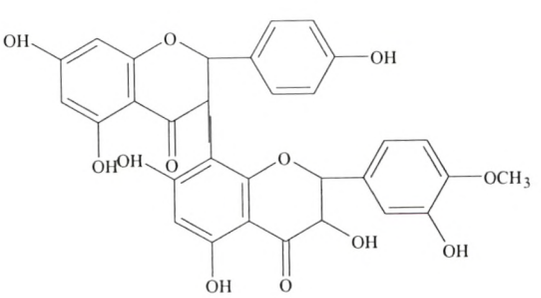


D-Glc- ß(1-->3)-D-Fuc

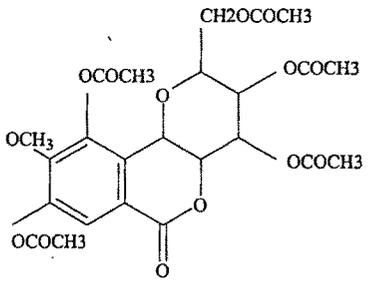
Saikosaponin a



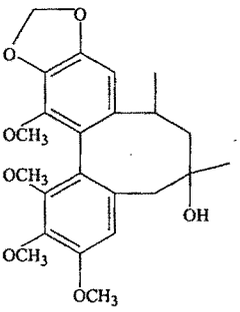
Silybin



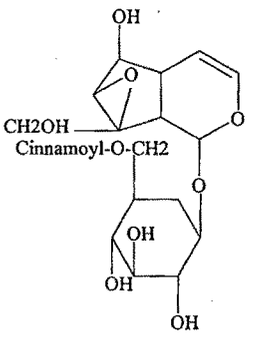
Kolaviron



Acetylbergenin



Gomosin A



Picroside I

The following are the some of the plants drugs having hepatoprotective claims.

The usefulness of silymarin from the extracts of *Silybum marianum* (*Compositeae*) in liver disorders was reported (Wagner et al., 1988).The usefulness of rhizomes of *Curculigo orchioides* (*Hypoxidaceae*) was reported (Rao and Mishra, 1996). The antihepatotoxic activity of *Capparis spinosa* was documented (Chhaya and Mishra, 1999). *Fumaria indica* (*Fumariaceae*) was found to possess antihepatotoxic activity (Rao and Mishra, 1998). The usefulness of the *Andrographis paniculata* (*Acanthaceae*) for hepatoprotective activity was reported (Handa and Sharma, 1990).The hepatoprotective activity of picroliv from *Picrorhiza kurrooa* (*Scrophulariaceae*) was established (Dwivedi et al., 1991).

The other plants that are reported in the literature possessing anti-hepatotoxic/hepatoprotective activities are *Eclipta alba* (*Asteraceae*), *Glycyrrhiza glabra* (*Leguminosae*), *Tinospora cardifolia* (*Menispermaceae*), *Uncaria gambier* (*Rubiaceae*), *Sida cordifolia* (*Malvaceae*), *Phyllanthus niruri* (*Euphorbiaceae*), *Inula racemosa* (*Asteraceae*), *Garcinia kola* (*Guttiferae*), *Boerhaavia diffusa* (*Nyctaginaceae*), *Phyllanthus amarus* (*Euphorbiaceae*), *Schizandra chinensis* (*Schizandraceae*) etc.

1.4 Review of selected plant drugs

Pergularia daemia, commonly known as Dustapu teega in Telugu and Uttaravaruni in Hindi is particularly used by the folklore people of the Chittoor district of the Andhra Pradesh state in India, to treat jaundice. Ayurveda

describes the usefulness of *Baliospermum montanum*, commonly known as Danti, in the treatment of jaundice. Literature survey of theses drugs revealed that scientific work on their hepatoprotective activity and medicaments are yet not well documented. Therefore these plants were selected for further investigations.

***Pergularia daemia*:**

Pergularia daemia (Forsk) Chiov, Syn. *Daemia extensa* R Br. (*Asclepiadaceae*) is a foetid smelling laticiferous twinner found in the plains throughout the hotter parts of India, ascending to an altitude of 1000 m in the Himalayas (The Wealth of India, 1966).

The synonyms of the *P. daemia* in various vernacular languages are as follows.

