EVALUATION OF HEPATOPROTECTIVE ACTIVITY OF CERTAIN INDIAN MEDICINAL PLANTS USING *IN VITRO* AND *IN VIVO* METHODS

Thesis submitted to
THE TAMIL NADU Dr. M. G. R. MEDICAL UNIVERSITY, CHENNAI

for the award of Degree of
DOCTOR OF PHILOSOPHY

in
PHARMACY

Submitted by
V. Subhadra Devi, M. Pharm.,

Under the guidance of
Dr. M. Gopal Rao, M. Pharm., Ph.D.

College of Pharmacy
Sri Ramakrishna Institute of Paramedical Sciences
Coimbatore-641044. Tamil Nadu, India.

JUNE 2016

CERTIFICATE

This is to certify that Ph.D dissertation entitled "**EVALUATION OF HEPATOPROTECTIVE ACTIVITY OF CERTAIN INDIAN MEDICINAL PLANTS USING *IN VITRO* AND *IN VIVO* METHODS"** being submitted to The Tamil Nadu Dr. M.G.R Medical University, Chennai, for the award of degree of **DOCTOR OF PHILOSOPHY** in the **FACULTY OF PHARMACY**, was carried out by **Ms. V. SUBHADRA DEVI,** in College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, under my direct supervision and guidance to my fullest satisfaction. The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma.

Dr. M. Gopal Rao, M. Pharm., Ph.D.
Vice Principal, College of Pharmacy,
Sri Ramakrishna Institute of Paramedical Sciences,
Coimbatore – 641044, Tamil Nadu.

Place: Coimbatore
Date:

CERTIFICATE

This is to certify that Ph.D dissertation entitled "**EVALUATION OF HEPATOPROTECTIVE ACTIVITY OF CERTAIN INDIAN MEDICINAL PLANTS USING *IN VITRO* AND *IN VIVO* METHODS"** being submitted to The Tamil Nadu Dr. M.G.R Medical University, Chennai, for the award of degree of **DOCTOR OF PHILOSOPHY** in the **FACULTY OF PHARMACY**, was carried out by **Ms. V. SUBHADRA DEVI,** in College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, under my co-guidance to my fullest satisfaction. The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma.

Dr. M. Uma Maheswari, M. Pharm., Ph.D.
Assistant Professor,
Department of Pharmacology,
College of Pharmacy,
Sri Ramakrishna Institute of Paramedical Sciences,
Coimbatore – 641044, Tamil Nadu.

Place: Coimbatore
Date:

CERTIFICATE

This is to certify that the Ph.D dissertation entitled "**EVALUATION OF HEPATOPROTECTIVE ACTIVITY OF CERTAIN INDIAN MEDICINAL PLANTS USING *IN VITRO* AND *IN VIVO* METHODS"** being submitted to The Tamil Nadu Dr. M.G.R Medical University, Chennai, for the award of **DOCTOR OF PHILOSOPHY** in the **FACULTY OF PHARMACY**, was carried out by **Ms. V. SUBHADRA DEVI** in College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, under the direct supervision and guidance of **Dr. M. GOPAL RAO, M. Pharm., Ph.D.** and the co-guidance of **Dr. M. UMA MAHESWARI, M. Pharm., Ph.D.** in College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore.

Dr. T. K. Ravi, M. Pharm., Ph.D., FAGE.
Principal, College of Pharmacy,
Sri Ramakrishna Institute of Paramedical Sciences,
Coimbatore– 641044, Tamil Nadu.

Place: Coimbatore
Date:

DECLARATION

I hereby declare that the Ph.D. dissertation entitled "**EVALUATION OF HEPATOPROTECTIVE ACTIVITY OF CERTAIN INDIAN MEDICINAL PLANTS USING *IN VITRO* AND *IN VIVO* METHODS"** being submitted to The Tamil Nadu Dr. M.G.R Medical University, Chennai, for the award of degree of **DOCTOR OF PHILOSOPHY** in the **FACULTY OF PHARMACY** was carried out by me under the direct supervision and guidance of **Dr. M. GOPAL RAO, M. Pharm., Ph.D.** and the co-guidance of **Dr. M. UMA MAHESWARI, M. Pharm., Ph.D,** in College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore. The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma.

V. Subhadra Devi

Place: Coimbatore
Date:

INTRODUCTION

1.1 Liver

Liver is the heaviest and the largest glandular organ in the body. It is a key organ of paramount importance that regulates homeostasis of the body[1, 2]. It is the chief site and plays an essential role in regulation of physiological processes for several fundamental functions such as intense metabolism and excretion. It is responsible for carbohydrate, protein and fat metabolism[4, 5]. It plays an important role in bile production, excretion of bilirubin, cholesterol, hormones and drugs.

A number of hepatotoxins such as viruses, bacteria, chemicals, free radicals, alcohol, xenobiotics, food additives and pollutants are prone to injure the liver and these are the major risk factors which lead to the manifestation of various liver disorders such as hepatitis, cirrhosis and alcoholic liver diseases. Inside the human body, free radicals are continuously generated as a result of exposure to either exogenous chemical in the environment or by endogenous hepatotoxins. Hepatic injury is also caused by the production of free radicals during biochemical reactions. One of the common disease conditions leading to serious consequences ranging from metabolic disorder to death is hepatotoxicity. Thus today it is one of the major causes of morbidity and mortality among all the fatal diseases[7]. So, there lies an urge to safeguard the liver.

1.2 Role of hepatoxicants in liver damage

In this regard, Paracetamol (PCM) (also called as acetaminophen), the most commonly used analgesic and antipyretic drug.. Paracetamol at higher doses causes severe hepatotoxicity, leading to fulminant hepatic and renal tubular necrosis. The most classic and widely used experimental model to study the hepatoprotective activity of therapeutic drugs is the paracetamol induced

hepatotoxicity[8]. Galactosamine is a hexosamine derived from galactose. D-Galactosamine is the best system induced hepatotoxicity among numerous models for experimental hepatitis. Hence, D-Galactosamine mediated hepatotoxicity was also chosen as the experimental model [9,10].

