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Studies on hepatoprotective activity of traditional ayurvedic formulation 'Vidakana Choornam' against carbon tetrachloride induced hepatotoxicity in albino rat

Jayarama Reddy[1], Gnanasekaran D.[2], Vijay D.*[3] and Ranganathan T.V.[1]
[1]St. Joseph's Post graduate and Research Centre, 36, Langford Road, Bangalore-560027
[2]Bharati College of Pharmacy, Bharati Nagara, Mandya, Karnataka
[3]Research Department of Plant Biology and Biotechnology, Arignar Anna Government Arts College, Viluppuram, Tamil Nadu

Abstract- The study was carried out to ascertain the hepatoprotective activity of traditional ayurvedic formulation 'Vidakana Choornam' against carbon tetrachloride induced hepatotoxicity in albino rat. Effect of carbon tetrachloride and herbal products on liver weights was studied. Bilirubin level in serum, Serum Glutamate Pyruvate Transaminase (GPT) level in serum, Serum Glutamate Oxaloacetate Transaminase (GOT) level in serum and ALP level in serum were estimated in both control treatment. Histopathological studies were also carried out. It was found that there was an increase in liver weight during carbon tetrachloride treatment which may be due to accumulation of fat. The results of Vidakana Choornam' treatment against carbon tetrachloride induced hepatotoxicity revealed that there was considerable reduction in Bilirubin level in serum, Serum Glutamate Pyruvate Transaminase (GPT) level in serum, Serum Glutamate Oxaloacetate Transaminase (GOT) level in serum and ALP level in serum. The hepatoprotective activity of this simple formulation was found to be as effective as Liv – 52, infact the activity on decreasing the serum bilirubin level was much higher when compared to Liv – 52.
Keywords: Vidakana Choornam, Bilirubin, Serum Glutamate Pyruvate Transaminase, Glutamate Oxaloacetate Transaminase, ALP level, hepatoprotective, Histopathology.

1. Introduction

Diseases of Liver

Disturbances of metabolism occurring in liver diseases are largely the result of failure of the parenchymal cells to carryout vital functions, because of

- Infection or presence of noxious agents
- Decrease mass of functioning cells
- Decreased blood supply
- Impaired nutrition and
- Reaction of other organs to liver damage e.g, Kidney, pancreas, adrenals, gonads and spleen.

Infectious diseases of the liver, such as viral hepatitis, are characterized by degeneration and necrosis of parenchymal cells and may be followed by complete disappearance of cells and destruction of the normal architecture of the lobule. Deposition of fat in the liver may occur from over nutrition, from dietary deficiencies or from secretion of toxic substances. Impaired nutrition may be an important factor contributing to liver diseases accompanying chronic addiction to alcohol.

Proliferation of connective tissue of the liver leads to disorganization of the liver structure and this leads to interference with the blood supply. The end result is a shrunken liver consisting largely of connective tissues with a markedly decreased mass of parenchymal, reticulo – endothelial and vascular tissue.

Damage to liver may be caused by many chemicals and drugs. The main site of action may be the parenchymal cells with wide spread necrosis which may prove fatal or it may be confined largely to relative benign effects upon the biliary secretory mechanism.

Liver disease may affect the metabolism and functions of other organs notably the brain and the kidneys.

Hepatotoxicity

A hepatotoxin might be defined as any chemical agent that can produce injury to the liver. A large number and variety of compounds have been identified as hepatotoxins of chemical or experimental relevance. They may be as simple as inorganic elements and compounds or as complex as heterocyclics, steroids or peptides.

The hepatic damage may involve mainly the hepatic parenchymal cells, cells of the excretory tree or both. In the case of some hepatotoxic agents, vascular structures are the primary focus of injury, acute hepatic injury may be translated into chronic liver diseases, expressed as cirrhosis or even as carcinoma.

The large volume of literature on experimental hepatotoxicity includes many facets relevant to clinical medicine. The studies conducted have been useful in understanding of the character and mechanism of hepatotoxic states in human (e.g., acute hepatic injury induced by carbon tetrachloride). Studies of experimental hepatotoxicity also provide increased understanding of chronic diseases that might be due to hepatotoxins. Experimental hepatotoxic states also have provided as useful models for study of the genesis of the tissue injury in general and the histogenesis, biochemical features and manifestations of spontaneous hepatic diseases (e.g., viral hepatitis). In particular, hepatic necrosis induced by the chemical agents has

been useful model for the study of biochemical pathology of the death of cells. Experimental hepatic injury is a fundamental tool for the development of diagnostic methods, and it has become traditional for tests of liver function and serum enzyme test to be evaluated in animals with experimental hepatic injury before assessing their usefulness in clinical basis [1].

Classification of Hepatotoxins

Clinical and experimental observations have led to the general agreement that there are two main categories of agents that can produce hepatic injury. One consists of agents that are predictable (true, intrinsic) hepatotoxins . Intrinsic hepatotoxins are by definition, experimental hepatotoxins and the other consists of agents that are not predictably hepatotoxic but produce hepatic injury in only a small portion of exposed individuals, who are uniquely susceptible (host idiosyncrasy).

Intrinsic Hepatotoxins

There are two types of intrinsic hepatotoxins

- Direct and
- Indirect

Direct hepatotoxins produce direct injury to the hepatocyte and its organelles, especially the endoplasmic reticulums. Carbon tetrachloride, the prototype, produces peroxidation of the membrane lipids or other chemical changes that lead to denaturation of the membranes. These changes which begin almost immediately after administration of the toxin are the first stages of the injury and culminates in the characteristic cytotoxic effect i.e., hepatic necrosis, steatosis or both.