Sanskrit: Uttaravaruni
Hindi: Utranajutuka
Telugu: Dustapu teega
Gujarati: Amaradudheli
Marathi: Mendhadudhi
Tamil: Achanimuli

The plant is a perennial wining herb, foetid when bruised and with much milky juice; stems clothed with spreading hairs. Leaves thin, 5-10 by 3.8-9 cm, broadly ovate or sub orbicular, acuminate, glabrous or more or less shortly pubescent above, usually velvety pubescent beneath, the margins ciliate, base deeply cordate, the basal lobes semi orbicular; petioles 2-6.3 cm long, pubescent. Flowers greenish yellow or dull white tinged with purple, in lateral cymes which are at first corymbose, afterwards racemose; peduncles pubescent, coming off from between the petioles, though not quite midway between them, 7.5-15 cm long; pedicels capillary, 2-3.2 cm long, pubescent. Calyx pubescent, divided to the base; sepals 3 mm long, ovate-lanceolate, acute, and ciliate. Corolla is narrowly campanulate. Follicles reflexed, 5-7.5 cm by 1.3 cm, lanceolate, attenuated into a long beak, echinate with soft spines. Seeds 4-8 mm, ovate, truncate at the apex, densely velvety-

pubescent on both sides, narrowly margined, crenate at the round base (Kirtikar and Basu, 1983).

The plant is pungent, cooling; anthelmintic, laxative, antipyretic; cures asthma, biliousness and ulcers; useful in eye troubles, urinary discharges, leukoderma, uterine complaints and inflammations; facilitates parturition (Kirtikar and Basu, 1983).

The root bark is mixed with cow's milk used as a purgative in rheumatic cases. The fresh leaves made into a pulp are used as a stimulating poultice in carbuncle, with good effect. A decoction is given as an anthelmintic. The juice of the leaves is used as expectorant in the treatment of catarrhal affections; it is given in asthma, and applied to rheumatic swellings in combination with lime or ginger. The juice is also used in the preparation of purgative medicinal oil given in rheumatism, amenorrhoea and dysmenorrhoea. In western India the plant has a general reputation as an expectorant and emetic. It is also used in infantile diarrhoea. The juice of the leaves is squeezed into sore eyes, which it is said to cure. The drug was strongly recommended for malarial intermittent fevers.

The plant extract is used for uterine and menstrual troubles and to facilitate parturition. It has a stimulant action on uterine and other involuntary muscles, simulating that of Pituitrin in many respects. Administration of extract causes rise in arterial blood pressure, increase in movement and tone of the urinary bladder and stimulation of gastric secretions (The Wealth of India, 1966).

The presence of cardenolides, alkaloids, triterpenes and saponins in the plant was reported (Sathish et al., 1998).

Various triterpenes and steroidal compounds like β-amyrin, α-amyrin, lupeol, oleanolic acid, β-sitosterol etc were reported in *P.daemia* (Aanjaneyulu et al., 1998; Talapatra et al., 1981).

The ethanolic extract of the plant was found to possess anti inflammatory, anti pyretic and analgesic activities (Sathish et al., 1998).

The alcoholic and aqueous extracts of the plant exhibited anti diabetic activity (Wahi et al., 2002).

Anti inflammatory activity of the crude ethanolic extract was reported (Hukkeri et al., 2001).

The presence of various cardenolides like, calotropin, uzarigenin, calactin, corotoxigenin, calotoxin etc. was also documented (Mittal et al., 1962)

The musculotropic action of polypeptide fraction isolated from ethanolic extract of *Pergularia extensa* was also reported (Roy et al., 1960).

The presence of various sterols in petroleum ether extract of *P. daemia* was reported (Rakhit et al., 1959).

The presence of various flavonoidal compounds in the *P. daemia* was reported (Samia et al., 2006).

The present studies were planned to assess the hepatoprotective activity scientifically in rats against carbon tetrachloride, paracetamol and thioacetamide as hepatotoxins to verify its claims in folklore practice against liver disorders and to isolate bio active molecules from the bio active guided extracts.

Baliospermum montanum:

Baliospermum montanum Muell Arg. Syn *Baliospermum axillare* Blume. of the family *Euphorbiaceae* is a leafy undershrub, distributed in outer range of Himalayas from Kashmir to Assam and in moist deciduous forests elsewhere in India.(The Ayurvedic Pharmacopoeia India, 2001)

The synonyms of the *B. montanum* in various vernacular languages are as follows.

Sanskrit: Danti

Hindi: Danti

Telugu: Adaviaamudamu

Gujarati: Jamalgota, Dantimul

Marati: Danti

Tamil: Kattamanakku

Malayalam: Dantika, Nagadanti.