Alcohol (ethanol) is considered as the most widely used psychoactive substance in the world since thousand years. In small doses, alcohol has great medicinal value. But when alcohol is administered in excess, it leads to liver damage as 80% of ingested alcohol is metabolized in the liver. Alcoholic liver disease (ALD) is one of the major serious health problems[11]. Hence ethanol-induced hepatotoxicity is used to study the hepatoprotective activity of curative drugs.

Free radicals are formed in many metabolic processes and attack the membrane lipids. It leads to a number of severe pathophysiological conditions. Antioxidants that are present in plants form the basis for the prevention and cure of a disease. These hepatotoxins acts as free radicals and the antioxidants that present in the plants scavenge the free radicals and perform a role as hepatoprotective agents. Antioxidants are the major line of defense and the most active universal components among the body's free radical neutralizing systems[12]. For the past three decades, in the maintenance of human health, the natural plant based antioxidants have played an important role for hepatic disorders[13]. It has been reported that the antioxidants scavenge free radicals by the process of interference with the oxidation and chelation of metal ions and provide protection against infections and degenerative diseases[14]. Thus by the action of antioxidants, oxidative stress is prevented[6].

1.3 Limitations of existing therapy

In spite of the invention of newer drugs in the treatment of chronic liver diseases, none of such drugs has been able to overcome the progression of hepatic diseases and also they are often associated with side effects. Usage of

modern medicines for hepatic ailments management often causes harmful side effects like vomiting, depression, insomnia and depression.[15,16] The recently developed drugs that are used for chronic liver disorder e.g., Rimonabant, corticosteroids or propylthiouracil possess harmful side effects. Therefore search for a drug to treat liver diseases without side effects continues in the present scenario.

1.4 Importance of medicinal plants in liver diseases

In the modern system of medicine there is no truly satisfactory liver protective drug which is effective and safe [17,18]. Medicinal herbs are widely used in hepatitis and cirrhosis[19]. Among the developing countries of the world for primary health care, medicinal plants have been valued due to lesser side effects. The traditional medicine is being relied by about 80% of the world population which is predominately based on plant material[20]. India is referred as "Medicinal Garden of the World" as it has been bestowed with huge wealth of medicinal plants[6]. India has a long history for the usage of herbal-based therapeutics from ancient times, traditional medicinal plants are used for liver disorders that could potentially substitute the chemical-based drugs and have proven to be an effective alternate treatment [21,22].

In recent years phytoconstitutents have received considerable attention, due to their diverse pharmacological property including hepatoprotective activity[23,24]. The most important representative of hepatoprotective effect of plants is phytoconstitutents. Medicinal plants are prospective sources of naturally occurring phytoconstitutents that has been used since long time to heal liver disorders.

In general, inspite of the noteworthy popularity of phytoconstitutents there are several herbal medicines. Therefore, in the recent scenario, globally there is profound interest, in the therapeutic potentials of medicinal plants which act as antioxidants and reduce the harmful effects of free radicals[7,20]. In this aspect, the rhizomes of *Alpinia speciosa* and roots of *Valeriana wallichii* were evaluated for their hepatoprotective property against various hepatotoxins.

2. AIM AND OBJECTIVES

To determine the hepatoprotective activity of rhizomes of *Alpinia speciosa* and roots of *Valeriana wallichii* by both *in vitro* and *in vivo* methods against liver damage.

The objectives of the research are as follows:

- **Literature review**
- **Collection and authentication of plants: *Alpinia speciosa* and *Valeriana wallichii***
- **Preparation of various extracts using the rhizomes of *Alpinia speciosa* and roots of *Valeriana wallichii***
- **Phytochemical screening of the various plant extracts**
- **Study of *in vitro* hepatoprotective activity of rhizomes of *Alpinia speciosa* and roots of *Valeriana wallichii* against Paracetamol - induced hepatotoxicity by liver slice model**
- **Acute toxicity studies using OECD guidelines**
- **Study of *in vivo* hepatoprotective activity of rhizomes of *Alpinia speciosa* and roots of *Valeriana wallichii* against**
    - **Paracetamol**
    - **D-Galactosamine**
    - **Ethanol**
- **Determination of**
    - **Serum biochemical parameters**
    - **Tissue protein, malondialdehyde and lipid hydroperoxides**
    - **Liver enzymatic and non enzymatic antioxidants**
- **Histopathological studies**
- **Isolation and identification of active constituents**

3. REVIEW OF LITERATURE

Review based on the selected plants used in the study

3.1 Review of *Alpinia speciosa*

Mohanasundari *et al*., 2015 evaluated the phytochemical screening of *Alpinia speciosa* and *Alpinia calcarata* rhizomes. In Ayurveda, the rhizomes of *Alpinia calcarata* and *Alpinia speciosa* has been used to treat different diseases such as antibacterial, anti inflammatory, analgesic, diuretic, anticancer etc. Both the plants were extracted by using different solvents in Soxhlet apparatus [26].

Lima *et al*., 2015 reported that the extract of *Alpinia speciosa* exhibited good photoprotection for a UV-filter substance and can be considered. These findings confirmed that the plant have an extensive role in anesthetics and in curing degenerative diseases[27].

Pharmacognostical, physicochemical and chromatographic estimation of *Alpinia speciosa* Roxb rhizomes and its oil were determined by Thenmozhi *et al*., 2013. Hydro distillation method was used to collect the volatile oil from the collected fresh rhizomes and purified. The physical parameters of the distilled volatile oils were observed[29].

Chompoo *et al*., 2012 determined the antiatherogenic properties of *Alpinia zerumbet* acetone seeds extract. Amongst all parts of the plant, the seed extract had strong activity. A steroid present could be responsible for the atherosclerosis activity[31].

Thenmozhi *et al*., 2011 evaluated the effect of analgesic of the plant. The analgesic activity was screened using *Wistar* rats. On oral administration of the extract exhibited anti nociceptive effect[32].