Indirect hepatotoxins are more selective. They are antimetabolites and related compounds that produce hepatic injury by interference with a specific metabolic pathway or process. The structural injury produced by indirect hepatotoxins appears to be secondary to a metabolic lesion (hence indirect), while in that produced by direct hepatotoxins, the metabolic derangement is secondary to structural injury. The hepatic damage produced by indirect hepatotoxins may be mainly cytotoxic expressed a s steatosis or necrosis or may be mainly cholestatic expressed as arrested bile flow with or without bile duct injury.

Host Idiosyncrasy

Two types of mechanisms are involved

- Hypersensitivity due to drug allergy
- Metabolic abnormality due to production of toxic metabolites.

A number of agents including chloroform, carbon tetrachloride, frusemide, phenacetin, acetaminophen etc., are directly toxic to the liver cells. These direct acting compounds shows a strict dose dependence with very high reproducibility experimentally and with a very high incidence of damage in exposed individual. The latency between drug administration and onset of damage is very short, of the order of few hours [1].

Mechanism of Hepatotoxicity of Carbon Tetrachloride

In the case of carbon tetrachloride poisoning the toxic metabolite is the trichloromethyl free radical, {CCl3} which can be “mopped up” by N-acetyl cysteine or cystomine given up to 12 hours after carbon tetrachloride administration. In contrast, drugs such as pyrazole or amino triazole, which prevent the free radical formation are protective only if given before or together with carbon tetrachloride, but cannot counteract against CCl. * if given after it has formed [2].

The injury caused by carbon tetrachloride can be demonstrated by :

1. Liver function tests
2. Histopathology

Liver Function Tests

The history of patients illness and the findings on clinical examination provide the essential basis on which to select further investigations of suspected liver disease and more specifically, to interpret their results. Both clinical chemistry and histological examination of the liver is usually needed for more precise diagnosis.

Hepatic function tests may be used to

- Establish the presence or absence of disease
- Differentiate the types of jaundice and
- Determine, by serial performances of function tests whether a disease process is remaining static, progressing or regressing.

Classification of Liver Function Tests

1. Tests depending primarily on hepatic secretion and excretion
   - Bile pigments
   - Clearance of foreign substances from the serum
2. Tests depending upon specific biochemical functions
   - Protein metabolism tests
   - Carbohydrate metabolism tests
   - Lipid metabolism tests
3. Tests depending upon the measurement of serum enzyme activity
   - GGTP or alkaline phosphates
   - Transaminases
   - Other enzymes

Serum Bilirubin Level

In general, rising serum bilirubin levels have unfavorable implications, and falling values are characteristic of remission of liver disease or biliary obstruction. Elevation may occur because of excessive destruction of haemoglobin. In viral hepatitis, bilirubin may be present in urine, and may increase slightly in serum. In viral hepatitis with jaundice high total and conjugated serum

bilirubin are found in serum and urine. In complete extrahepatic obstruction marked elevation of bilirubin is observed.

Plasma Enzyme Tests In Liver Diseases

Three patterns of altered plasma enzyme activity may be seen in patients with liver diseases, due to the following derangement's –

• Release of soluble cytoplasmic enzymes and to a lesser extent, mitochondrial enzymes. This occurs in hepatic cellular damage.
• Release of membrane associated enzymes.
• Impaired synthesis of certain enzymes (e.g. cholinesterase)

The third group is rarely used for assessing the severity of liver diseases.

Soluble Cytoplasmic Enzymes

Measurement of plasma amino transferase (SGPT and SGOT) activity is the standard tests of hepato cellular damage. Level of both transaminases is elevated in sera of patients with acute hepatic disease. Both SGPT and SGOT(ALT and AST) are located in the cytosol of the liver cell. SGOT in addition is also found in mitochondria. These enzymes are of majot importance in assessing and monitoring the degree of liver cell inflammation and necrosis, which results in the release of these enzymes into circulation, due to the increased permeability of the cell membrane or breakdown of the cells.

Membrane Associated Enzymes

Many enzymes are anchored to cell membranes, in particular to the biliary canaliculus. These include alkaline phosphatase and gamma glutamyl transpeptidase. Alkaline phosphatase is used as one of the routine liver function tests in almost all hospitals and laboratories. Changes in the activity of other membrane associated enzymes usually accompany the changes in plasma alkaline phosphatase in cholestatic liver disease [3,4,5].

Histopathology

Since carbon tetrachloride causes acute hepatic injury, especially cytotoxic form of damage, the characteristic features seen are necrosis and steatosis (fatty liver). Carbon tetrachloride induces centrizonal necrosis. Degenerative changes including formation of free (sinusoidal) acidophilic bodies, acidophilic degeneration, ballooning, precede centrolobular necrosis. Lesions of smooth or rough endoplasmic reticulum, changes in chromatin, nucleus, nucleolus, lysozomes and mitochondria are induced by toxic agents like carbon tetrachloride and can be studied by hepatic pathology [1].

Hepatoprotective Formulations

About 600 commercial preparations with claimed liver protecting activity are available all over the world. In India about 33 patent herbal formulations are availablr for liver ailments and these preparations represent a variety of combinations out of 100 Indian medicinal plants belonging to about 40 families. Patent Indian herbal preparations sold for liver ailments are Acilvan, Adliv, Biligen, Hepa – 10, Hepex, Hipex, Kalmegh Compound, Liv – 52, Liv – 77, Liva -16, Livarin, Livatone, Livergen, Livodin, Livomyn, Neoliv – 100, Stimuliv, Syliv, Tefroli, Vimiliv etc,. The marketed preparation selected for present investigation is Liv – 52, activity of which is used for comparing the activity of traditional Ayurvedic formulation 'VIDAKANA CHOORNAM'.