The plant is a stout, usually monoecious undershrub, 0.9-1.8 m height, with many shoots arising from the base, distributed almost throughout India from Kashmir eastwards to Arunachal Pradesh, up to an elevation of 1,000 m and southwards to peninsular India, ascending to an altitude of 1,800 m in the hills of Kerala. Bark is light brown in color, fracture is difficult; upper leaves are small, lanceolate, lower leaves are large, up to 30 cm, ovate, palmately lobed, petioles with a pair of stipular glands; flowers are small, green, in axillary racemes or panicles, all male or with a few female flowers below; capsules obovoid, 3-lobed, 0.8-1.3 cm long; seeds oblong, smooth, shiny, 0.6-0.8 cm long, mottled; endosperm oily. The plant is common in shady places and often reproduces by root suckers (The Wealth of India, 1988).

In Ayurvedic system of medicine the roots are useful as purgative, anthelmintic, diuretic. They are also useful in jaundice, disease of the skin and of the abdomen, piles, wounds, enlarged spleen, inflammations, anaemia, leukoderma, and in pains (Kirtikar and Basu, 1983).

The seeds are used as a drastic purgative. They are also used externally as a stimulant and rubefacient. The oil is a powerful hydragogue cathartic, and is useful for external application in rheumatism (Kirtikar and Basu, 1983).

The usefulness of the plant in abdominal tumours and cancer was reported (Hartwell, 1969).

Phytochemically the leaves of the plant have been investigated for steroids, terpenoids and flavonoids (Mukherjee and Ray, 1980).

The presence of phorbol esters like Baliospermin, Montanin, Phorbol 12-deoxy 13-O-palmitate etc which are diterpenes, in the ethanolic extract of roots and their anti tumour activity was reported (Ogura et al., 1978).

The usefulness of macerated aqueous extract of leaves in asthma was also reported (Panthong et al., 1986).

Hypotensive activity of the hydro alcoholic extract of entire plant was carried out (Bhakuni et al., 1971).

The usefulness of the hot aqueous extract of plant as a purgative and stimulant was documented (Kapur, 1983).

The usefulness of the aqueous extract of the leaves from plant in the treatment of scabies was also reported (Alam, 1992).

The usefulness of the stems in toothache and dried seeds as purgative was reported (Shah and Gopal, 1985).

Based on the above literature the plant was selected for the study to assess the hepatoprotective activity scientifically in rats against carbon tetrachloride, paracetamol and thioacetamide as hepatotoxins to verify its claims in traditional medicine (Ayurveda).

1.5 Research envisaged

Survey of literature on selected plants i.e. *Pergularia daemia* and *Baliospermum montanum* revealed that the scientific data are unavailable as regards to their hepatoprotective activity is concerned although these are used in traditional system of medicine for liver disorders. These plants therefore offer scope for investigations on the hepatoprotective potential, isolation and characterisation of bioactive molecules responsible for activity, in order to verify, claims in the traditional system of medicine for their efficacy.

Therefore, studies on the standardization of the selected plants were planned in the following manner, taking WHO guidelines for quality control methods for medicinal plant materials into consideration.

A. Pharmacognostic studies:

1. Collection and identification of plant material
2. Macroscopic evaluation.
3. Microscopic evaluation.

B. Proximate analysis.

1. Loss on drying.
2. Determination of foreign organic matter.
3. Determination of extractive values.
4. Determination of ash values.
5. Determination of foaming index.
6. Estimation of heavy metals.
7. Estimation of total phenolic content.
8. Estimation of total flavonoid content.

F. Phytochemical studies.

1. Successive solvent extraction of plant drugs.
2. Qualitative evaluation of successive extracts.
3. TLC studies of successive extracts.
4. Preparation of selective extracts and fractionation of them.
5. TLC studies of selective extracts and their fractions.

G. Biological studies.

1. Acute toxicity studies.
2. In vivo screening of extracts and fractions for hepatoprotective activity
    a. Carbon tetrachloride model
    b. Paracetamol model.
    c. Thioacetamide model.
3. Histopathological studies.
4. In vitro screening of fractions for hepatoprotective activity.
    a. Hepatic cytotoxicity testing.
    a. Carbon tetrachloride model
    b. Paracetamol model.
    c. Thioacetamide model.

H. Isolation and characterisation of bio active molecules from bio active guided fractions and their in vitro screening for hepatoprotective activity.

I. High performance thin layer chromatographic studies.

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