Santos *et al*., 2011 examined the cardio depressive effect of *Alpinia speciosa*. Left atria isolated from adult rats spontaneous – beating and electrically stimulated left atria were conducted. There was decrease in the cardiac rhythm. Thus the oil of the plant in rat heart inhibited the L-type Ca^{2+} channels that had been involved in the cardio depressive effect[33].

Wong *et al*., 2009 performed DPPH, reducing power, ferrous ion chelating and β carotene bleaching assays. *Alpinia speciosa* leaves showed high antioxidant activity in DPPH and reducing power assays[37].

Mendonca *et al*., 1991 determined pharmacological and toxicological evaluation of *Alpinia speciosa*. When hydroalcoholic extract was injected in a dose range of 100-1400 mg/kg in mice, it produced writhing, psychomotor excitation, hypo kinesis and pruritis. The hydroalcoholic extract produced a dose-dependent fall in blood pressure at a dose 10-30mg/kg[39].

3.2 Review of *Valeriana wallichii*

Srivastav *et al*., 2016 determined the anticonvulsant activity of *Valeriana wallichii* Linn. root extracts in experimental rats. The study was carried out in the extracts of the roots of *Valeriana wallichii* on the rats, induced both chemically and electrically. Maximal electroshock (MES) and pentylenetetrazole (PTZ) induced convulsions in rats were the models chosen for the study. In PTZ induced seizures, onset of clonic convulsions were observed while in MES model, reduction in the mean duration of extensor phase was carried out. Both the extracts showed anticonvulsant activities against MES and PTZ animal models[41].

Sridharan *et al*., 2014 demonstrated neuroprotective effect of *Valeriana wallichii* rhizome extract in SH-SY5Y cells. *Valeriana wallichii* rhizome extract was evaluated for cell viability analysis and determination of IC_{50} by MTT assay. Rhizome extract of *Valeriana wallichii* showed good free radical scavenging properties. The chemical induced cell shrinkage were inhibited by treatment with 1 mg/ml of *Valeriana wallichii* rhizome[43].

Bhandarkar *et al*., 2014 compared the anxiolytic activity of *Valeriana wallichii* petroleum ether extract from different accessions in experimental animals. In mice different accessions by using various anxiolytic activities were carried out and sodium thiopental induced sleep were evaluated. The spontaneous locomotor activity count was decreased at 120 min after administration of the plant extract. The study concluded that the *Valeriana wallichii* extract possessed anxiolytic activity[44].

Shi *et al*., 2014 investigated the anxiolytic effects of Valtrate; a principle compound isolated from *Valeriana wallichii* in rats involving the changes of corticosterone levels. For 10 continuous days valtrate (5, 10 and 20 g/kg daily) was exposed to anxiolytic activity. By enzyme – linked immunosorbent assay the cortisone levels in the rat serum were measured[45].

Joseph *et al*., 2013 evaluated anticonvulsant property of *Valeriana wallichii* in Swiss albino mice. Effects of the *Valeriana wallichii* root extract was tested for motor co ordination with rota rod before and after dosing with the extract showed a dose dependant reduction of hind limb in comparison with the standard and control groups. Thus the root proved a better effect profile when compared with the standard antiepileptic drugs[46].

Khuda *et al*., 2013 evaluated the topical preparation of *Valeriana wallichii* leaves for its anti-inflammatory activities. Carrageenan induced hind paw was tested for their effect on both the phases of inflammation models in male *Wistar* rats. The ethyl acetate fraction showed considerable activity with that of the standard[47].

Nabi *et al*., 2013 evaluated the effect of *Valeriana wallichii* DC on learning and memory impairment in streptozotocin induced diabetic animal. Extent of improvement of memory was measured at the end of the study by Morris water maze and elevated plus maze. Biochemical parameters were measured in brain homogenate after the animals were sacrificed. The diabetic rats developed impairment in learning and memory as compared to control rats. Moreover, lipid peroxidation levels were increased. Attenuation of hyperglycemia, cholinergic dysfunction and decreased MDA levels were attained after 30 days treatment with *Valeriana wallichii* (50 mg/kg, 100 mg/kg, 200 mg/kg) and vitamin C (100 mg/kg). A beneficial effect in memory deficits of rats was exerted by *Valeriana wallichii* possibly though multiple actions specifically through potential anti-oxidative effect[48].

Sahu *et al*., 2012 investigated the sleep quality and modulating ability of *Valeriana wallichii* in rat's brain. Duration of non rapid eye movement was increased and proved that the plant extract has sleep improving quality[49].

Sudhanshu *et al*., 2012 determined the ability to scavenge the free radicals of the methanolic extract of *Valeriana wallichii* roots. It was found to contain alkaloids, flavonoids, tannins, saponins, glycosides. The result revealed that the plant's extract had the capability to scavenge the free radicals[50].

Katoch *et al*., 2012 examined the radioprotective property of aqueous extract of *Valeriana wallichii.* To protect against radiation – injury in DNA and cultured human fibroblast cells hesperidin, one of the major constituent of *Valeriana wallichii* was evaluated. The extract was found to counter radiation-

induced free radicals at 4 hr after irradiation, increase in mitochondrial mass was lead by reduced prolonged oxidative stress, enhanced reproductive viability of cultured cells and protection against radiation induced DNA damage[51].

Mhaske *et al*., 2011 determined the *Valeriana wallichii* roots and rhizomes for antimicrobial activity. The roots and rhizomes of various extracts were tested for various micro organisms. To determine the antimicrobial activity of the plant extract, agar diffusion method was used. By turbimetric method, MIC values were used to study the micro organisms. A good inhibition against various bacterial strains (MIC between 10 and 80µgm/ml) was exhibited with the methanolic extract of *Valeriana wallichii* roots[52].