LIV – 52, a product of Himalaya Drug Company, Bombay is composed of

Achillea millefolium
Capparis spinosa
Cassia occidentalis
Cinchorium intybus
Solanum nigrum
Tamarix gallica
Terminalia arjuna
Eclipta alba
Phyllanthus niruri
Boerhavia diffusa
Phyllanthus embilica
Fumaria officinalis
Terminalia chebula
Tinospora cordifolia
Andrographis paniculata

Liv -52 is a mixture of extracts from 18 herbaceous plants. It has been reported that Liv -52 protects the liver form the hepatotoxicity of paracetamol, anticancer drugs, antibiotics, oral contraceptives, alcohol and carbon tetrachloride. Liv -52 enhances the activity of microsomal drug metabolizing enzymes. Further studies revealed that Liv -52 protects mice liver against cadmium intoxification [6,7,8]. Comparative efficacy of Liv – 52 and andrographics paniculata was evaluated in carbon tetrachloride induced liver damage in rabbits. Animals treated with these drugs showed marked clinical recovery from liver damage as compared to control animal. But recovery was found to be rapid in the Liv – 52 treated animals. Pathology reports showed remarkable regenerative changes in Liv – 52 treated animals where as animals treated with Andrographics paniculata did not show rapid regenerative changes [9].

Liv – 52 has got a marked beneficial effect in the early cases of hepatic cirrhosis. It was found useful in cases of hepatic cirrhosis having steatorrhoea. Liv – 52 was found to be an useful drug for therapy of acute viral hepatitis. It was also revealed that Liv – 52 increased the LD50 of Beryllium salts in rats by many times and thus induced a significant protective index [10,11,12].

INGREDIENTS OF VIDAKANA CHOORNAM

Moringa oleifera

Family
Moringaceae
Vernacular Names
Sanskrit Shobanjana
Hindi Sainjna
Telugu Mulaga
Tamil Murangae
Kannada Nugge
Malayalam Muringa
Habitat
A small or medium sized tree, about 10mts high, found wild in the sub-Himalayan tract and cultivated all over the plains of India.

Constituents

The root bark contains two alkaloids (total alkaloid 0.1%) Moringine (identical with benzylamine) and moringinine. An alkaloid, named spirochin, has been isolated from root bark. Root bark contains traces of essential oil with pungent smell, phtosterols, waxes and resins.

Uses

All parts of *Moringa oleifera* are used in Ayurvedic medicine. The leaf is considered as an important eye nutrient, is particularly rich n Vitamin A and also used as an aphrodisiac. It also has antibiotic and antifungal properties. Also used as an antitubercular drug, although ayurvedic medicine uses the root for liver disorders. Internally a decoction or infusion of root (1 in 20) with the addition of mustard seed bruised is useful in doses of 1-2 ounces in ascites due to diseases of liver and spleen. Medicines made from Moringa are also gynecologically valuable both to induce abortions and in child birth as an aid for difficult deliveries [13].

Embelia ribes

Family
Myrsinaceae
Vernacular Names
Sanskrit Vidanga
Hindi Waranga
Telugu Vellal
Kannada Vauvidanga
Malayalam Vizhalari
Habitat
These climbers are found in hilly parts from the central and lower Himalayas down to Ceylon and Singapore.
Parts Used
Berries (fruits), leaves and root bark.

Constituents

It contains embelic acid, volatile and fixed oil, coloring matter, tannins, a resinoid and alkaloid called christambine.

Uses

Dried berries are useful to expel intestinal worms especially tape worm, prevents flatulence and useful in dyspepsia.

Piper longum

Family
Piperaceae
Vernacular Names
Sanskrit Pippali
Hindi Pipal
Malayalam Tippali
Kannada Hippali
Telugu Pippali
Tamil Pipilli
Habitat
This plant is indigenous to north eastern and southern India and Ceylon and cultivated in Eastern Belgaum.
Parts Used
Immature berries (unripe fruits or fruiting spikes) and stems

Constituents

Resins, volatile oil, starch, gum, fatty oil, inorganic matter and an alkaloid – piperine – 1-2%

Uses

Infusion is stimulant, carminative, tonic, aphrodisiac, diuretic, vermifuge and emmenagogue. Also used in bronchitis, gout, paralysis, epilepsy and obstruction of liver and spleen.

2. MATERIALS AND METHODS

2.1 Collection of Materials

Constituents of the formulation namely the bark form the root of *Moringa oleifera*, seeds of *Embelia ribes* and berries of Piper longum, were collected from the plantation maintained by Gopalakrishnan (Ayurvedic practitioner). The parts collected were washed to free it from adhering impurities and dried in shades. The formulation was then prepared as per the text under the guidance of Gopalakrishnan (Ayurvedic practitioner).

2.2 Preparation of the Formulation

Ingredients

Moringa oleifera (Muringa)	-100gms
Embelia ribes (Vidang)	-100gms
Piper longum (pippali)	-100gms

Preparation

Moringa oleifera – Barks from the roots were washed, dried in the shades and powdered.

Embelia ribes – The dried fruits were taken and the testa was removed. The endosperm was dried again under shade, powdered and sieved through muslin cloth.

Piper longum – The dried and unripe fruits were powdered and sieved through muslin cloth. All the three powders were mixed thoroughly in

equal proportions and preserved in air tight containers.

Indications
Jaundice, Steatosis (fatty liver).

Dose
One teaspoonful along with milk to be taken twice a day, during earlier stages of disease. During severe conditions one teaspoonful up to 4 times a day along with milk.

2.3 Stability Studies
The importance of stability testing in the development of pharmaceutical dosage form is well recognized in the pharmaceutical industry. The FDA regulation requires a complete description of the data derived from studies of the stability of the drug including information showing the suitability of the analytical method used. Various stress tests are performed on solid and solution samples to establish the effect of heat, light, oxygen and pH on drug substance stability.

Heat Stability
Heat stability of a drug substance will have a major influence on the marketable physical form of the drug product as well as processing parameters allowable. Drugs that are not stable in solution require refrigerated storage or lyophilization. Lyophilized products are limited to constitution and use within a short period of time, whereas the need for refrigerated storage is economically undesirable and a marketing disadvantage unless completely justifiable. Samples were stored at different temperatures like 4C, 25C, 37C and 50C. At the end of 7, 14, 21 and 30 days all the samples stored under different temperatures were observed for any physical change and TLC pattern.