Sah *et al*., 2010 elucidated mechanism of analgesic action of *Valeriana wallichii* DC chemotype in mice. Acetic acid induced writhing and tail flick models were used for screening of analgesic effect. The methanolic extract of *Valeriana wallichii* exhibits only central analgesic activity. These data suggested that methanolic extract of essential oil exerted good activity[56].

Rehni *et al*., 2007 reported anxiolytic activity were attenuated in cerebral damage in the extracts of chlorophyll and aqueous extracts of *Valeriana wallichii*[57].

Gilani *et al*., 2005 determined the ability of hypotensive effects and antispasmodic capacity of the plant extract through K^+ channel activation. Fall in arterial blood pressure and by the complete relaxation of *Valeriana wallichii* indicated the antispasmodic and hypotensive effects possibly through K_{ATP} channel activation[58].

Lefebvre *et al*., 2004 demonstrated root of valerian inhibited the liver microsomal enzymes when taken with other medicines[59].

3.3 Review of *in vitro* hepatoprotective activity

Wormser *et al*., 1990 determined the liver slice culture method. Estimation of lactate dehydrogenase was carried out for the supernatant [60].

Jyothi *et al*., 2014 determined paracetamol-induced hepatotoxicity in *Limnanthemum indicum* (whole plant) using Brl-3a cell line. Brl-3a cell line was tested on rat liver cell line. The inhibitory effect on Brl-3a was tested with various solvent extracts. The percentage viability of 0% was observed in the cells that were exposed only with paracetamol but when treated with extract exhibited an increase in percentage viability. The standard drug silymarin showed good activity of cell protection[61].

Naik *et al*., 2004 demonstrated the hepatoprotective activity of curcumin. The release of LDH and lipid peroxidation had reduced the oxidative stress [63].

3.4 Review of *in vivo* hepatoprotective activity

Review of paracetamol-induced hepatotoxicity

Hanafy *et al*., 2016 evaluated the hepatoprotective property of *Adansonia digitata* extract .The extract offered good protection aganist the toxic dose of paracetamol in rats [64.]

Hafez *et al*., 2015 studied the protective effect of *Euryops arabicus Euryops arabicus.* The degree of protection was observed in the indices of the liver of the serum enzymes in *Euryops arabicus* extract.

Fogha *et al*., 2015 demonstrated that *Morinda lucida* stem bark protected paracetamol-induced liver damage. Once daily orally for 15 days the treatment was given. The measurement of ALT, AST, cholesterol, triglycerides and glucose concentration was determined. Then the animals were sacrificed

and blood was collected. The best protective effect was shown in *Morinda lucida* [67].

Rajasekhar *et al*., 2015 investigated the protective effects of *Solanum dulcamara* L. and *Nephrolepis cordifolia* (L) C. Presl. Hydroalcoholic extract of both the plants decreased the activity of serum enzymes and also decreased the TG, TC, LDL levels and in tissue LPO and elevated the antioxidant enzymes [68].

Santiago *et al*., 2015 determined protective effects of *Ficus Pseudopalma Blanco*. The plant extract at a higher dose decreased the serum enzymes and increased the antioxidant enzyme levels[69].

Qadir *et al*., 2014 demonstrated the *Cestrum nocturnum* leaves for its hepatoprotective effect by using aqueous ethanol extract against paracetamol-induced hepatotoxicity in mice. Maceration technique was employed to prepare the aqueous extract of *Cestrum nocturnum*. The hepatoprotective activity of the leaves may be because of the active constituents present in them.

Mahmood *et al*., 2014 investigated the paracetamol-induced hepatotoxic activity in methanolic extract of *Muntingia calbura* L. leaves. Rats were divided into groups with plant extract of two different doses. The rats received DMSO (negative) control, N-acetyl cysteine and paracetamol respectively. Bio chemical and histopathological studies were carried out in the blood and liver tissues. The extract exhibited good hepatoprotective activity[2].

Arote *et al*., 2014 assessed an herbal formulation against paracetamol-induced hepatotoxicity for its hepatoprotective activity in rats. Liver injury was induced by paracetamol in *Wistar* rats. The study was carried out in serum enzymes. A reduced sign of paracetamol-induced hepatotoxicity was observed in histological studies. The histological studies the formulation confirmed that it is a hepatoprotective agent[72].

Ogbonnaya *et al*., 2014 evaluated the ethanolic extract of *Gongronema latifolium* leaves. Six groups of four animals per group were randomly divided and three experimental groups received paracetamol before, after or simultaneously with the administration of ethanolic extract *Gongronema latifolium.* After 6 days the animals were sacrificed and observed for liver function parameters. There was an elevation in the antioxidant enzymes levels and fall in the serum biochemical levels indicating that *Gongronema latifolium* ethanolic extract have hepatoprotective effect against paracetamol-induced toxicity in rats[73].

Baig *et al*., 2013 determined the antihepatotoxic activity *Caesalpinia decapetala* in rabbits. Determination of hepatoprotective activity was carried out. Polyphenols and flavonoids were revealed by phytochemical analysis of the plant extract. The groups that received paracetamol were found to have increased levels of liver enzyme levels and were reduced in rabbits who received *Caesalpinia decapetala* ethanolic extract. This concluded the hepatoprotective effect of *Caesalpinia decapetala* ethanolic extract against paracetamol-induced hepatotoxicity[74].

Thummala *et al*., 2013 investigated the hepatoprotective activity of *Mussanda frondosa*. The decrease in the serum enzyme levels and proved that the *Mussanda frondosa* possessed hepatoprotective activity[75].

Shrivatsava *et al*., 2013 determined the paracetamol-induced hepatotoxic activity of hydro alcoholic extract of *Scindapsus officinalis* fruit for its hepatoprotective activity. In paracetamol treated groups the serum enzyme levels SGPT, SGOT, ALP, bilirubin, total protein were increased. The hydroalcoholic extract of *Scindapsus officinalis* fruit decreased the above parameters proved that the plant having hepatoprotective activity[76].

3.5 Review of D-Galactosamine-induced hepatotoxicity

Kim *et al*., 2015 determined the protective effect of wild *Ginseng*. It was found that there was an increase in serum aminotransferase activity. *Ginseng* exhibited the increase in serum interleukin–10 and hepatic heme oxygenase -1 protein and mRNA suggesting *Ginseng* protected liver against D-GalN induced hepatotoxicity in rats[79].

Roy *et al*., 2015 evaluated the protective effects of *Madhuca longifolia* bark. Pretreatment with different doses of the plant extracts brought the distorted levels to normal. These two doses of the extract were also confirmed by the histopathological studies[9].

Jayachitra *et al*., 2015 determined the antioxidant activity of *Nyctanthes Arbor tristis* L. The plant extract decreased the serum enzyme levels and elevated the antioxidant levels[80].

Kadam *et al*., 2015 determined the hepatoprotective activity of *Boswellia ovalifoliolata*. Various studies involved with the liver were conducted. Reduced levels of elevated biochemical markers and histopathological observation proved that the treatment with *Boswellia ovalifoliolata* had protected the liver from hepatotoxicity[81].

Padmanabhan *et al*., 2014 determined the hepatoprotective potential of herbal preparation (HP-4). Herbal preparation is a combination of alcoholic extract of few plant extracts that had been studied to determine its efficacy on mice model of the above mentioned toxicity in mice. The studies concluded that the formulation offered protection[82].

Gani *et al*., 2013 determined the antihepatotoxic effect of alcoholic extract of *Nigella sativa* L. These findings indicated that *Nigella sativa* had hepatoprotective efficiency against D-GalN induced toxicity in rats[83].

Pandian *et al*., 2013 examined the hepatoprotective activity of methanolic extract of *Oldenlandia herbacea*. On oral administration for 8 days at a dose of 100 and 200 mg/kg/day methanolic extract of the whole plant *Oldenlandia herbacea* was administered. The standard drug used was silymarin (100mg/kg). Prevention of D-GalN induced hepatotoxicity by the extent was confirmed in the histopathology of the liver sections[85].

Ganesan *et al*., 2013 investigated protective effect of *Coldenia procumbens* Linn. It was found that the D-Galactosamine treated groups were found to increase in all the serum liver marker enzymes; whereas the plant extract treated groups were normal proving the plant had hepatoprotective activity[86].

Gupta *et al*., 2013 evaluated hepatoprotective and antioxidant activity of *Nardostachys jatamansi* against D-Galactosamine-induced in rats. In rat liver *in vivo* antioxidant enzymes such as lipid peroxidation, reduced glutathione, SOD, CAT were estimated along with ATPase. The results clearly demonstrated that the protective effect of the plant against D-GalN-induced hepatotoxicity[87].

Wu *et al*., 2012 evaluated hepatoprotective potential of *Laggera alata* extract. The principle component isochlorogenic acids were isolated from *Laggera alata*. The oral administration of *Laggera alata* at different doses reduced the serum enzymes in liver sections[88].

Shukla *et al*., 2011 determined the antihepatotoxic activity of piperine. Piperine (100 mg/kg) was orally administered to the animals which were induced by D-Galactosamine. It was found that combination of silymarin along with piperine was better in curing liver damage induced by D-Galactosamine. It can be confirmed that combination of piperine along with silymarin acts as a good hepatoprotective agent[90].

Srividhya *et al*., 2010 evaluated both hepatoprotective activity of *Gymnema sylvestre*. Altered biochemical parameters were restorated towards the normal when compared to D-Galactosamine treated groups and found to be dose dependent. These changes concluded that the *Gymnema sylvestre* proved hepatoprotective activity[91].

Jaishree *et al*., 2010 examined the antihepatotoxic effect of sertiamarin. Sertiamarin isolated from successive ethyl acetate extract of the plant *Enicostemma axillare*, at a dose of 100 and 200 mg/kg body weight was carried out against intraperitoneally administered. Alternation of several hepatic parameters is the result of D-Galactosamine-induced hepatotoxicity [92].

Adaramoye *et al*., 2006 demonstrated the hepatoprotection of *Garcinia kola* seeds. Three fractions of Kolaviron were separated by thin layer chromatographic technique. All the fractions reduced the serum enzyme levels proving that the fractions possessed good hepatoprotective activity against D-Galactosamine-induced hepatotoxicity[93].

3.6 Review of ethanol-induced hepatotoxicity

Rao *et al*., 2015 determined the ethanol-induced hepatotoxicity of herbal extract mixture in rats. The herbal formulation includes various parts of the plants of *Phyllanthus amarus, Ricinus communis, Terminalia chebula, Cichorium intybus, Aloe vera* and *Vitex negundu*[95].

Varalakshmi *et al*., 2015 examined the protective effect of *Curcuma longa* on ethanol. Histopathological examination of liver sections also proved for the biochemical parameters. The standard known hepatoprotective reference drug, silymarin was used to compare with the activity of the extract[96].

Rojin *et al*., 2015 evaluated hepatoprotective effect of *Mussaenda erythrophylla* and *Aegle marmelos* against ethanol. Biochemical parameters

such as ALT, AST and total bilirubin was determined. Treatment with the plant extract increased the antioxidant levels[97].

George *et al*., 2014 determined the antihepatotoxic effect of *Ocimum basilicum* L. methanolic extract. The results indicated that the extracts possessed a significant protection against ethanol-induced hepatotoxicity in rats[99].

Amrani *et al*., 2014 evaluated butanolic extract of *Chrysanthemum fontanesil* for its hepatoprotective property by ethanol. Leaves of butanolic extract of *Chrysanthemum fontanesil* were administered from the 6th to 17th day of gestation. Ethanol orally was given along with plant extract, vitamin E and C. On the 18th day pregnant mice different organs were removed. In all biomarkers severe alternations were observed after injury with ethanol. Butanolic leaf extract has found to decrease the extent of lipid peroxidation concluding *Chrysanthemum fontanesil* possessed hepatoprotective activity[100].