2.4 Pilot Study to Determine the Dose of Carbon Tetrachloride
Procedure
1. 28 Male albino rats weighing between 150 – 200gms were taken and divided into seven groups of four animals each.
2. Group I animals were maintained as normal control without carbon tetrachloride treatment.
3. All the animals were maintained at normal diet.
4. Group II, III, IV, V, VI and VII rats were treated with carbon tetrachloride subcutaneously at a dose of 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 ml/kg body weight respectively with equal volume of liquid paraffin for two successive days.
5. 24 hours after the second dose the rats were sacrificed and liver was removed and subjected to histopathological studies to determine the extent of carbon tetrachloride induced damage.

2.5 Effect of Carbon Tetrachloride and Formulations on Liver Weight
1. 24 male albino rats weighing between 150 – 200 gms were divided into 4 groups of six animals each. The animals were maintained on a uniform diet throughout the experiment.
2. Group I was maintained as normal control without any treatment.
3. All the other groups were administered with carbon tetrachloride with a dose of 3ml/kg body weight with equal volume of liquid paraffin subcutaneously for two successive days.
4. Group II animals were maintained on carbon tetrachloride control without any drug treatment.
5. Group III and IV animals received Vidakanachoornam and Liv – 52 respectively in a dose of 300mg/kg body weight. (Liv -52; Batch No: 20603; Date of manufacture: March 1996). The drug treatment was carried out orally for a period of four days.
6. 24 hours after the last day's drug treatment, the blood was collected by intra cardiac puncture.
7. Serum was separated and tested for Bilirubin, SGPT, SGOT and ALP levels.
8. After collecting the blood, the animals were sacrificed and liver was isolated, weighed and preserved in 10% formalin solution for histopathological studies.

2.6 Estimation Of Bilirubin
Total Bilirubin reagent (ERBA – TEST, TRANSASIA BIOMEDICALS PVT. LTD.) was used for invitro qualitative estimation of total bilirubin in serum.
Principle
The assay of bilirubin was based on the reaction with diazotized sulphanilic acid to form a colored azobilirubin. In aqueous solution, only conjugated bilirubin will react and to measure the total bilirubin it is necessary to add an "Accelerator" or solvent.
The intensity of purple color formed was proportional to the bilirubin concentration and was measured photometrically between 540-600nm with a broad spectrum peak at 560nm.

Reagent Composition
Active Ingredient
Concentration
1. Sulphanilic acid
29mmol/lt.
2. Hydrochloric acid
165mmol/lt.
3. Dimethyl sulphoxide (DMSO)
4400mmol/lt.
4. Sodium nitrite
0.72mmol/lt.

Procedure
1ml of working reagent was added to each cuvette. 0.1ml each of distilled water, standard and sample was added to the blank, standard

and sample tubes respectively and kept for 6 minutes and the absorbance was read at 550nm.

System Parameters

Temperature	Room temperature
Wavelength	550nm
Absorbance	0 – 2 A
Cuvette path length	1 cm
Reagent volume	1ml
Sample volume	100 UL(0.1 ml)

Calculation

Total Bilirubin = Absorbance of unknown / Absorbance of control sera x value of control sera (mg%)

2.7 Estimation of Serum Glutamate Pyruvate Transaminase

The SGPT reagent kit (auto pak AMES) was used for in-vitro quantitative determination of GPT activity in serum.

Principle

GPT catalyzes the transfer of the amino group from L-alanine to alpha keto glutarate to yield pyruvate and L-glutarate. Lactate dehydrogenase (LDH) then converts pyruvate and NADH into lactate and NAD. The conversion of NADH to NAD decreases the absorption at 340 nm. The rate of decrease in absorbance is proportional to the GPT activity.

Reagent

1. Alanine / Buffer
   1A. NADH and LDH
2. Alpha – keto glutarate

The SGPT reagent kit consists of 2 bottles of reagent 1 and two vials each of reagent 1A and 2.

Preparation of Working Solution

Reagents were allowed to attain the room temperature. The content of reagent vial 1A was quantitatively transferred to bottle 1 and mixed until completely dissolved (solution1), 7 ml of distilled water was added to the vial no.2 and mixed until completely dissolved (solution 2).

The working solution was freshly prepared in the proportions given below –

Solution 1	3.0ml
Solution 2	0.3ml

Freshly prepared working solution should be stored at 2-8 degree C and used within 8 hours of preparation.

Procedure

1ml of working solution was added to each cuvette, followed by addition of 10 ml each of distilled water (blank), sample and standard to the respective tubes and the absorbance was read at 340 nm.

General System Parameters

Reaction type	Kinetic
Wavelength	340 nm
Flowcell temperature	37C
Sample volume	00ml
Reagent volume	cm
Path length	1cm
Factor	1749
Zero setting with	Distilled water

Calculation

TV = total volume of assay(1.1ml)
6.22 = Molar extinction co-efficient of NADH
1000 = To convert units/ml to units/litre
SV = Sample volume (0.1ml)
LP = Light path in cm (1cm)
1749 = Absorbance/minute has to be multiplied by this factor.

2.8 Estimation of Serum Glutamate Oxaloacetate Transaminase

RAICHEM SGOT Reagent kit was used for in-vitro quantitative determination of GOT activity in serum (Reagent Applications Inc. San Diego)

Principle

GOT catalyses the transfer of the amino group from L-aspartate to alpha keto glutarate to yield oxaloacetate and L-glutamate. Malate dehydrogenase (MDH) then converts oxaloacetate and NADH to NAD and L-malate. The conversion of NADH to NAD decreases the absorbance at 340 nm, the rate of which is proportional to the GOT activity.