Padmanabhan *et al*., 2014 investigated a herbal preparation for its anti-hepatotoxic activity. Herbal preparation consisting of 80% alcoholic extract leaves and rhizomes were prepared. Histopathological studies of the herbal preparation exhibited a potential hepatoprotective activity.

Adaramoye *et al*., 2010 evaluated the protective effect of *Cnidoscolus aconitifllius* against ethanol damage. It was found that the ethanol toxicity lowered the antioxidant defense indices and elevated the serum enzyme levels, which were reversed by the action of *Cnidoscolus aconitifllius,* proved that the plant possessed hepatoprotective property[103].

Gao *et al*., 2013 investigated the hepatotoxic effect of acute alcohol-induced liver injury. It was found that the extract had a protective effect in reducing all the biochemical parameters. The liquor also elevated the

antioxidant enzyme levels suggested that the Zhuyeqing liquor possessed good hepatoprotective activity[104].

Rekha *et al*., 2013 evaluated the protective effect of *Vitis vinifera* L. seed extract. Every day for 4 weeks liver injury was induced by ethanol (5g/kg/day, 20%w/v), seed extract (200 mg/kg/b.w) and silymarin (100 mg/kg b.w) was administered. Animals were sacrificed and various biochemical parameters were assessed. The toxic effect of ethanol on the serum markers were determined and elevated hepatic DNA and decreased hepatic SOD, catalase, GPx levels were decreased by the *Vitis vinifera* seed extract and silymarin decreased the toxic effect of ethanol[105].

Kumar *et al*., 2013 evaluated *in vivo* hepatoprotective activity of *Allium cepa* bulb. The toxic effect of ethanol on the above parameters was prevented by the administration of the plant extract and as well as the silymarin treatment[107].

Basu *et al*., 2013 evaluated the antihepatotoxic activity of *Amorphophallus campanulatus* ethanolic extract against ethanol-induced hepatotoxicity. Ethanolic extract of the plant extracts at two doses body weight were administered for 30 continuous days. The plant extract and the silymarin prevented the toxic effects of ethanol. It is strongly indicated that the *Amorphophallus campanulatus* has good hepatoprotective activity[108].

Sudir *et al*., 2013 determined the ethanol-induced liver damage of *Commelina benghalensis* Linn (root) extract in rats. The alcoholic and aqueous extracts of *Commelina benghalensis* Linn (root) extract were taken into the study. The beneficial role of the plant extract proved the hepatoprotective activity in ethanol-induced hepatotoxicity in rats[109].

Maity *et al*., 2012 determined the alcohol-induced hepatotoxicity against the effects of *Milkania scandens* (L) willd in rats. Elevated levels of

AST, ALP, ALT and lipid peroxidation were elevated on alcohol administration. Catalase, glutathione reductase and superoxide dismutase were also decreased. The hepatotoxic action of ethanol was found to be protected when treated with silymarin (100 mg/kg).

Hoskeri *et al*., 2012 examined the prophylactic effects against ethanol-induced hepatotoxicity of *Flaveria trinervia* extract in rats. Orally methanolic extract of 3 different doses was administered during 7 days study. Biochemical tests with respect to the hepatotoxicity markers were performed. By the treatment of methanolic extract the substantially elevated serum enzymatic levels of AST, ALT, ALP and bilirubin in ethanol treated animals were restored towards normalcy[112].

3.7 Review of isolated compounds of *Alpinia speciosa*

Hammouda *et al*., 2015 determined phenolic compounds isolated from *Alpinia zerumbet* (Pers.) B. L. grown in Egypt. Three phenolic compounds were isolated from 80% methanolic extract of *Alpinia speciosa*. Two compounds were isolated. The isolated compounds were identified by PC, TLC, EI/MS and ^{1}H-NMR[116].

Rajalakshmy *et al*., 2014 determined phytochemical and biomarker evaluation from a poly herbal formulation. Various physicochemical characteristics were evaluated. The phytochemical consitutents were determined by phytochemical analysis. By using column chromatography biomarker compound was isolated. Physicochemical analysis showed presence of about 18% water soluble components and about 14% alcohol soluble components. Quantitative analysis showed presence of various components.