Reagent

1. L-Aspartate/Buffer
   1A. NADH and MDH
2. Alpha-keto glutarate.

Procedure

Reagent was brought to incubation temperature. 1ml working solution was added to each cuvette, followed by addition of 0.1ml each of blank (distilled water) standard and sample to the respective tubes, mixed and incubated for 1 minute at reaction temperature. The instrument was adjusted to zero absorbance with the blank. Absorbance reading was taken at 30 seconds interval. The absorbance/minute was determined from the linear part of the assay.

System Parameters

Wavelength	340nm
Temperature	30o C
Blank	Distilled water
Sample volume	.1 ml
Reagent volume	ml
Path length	1cm
Factor	768

Calculation

A/min X(TV X 1000) / (6.22 X SV X LP) = GOT/L of sample where

TV = total volume of assay (1.1 ml)
6.22 = Molar extinction coefficient of NADH

1000 = to convert units/ml to units/ litre
SV =Sample volume (0.1 ml)
LP = Light path in cm (1cm)
1786 = Absorbance/minute has to be multiplied by this factor.

2.9 Estimation of ALP Level
Procedure
The samples and the working solution were brought to room temperature prior to use.
System Parameters

Reaction type	Kinetic
Wavelength	405nm
Flow cell temperature	25 degree C
Delay time	60 seconds
Number of reading	4
Internal	30 seconds
Sample volume	30.0 UL
Reagent volume	1.0 ml
Path length	1 cm
Factor	1826
Zero setting with	Distilled Water

2.10 HISTOPATHOLOGICAL STUDIES
Processing of Isolated Liver
The animals were sacrificed and the livers were isolated. The isolated liver was cut into small pieces and fixed in buffer neutral formalin (10%) solution for atleast two days. Following this was the washing step where by the liver pieces were washed in running water for 12 hours. This was followed by dehydration with alcohol of increasing strength (70%, 80% and 90%) for 12 hours each. Then the final dehydration was done using absolute alcohol with about three changes at 12 hours interval.
Clearing was done by using xylene with two changes for 15 to 20 minutes each. After clearing, the organ pieces were subjected to paraffin infiltration in automatic tissue processing unit.
Hard paraffin was melted and the hot paraffin was poured into L shaped blocks. The liver pieces were then dropped into the melted paraffin and allowed to cool quickly. The blocks were cut using microtone to get sections of thickness of 5u. the sections were taken on a microscopic slide on which egg albumin (sticky substance) was applied. The sections were allowed to remain on the slide for 3 days till it sticks firmly onto the slide.

Staining
Eosin is an acid stain. Hence it stains all the cell constituents, which are basic in nature, red (e.g., cytoplasm). Hemotoxylin is a basic stain which stains all the acidic cell components blue (e.g., DNA,RNA in nucleus).

Staining Procedure
1. The sections were washed with xylene for about 15 minutes to deparaffinize the section.
2. The sections were then washed with alcohol of decreasing strength (100%, 90%, 80%, 70%) to hydrate the section.
3. Finally sections were washed with water.
4. Sections were stained with Hematoxylin for 15 minutes followed by rinsing in water.
5. The slides were then dipped quickly in acid alcohol for 3 to 10 times for differentiation. Checked the differentiation with a microscope. Nuclei were distinct as the background was very light / colorless.
6. Slides were washed in water briefly and dipped in ammonia water until the sections becomes bright blue (3-5 dips).
7. Sections were then washed in running water for 10 to 20 minutes; if washing is inadequate eosin will not stain evenly.
8. The sections were then stained with eosin for 15 seconds to 2 minutes depending on the age of eosin and depth of the counter stain required.
9. Sections were cleared with xylene, 2 changes of 2 minutes each and mounted in DPX (Diphenyl xylene)

3. RESULTS AND DISCUSSION
3.1 Stability Studies
The importance of stability testing in the development of pharmaceutical dosage form is well recognized in the pharmaceutical industry. The FDA regulation requires a complete description of the data derived from studies of the stability of the drug including information showing the suitability of the analytical method used.
Heat stability of a drug substance will have a major influence on the marketable physical form of the drug product as well as processing parameters allowable. Samples were stored at different temperatures like 4C, 25C, 37C and 50C. At the end of 7, 14, 21 and 30 days all the samples stored under different temperatures were observed for any physical change and TLC pattern.

3.2 Effect of Carbon Tetrachloride and Herbal Products on Liver Weight

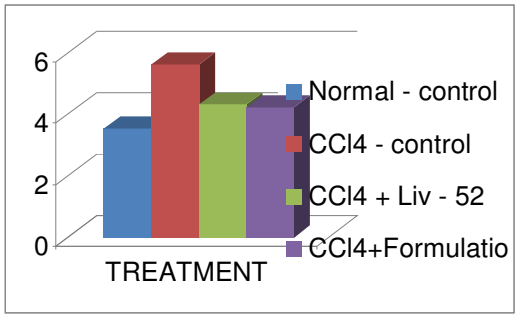

Fig. 1- Effects of Carbon Tetrachloride and Herbal Products on Liver Weight

From the results in Table-2 it can be observed that there was an increase in liver weight of carbon tetrachloride treated animals to the extent of 2.07 gms /100gms body weight. The increase in liver weight can be attributed to the accumulation of fat. Liv – 52 and Vidakanachoornam formulations were found to counteract the increase in liver weight. When compared to carbon tetrachloride treated group the increase in liver weight of Liv – 52 treated groups was only 0.78 gms/100 gms body weight. Vidakanachoornam treated showed an increase of 0.68gms/100gms body weight, clearly indicating liver protection towards fat accumulation. There was significant reduction in liver weight in Vidakanachoornam treated group (Figure-1)

3.3 Serum Bilirubin Level (Mg%) after 4 Days of Drug Treatment

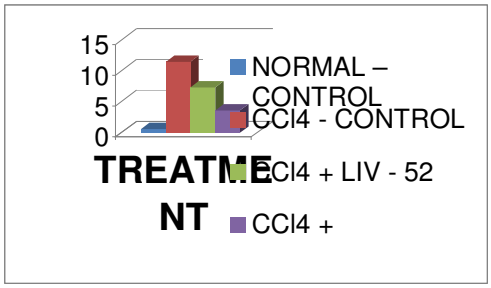

Fig. 2- Effects of Carbon Tetrachloride and Herbal Products on Serum Bilirubin - Level

The severity of the functional disorders of liver is reflected by elevation in the level of serum bilirubin. When compared to the normal control group, carbon tetrachloride control group showed a very significant rise in serum bilirubin level from 0.67mgs% to 11.5 mgs%. Liv – 52 treated group showed an increase in bilirubin level from 0.67 mgs% to 6.7 mgs %, thereby showing a decrease of 44.32% when compared to carbon tetrachloride treated group (Table-3). Vidakanachoornam, on the other hand showed an increase from 0.67mg% to only 3.08mg%, showing a percentage reduction in bilirubin level of 77.75%. This was indicated by graphical picture (Figure-2).

3.4 Serum GPT Levels (Iu) after 4 Days of Drug Treatment

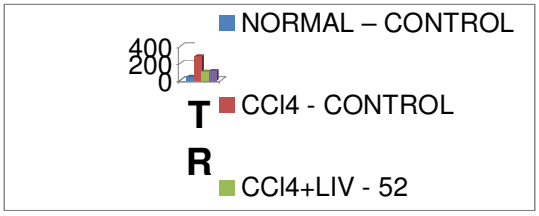

Fig. 3- Effects of Carbon Tetrachloride And Herbal Products on Serum Gpt – Level

Elevated SGPT level in serum, points out a high degree of hepatocellular damage in carbon tetrachloride treated group. When compared with normal control group, carbon tetrachloride treated group showed a phenomenal rise in seum GPT level from 51.17 IU(normal) to 288.00 IU. The serum GPT levels of Liv – 52 and Vidakanachoornam treated group at the end of 4 days of drug treatment were decreased significantly from 288.00 IU (CCl4 control) to 108.33 IU and 116.00 IU respectively (Table-4). The percentage reduction of SGPT level in Liv – 52 treated group Vidakanachoornam treated group was found to be 75.93 % and 72.71% respectively. Graphical picture indicated that there was reduction in serum GPT levels of Vidakanachoornam treated group (Figure-3). Based on statistical analysis significant reduction in serum GPT levels was also observed (Table-5).

3.5 Effects of Carbon Tetrachloride and Herbal Products on Serum GOT Level

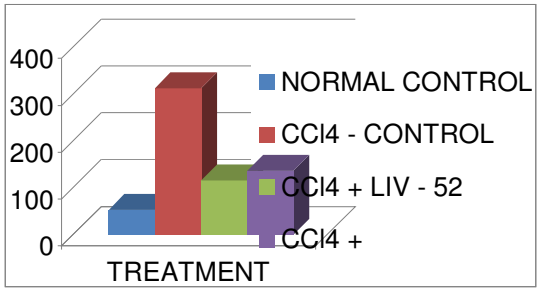

Fig. 4- Effects of Carbon Tetrachloride and Herbal Products on Serum Got Level

Elevated serum GOT activity can be observed soon after the exposure to hepatic infection. It was found that there was a marked rise in SGOT levels from 53.18 IU (normal control) to 3121.00 IU in the CCl4 treated group. The serum GOT levels of Liv – 52 and Vidakanachoornam treated groups at the end of 4 days of drug treatment were 115.67 IU and 136.67 IU respectively (Table-6). The percentage reduction in SGOT levels in Liv – 52 and Vidakanachoornam treated group, when compared with carbon tetrachloride control group, was found to be 75.93% and 67.85% respectively. Graphical picture indicated that there was reduction in serum GOT levels of Vidakanachoornam treated group (Figure-4). Based on statistical analysis significant reduction in serum GOT levels was also observed (Table-7).

3.6 Serum ALP Levels (Iu) after 4 Days of Drug Treatment

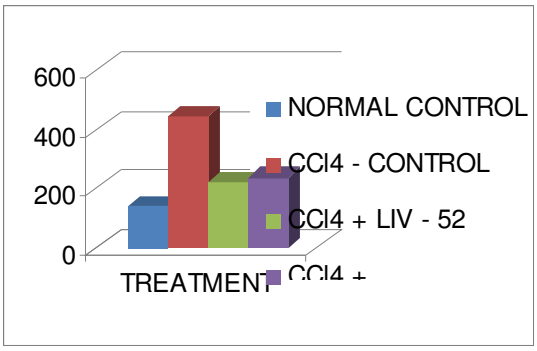

Fig. 5- Effects of Carbon Tetrachloride and Herbal Products on Serum Alp Level

Elevated serum ALP level indicates a high degree of hepatocellular damage.When compared to the normal control group, carbon tetrachloride treated group showed a significant rise in serum ALP levels from 142.83 IU to 446.83 (Table-8). It was found that the serum ALP levels of Liv – 52 and Vidakanachoornam treated group after 4 days of treatment were decreased significantly from 446.83 IU(for CCl4 control) to 224.33 IU and 239.83 IU respectively. The percentage reduction in serum ALP level in Liv - 52 and Vidakanachoornam treated group when compared to carbon tetrachloride treated group was found to be 73.19% and 68.09% respectively. Graphical picture indicated that there was reduction in serum GOT levels of Vidakanachoornam treated group (Figure-5). Based on statistical analysis significant reduction in serum GOT levels was also observed (Table-9).

3.7 Histopathological Studies

1. Normal control

Microscopic section shows normal hepatic architecture

2. Carbon tetrachloride control

The liver sections from the rats which received Carbon tetrachloride alone showed microscopically varying degress of fatty changes and necrosis with congested vessels and sinusoids with loss of architecture in general.