3.8 Review of isolated compounds of *Valeriana wallichii*

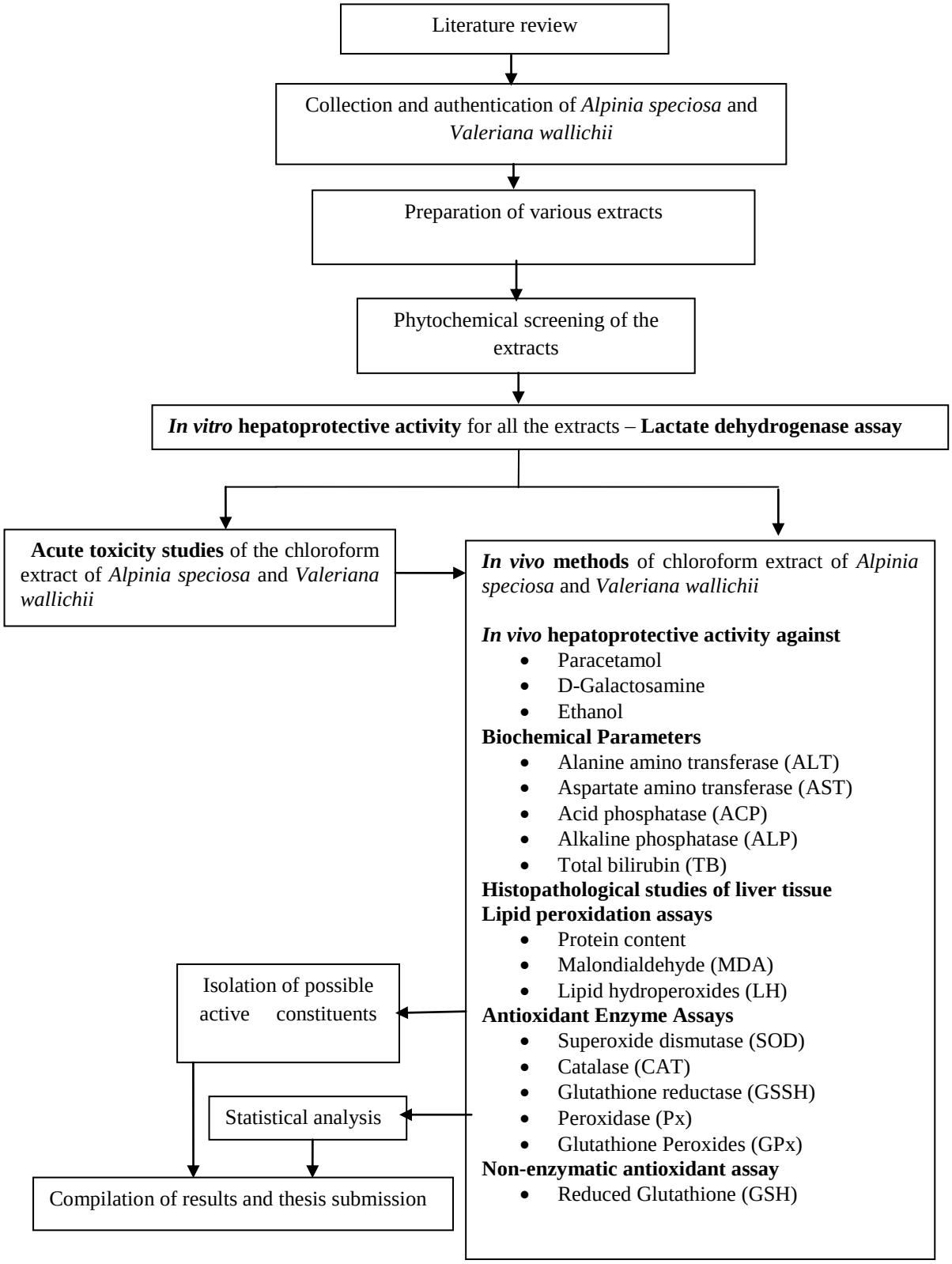
Thusoo *et al*., 2014 evaluated antioxidant activity of essential oil and extracts of roots of *Valeriana jatamansi*. In essential oil seven major components were determined, namely, β vatirenene, β - patchoulene, dehydroarmodendrene, β - gurjunene, patchoulic alcohol, β guaiene and α muurloene. Polyphenols and flavonoid contents were analyzed by preparing different extracts, namely, methanolic, aqueous and chloroform. DPPH radical scavenging and chelation power assay. The presence of rich amount of polyphenols and flavonoid content of the extract may be contributed for the antioxidant activity from the methanolic extract of *Valeriana wallichii*[118].

Joseph *et al*., 2013 evaluated the supraadditive effect of the plant extract and phenobarbitone against maximal electroshock. Intra peritoneal injection of two different doses was administered. The plant extract at a higher dose reduced the duration of maximal electroshock seizure in mice. Many active constituents such as valpotriates, sesquiterpenes, sesquiterpenes carboxylic acids, GABA, lignans, monoterpenes, flavonoids such as 6-methylapigenin and 2s (-) hesperidin had been isolated from the plant[6].

Saklani *et al*., 2012 determined the biological activities of *Valeriana wallichii* DC. It is used as ethno medicine for the treatment of habitual sleeplessness, to prevent convulsions, neurosis, anxiety, to increase urine output, liver diseases, to relieve pain and cytotoxic, muscle spasm, to reduce inflammation. Several phytoconstituents contain volatile oils, iridoids, alkaloids and flavonoids[119].

PLAN OF WORK

Schematic representation of work



PROFILE OF THE PLANTS

4.1. Profile of *Alpinia speciosa*

Family	:	Zingiberaceae
Common name	:	Shell Ginger
Vernacular name		
Languages		**Names**
English	:	Shell flower
Tamil	:	Sangarathai
Biological source	:	Rhizomes of *Alpinia speciosa*
Distribution	:	India, China and Asia

4.1.1 Description of the plant

It is an evergreen tropical perennial plant. It produces fleshy rhizomes resembling ginger like smell. It's also known as shell ginger because their individual shell pink flower, particularly when in bud, resembles sea shells[34].

4.1.2 Phytochemical Constituents of *Alpinia speciosa*

The rhizome was found to contain phenolic compounds such as cardomonin and alpinietin[28]. Phytochemical analysis of the leaves demonstrated the presence of catechuic tannins, phenols and alkaloids and also some essential oils[39]. Assay guided isolation gave three antioxidants from the rhizomes, an important food of Japan[36].

4.1.3 Traditional / Medicinal uses of various parts of the plant

Alpinia speciosa is used as an ornamental plant. It is also used for food and herbal medicine. In Northeast Brazil in the form of tea decoction is used for diuretics. Their rhizomes are useful in rheumatism and catarrhal afflictions. In addition the rhizomes also possess ulcer curing property.

The essential oils of *Alpinia* were also useful in the treatment of respiratory illnesses and have been used as a flavoring agent for beverages. It has also profound use as myorelaxant and antispasmodic actions in rat ileum[34,122]. The essential oil from its leaves and the flowers and seeds of the plant possessed antioxidant activity.

In folk medicine, it is used for its anti inflammatory, bacteriostatic and fungistatic properties. The plant is used as antinociceptive, anxiolytic, antipsychotic and antioxidant property.

4.1.4 Reported activities

The species of *Alpinia* possesses numerous pharmacological activities like pain relief, fight against micro organisms, to reduce inflammation, fight against free radicals, antiparasitic, antiplatelet, antispasmodic, antiulcer activity, to increase urine ouput, to decrease urine output, insecticidal, relaxation of the muscles and stimulation of the uterus.