3. Carbon tetrachloride and formulation treated

The liver sections from the rats which received Carbon tetrachloride and formulation, showed microscopically moderate fatty changes with mild degeneration of hepatocytes with attempted regeneration and intra portal band formation.

4. Carbon tetrachloride with Liv – 52 treated

The liver sections from the rats which received Carbon tetrachloride and formulation Liv – 52 showed microscopically centered and sinusoidal changes with mild fatty change of hepatocytes.

4. SUMMARY

Vidakanachoornam is a popular traditional Ayurvedic formulation, extensively prescribed by renowned traditional practitioner in Allapuzha District, Kerala, for liver disorders especially jaundice and steatosis (fatty liver). The formulation is used by the local population for liver disorders and was found to give excellent clinical benefits. The present work was carried out with a view to validate its therapeutic efficacy by testing the preparation on rats with experimentally induced liver damages.

Liv – 52, a patented herbal formulation popularly used for liver ailment, was selected as positive control for comparing the activity of Vidakanachoornam.

In the present study following parameters have been determined for evaluating the hepatoprotective activity of the herbal products:

1. Pilot study to determine the dose of carbon tetrachloride.
2. Effect of carbon tetrachloride and herbal products on liver weights.
3. Estimation of biochemical parameters:
   - Bilirubin level in serum.
   - GPT level in serum.
   - GOT level in serum.
   - ALP level in serum.
4. Histopathological studies.

For determining the dose of carbon tetrachloride, different groups of rats were administered with different doses of carbon tetrachloride. The group of animals which received 1.5, 2.0 & 2.5 ml/kg body weight of carbon tetrachloride with equal volume of liquid paraffin showed only moderate histopathological changes when compared with normal rats. But the sections of livers of rats treated with 3.0, 3.5 & 4.0 ml/kg body weight of carbon tetrachloride showed severe necrosis steatosis and haemorrhage. The damage caused by the dose of 3.5 & 4.0 ml/kg body weight of carbon tetrachloride was found to be severe and irreversible, where as that produced by a dose of 3 ml/kg body weight of carbon tetrachloride was found to be severe and reversible. Hence this dose was considered as the effective hepatotoxic dose and used as control throughout the investigation.

It was found that there was an increase in liver weight during carbon tetrachloride treatment which may be due to accumulation of fat. Liv – 52 and Vidakanachoornam were found to counteract the increase in liver weight by showing a significant reduction in liver weights of treated groups when compared to carbon tetrachloride treated group. The percentage of reduction in liver weight from carbon tetrachloride control group was found to be 62.32% and 67.15% respectively. Serum bilirubin level was raised considerably during carbon tetrachloride treatment. Both Liv-52 and Vidakanachoornam were found to be effective in reducing the raised

serum bilirubin level. In this respect, Vidakanachoornam was found to be more effective since it produced 77.75% reduction in serum bilirubin level when compared to 44.32% reduction by Liv – 52.

Serum GPT and GOT levels were significantly increased in carbon tetrachloride treated group when compared to normal control group. Both Liv – 52 and Vidakanachoornam showed significant reduction in elevated serum GPT and GOT levels. Both Liv – 52 and Vidakanachoornam were found to be effective in decreasing the elevated serum ALP levels. Liv – 52 showed 73.19% reduction where as Vidakanachoornam showed 68.09% reduction in elevated serum ALP level.Based on the results of the histopathological investigations it can be seen that both Liv – 52 and Vidakanachoornam were helpful in repairing the damaged liver parenchyma.

5. CONCLUSION

Vidakanachoornam is a very simple herbal preparation containing only 3 herbal ingredients namely *Morigna oleifera*, *Embelia ribes* & *Piper longum*. None of these herbs are present in the market. Liv -52 formulation as well as other popular marketed liver tonics like, Tefroli, Livergen, Stimuliv etc.

The hepatoprotective activity of this simple formulation was found to be as effective as Liv – 52, infact the activity on decreasing the serum bilirubin level was much higher when compared to Liv – 52. This indicates that this formulation can be more useful in treatment of Jaundice.

Also being a simple formulation of easily available ingredients, the formulation definitely is cost effective and can be popularized for treatment of liver disorders. Further studies on adaptogenic characters will be useful to assess the potential of this formulation.

6. REFERENCES

[1] Zimmerman H. (1979) *Med Clin North Am.*, 63:567-82.

[2] Halliwell Barry, Gutteridge, *John Free Radicals in Biology and Medicine*, *3rd Edition*.

[3] Coles E.H. (1986) *Veterinary Clinical Pathology. In: Health Science Division, Saunders, W.B. (Ed.). Elsevier Science, Philadelphia, USA.,* ISBN: 0721618286, pp: 10-42.

[4] Moran D.P. (1989) *Recogntion and managent of pesticide poisoinings. Fourth edtion. Health effects division, Office of pesticide programmes, U.S. EPA. Washington, DC*.

[5] Moudgil K.D. and Narang B.S. (1989) *The Liver and the Biliary System. In: Textbook of Biochemistry and Human Biology, 2nd Edn., Talwar, G.P., L.M. Srivastava and K.D. Moudgil (Eds.). Prentice-Hall of India Limited, New Delhi, India,* ISBN: 8120319656, pp: 271-273.

[6] Rathore H.S. and Saraswat V. (1986) *Probe,* 3, 257.

[7] Saxena A. and Garg N. K. (1979) *Ind. J. Exp. Biol.,* 17, 662.

[8] Piyush Bardhan, Sharma S.K. and Garg N.K. (1985) *Indian Journal of Medical Research,* (82), Oct., 359.

[9] Dwivedi S.K., Sharma M.C., Mukherjee S.C., Jawaharlal and Pandey N.N. (1987) *Indian Drugs,* (25), 1, 1.

[10] Patney N.L. and Ashok Kumar (1978) *Probe,* (XVII), 2, 132.

[11] Sama S.K., Benz W., Aach R., Mackir E. and Kaplan M. (1973) Lancet, 1, 14.

[12] Mathur R., Prakash A.O. and Mathur S. (1987) *Industrial Health,* 25, 131.

[13] Naveen Patnaik (1993) *The garden of life: An introduction to the healing plants of India.*

Table 1- Stability Studies

PARAMETERS	APPEARANCE	COLOUR	ODOUR	TASTE	TLC PATTERN
7TH DAY 4C	--	--	--	--	--
R T	--	--	--	--	--
37C	--	--	--	--	--
50C	--	--	--	--	--
14TH DAY 4C	--	--	--	--	--
R T	--	--	--	--	--
37C	--	--	--	--	--
50C	--	--	+	--	--
21ST DAY 4C	--	--	--	--	--
R T	--	--	--	--	--
37 C	--	--	--	--	--
50 C	--	--	+	--	--
30TH DAY 4 C	--	--	--	--	--
R T	--	--	--	--	--
37 C	--	--	--	--	--
50 C	--	--	--	+	--
R T = Room temperature (25 C – 30 C); -- = No change; + = Slight change					

Table 2- Effect of carbon tetrachloride and herbal products on liver weight

TREATMENT	**AVERAGE LIVER WEIGHT IN gms/100gms BODY WEIGHT**
Normal – Control	3.55 +/- 0.198
Carbon tetrachloride – control	5.62 +/- 0.185
Liv – 52	4.33 +/- 0.204
Vidakanachoornam	4.23 +/- 0.240
Animal – Rats (150-200gms) ; Routes – Carbon tetrachloride – S C; Drug - Oral	

Table 3- Serum bilirubin level (mg%) after 4 days of drug treatment

SERIAL NO.	**NORMAL – CONTROL**	**CARBON TETRACHLORIDE-CONTROL**	**CARBON TETRACHLORIDE+LIV - 52**	**CARBON TETRACHLORIDE + VIDKANACHOORNAM**
1	0.7	11.4	7.4	3.6
2	0.6	12.4	6.8	2.8
3	0.6	11.3	6.4	4.1
4	0.8	14.5	7.2	1.6
5	0.7	9.1	5.9	2.5
6	0.6	10.3	6.5	3.9
AVG.+/-SEM	0.67+/-0.033	11.5+/-0.750	6.7+/-0.225	3.08+/-0.391
% PROTECTION	--	--	44.32	77.75
Animal - Rats (150-200gms); Routes - Carbontetrachloride – S C; Drug -Oral				

Table 4- Serum Gpt Levels (Iu) after 4 Days of Drug Treatment

SERIAL NO.	**NORMAL – CONTROL**	**CARBON TETRACHLORIDE-CONTROL**	**CARBON TETRACHLORIDE+LIV - 52**	**CARBON TETRACHLORIDE + VIDKANACHOORNAM**
1	40	282	102	112
2	55	298	97	108
3	42	308	125	132
4	60	275	110	117
5	58	315	117	98
6	52	254	99	129
AVG.+/-SEM	51.17+/-3.40	288+/-9.28	108.33+/-4.51	116+/-5.26
% PROTECTION	--	--	75.93	72.71
Animal - Rats (150-200gms); Routes- Carbontetrachloride – S C; Drug - Oral				

Table-5 Student 'T' Test for Serum Glutamate Pyruvate Transaminase Activity

GROUP	Carbon tetrachloride control with Liv- 52 treated group	Carbon tetrachloride with Vidkanachooranam treated group
CALCULATED 't' VALUE	17.4803	16.1847
TABLE 't' VALUE	4.587	4.587
REMARKS	Significant	Significant

Table-6 Effects of Carbon Tetrachloride and Herbal Products on Serum Got Level

SERIAL NO.	NORMAL – CONTROL	CARBON TETRACHLORIDE- CONTROL	CARBON TETRACHLORIDE+LIV - 52	CARBON TETRACHLORIDE + VIDKANACHOORNAM
1	56	325	115	127
2	48	318	108	146
3	47	298	126	136
4	59	271	122	118
5	52	321	105	142
6	57	344	118	151
AVG.+/-SEM	53.18+/-2.02	312+/-10.30	115.67+/-3.29	136.67+/-5.04
% PROTECTION	--	--	75.93	67.85
Animal - Rats (150-200gms); Routes - Carbon tetrachloride – S C; Drug -Oral				

Table-7 Student 'T' Test for Serum Glutamate Oxaloacetate Transaminase Activity

GROUP	Carbontetrachloride control with Liv-52 treated group	Carbontetrachloride control with Vidkanachoornam treated group
CALCULATED 't' VALUE	18.2434	15.3657
TABLE 't' VALUE	4.587	4.587
REMARKS	Significant	Significant

Table-8 Serum Alp Levels (Iu) After 4 Days of Drug Treatment

SERIAL NO.	NORMAL CONTROL	CARBON TETRACHLORIDE - CONTROL	CARBON TETRACHLORIDE+LIV - 52	CARBON TETRACHLORIDE+ VIDKANACHOORNAM
1	125	415	216	256
2	162	475	238	214
3	102	507	206	231
4	148	438	242	236
5	175	390	226	204
6	145	456	218	298
AVG.+/-SEM	142.83+/-10.67	446.83+/-17.15	224.33+/-5.62	239.83+/-13.77
% PROTECTION	--	--	73.19	68.09
Animal - Rats (150-200gms); Routes - Carbon tetrachloride – S C; Drug - Oral				

Table 9- Student 'T' Test for Serum Alkaline Phosphate Activity

GROUP	Carbon tetrachloride control with Liv-52 treated group	Carbon tetrachloride control with Vidakanachoornam treated group
CALCULATED 't' VALUE	12.33997	9.4185
TABLE 't' VALUE	4.587	4.587
REMARKS	Significant	Significant