Figure 1: Rhizomes of *Alpinia speciosa*

4.2 Profile of *Valeriana wallichii*

Family	:	Valerianaceae
Common name	:	Indian Valerian
Vernacular name:		
Languages		**Names**
Tamil	:	Thakkaram
Malayalam	:	Tagara
Hindi	:	Dabha
Biological source	:	Dried roots of *Valeriana wallichii*
Distribution	:	India, Belgium, England and Germany

4.2.1 Description of the plant

Valeriana wallichii is a small 14-45cm height perennial herb, Valerian is a widely used plant–based medicin [51,53,59]. It is an ingredient of herbal medicines in Indian systems of medicine[54]. The odour is powerfully Valerianceous.

4.2.2 Phytochemical constituents

The major known active principles of this herb are valepotriates, dihydrovaltrate, isovalerinate, 6-methylapigenin, hesperidins and sesquiterpenoids.

4.2.3 Medicinal uses of the various parts of the plant

In modern rational phytotherapy also the reputation of *Valeriana* species is mainly for treating nervous tension and temporary sleeping problems[53].

4.2.4 Reported activities

It is used in the treatment of jaundice, pain conditions, neurosis, and also for cytotoxicity. For the treatment of habitual constipation the rhizomes of *Valeriana wallichii* have been studied. The herb has been used successfully in traditional systems of medicine like Ayurveda and Unani against leishmania, diseases of eye and liver, hysteria, hypochondriasis, nervous unrest and emotional arrest. The plant is used in habitual constipation, antispasmodic and as cytotoxic. The plant has been effective in dyspeptic symptoms. It's effective against micro organisms[59].

Figure 2: Roots of *Valeriana wallichii*

MATERIALS

Chemicals	Manufacturer
Acetic anhydride	Fischer scientific Pvt. Ltd, Mumbai
Silymarin	Microlabs, Bengaluru
Calcium chloride	SD fine chemical Ltd, Mumbai
AST	Agappe diagnostics Ltd, Kerala
ALT	Agappe diagnostics Ltd, Kerala
ACP	Agappe diagnostics Ltd, Kerala
ALP	Agappe diagnostics Ltd, Kerala
Bilirubin	Agappe diagnostics Ltd, Kerala
Bovine serum albumin	
Thiobarbituric acid	Sisco Research Laboratories, Mumbai
Carboxyl methyl cellulose	Loba chemie
Chloroform	
Glacial acetic acid -	
Glucose	
Hydrochloric acid	
Hydrogen peroxide	Fisher scientific Pvt. Ltd, Mumbai
Potassium phosphate	Hi Media laboratories, Chennai
Potassium dihygrogen phosphate	
Potassium chloride	
Sodium chloride	
Sodium hydroxide	

Silymarin	The pharmaceutical and chemical, Chennai
Protein	Agappe diagnostics Ltd, Kerala
Ether anesthetic	Hi-Pure Chemical Industries, Chennai
Hepes Buffer	Sisco research laboratory, Mumbai
Glucose	Qualigens Fine Chemicals, Mumbai
Magnesium sulphate	
Nicotinamide Adenine dinucleotide	
Dinitrophenyl hydrazine	
Lithium lactate	
Lead acetate	
Sodium Pyruvate	
Methanol	

Instruments

Centrifuge	Remi
Uv	Jasco v 530 model
Auto analyser	Mispa excel
Melting point	
H-NMR spectra	JEOL spectrometer at 500MHz

Mass spectra JEOL	Accu TOF JMS-T 100 LC Mass spectrometer
Column chromatography	Merck

EXPERIMENTAL METHODS

5.1 Experimental animals

The animals used for the study were male *Wistar* rats. The weights of the rats were between 150-200 g and were procured from College of Veterinary and Animal Sciences, Thrissur, Kerala. The animals were acclimatized for seven days. The feed given to the animals were rat pellets and and appropriate quantity of drinking water. They were approved by Institutional Animal Ethical Committee (IAEC), College of Pharmacy, SRIPMS, bearing the Approval no. 1559/PO/a/11/CPCSEA.

5.2 Plant materials

The dried rhizomes of *Alpinia speciosa* and roots of *Valeriana wallichii* were used for the experiment. *Alpinia speciosa* and *Valeriana wallichii* belongs to the family Zingiberaceae and Valerianaceae respectively.

5.3 Collection and authentication of plants

The rhizomes of *Alpinia speciosa* and roots of *Valeriana wallichii* used for this study were collected. The rhizomes of *Alpinia speciosa* was identified and authenticated by Dr. M. Palanisamy, Scientist and roots of *Valeriana wallichii* by Dr. G. V. S. Murthy, Scientist, TNAU. The specimens BSI/SRC/5/23/2013-14Tech/1884 and BSI/SRC/5/23/2014-15Tech/584 respectively for both the plants has been deposited in the institution.

5.4 Preparation of various plant extracts

Using appropriate machines, the rhizomes of *Alpinia speciosa* and the roots of *Valeriana wallichii* were powdered and were stored in suitable containers respectively. The powders of these plants were kept ready to use for the experiment.

5.5 Extraction of *Alpinia speciosa*

Soxhlet extraction technique was used individually on petroleum ether, chloroform, ethyl ether and ethanol for the extraction of the rhizomes of *Alpinia speciosa*. To 250 ml of each of the above mentioned solvents about 100 gm powder of the plant was mixed and extracted for 8 hours. The filtrates were concentrated to dryness under controlled temperature and pressure.

5.6 Extraction of *Valeriana wallichii*

Extraction of the roots of *Valeriana wallichii* was carried out similarly by using the above mentioned technique.

5.7 Phytochemical screening of various plant extracts

5.7.1 Preparation of reagents used for the phytochemical screening of the plant extracts:

Mayer's reagent: Mercuric iodide of weight 1.36 gm was mixed with 60 ml water. To this solution 20 ml of water and mixed with potassium iodide weighing 5 gms.

Libermann-Burchard's reagent: Approximately weighed quantity of 5 gm of acetic anhydride was mixed with 5 ml of concentrated sulfuric acid. This was further added to absolute ethanol on cooling.

Dragendroff's reagent: Exactly weighed quantity of 1.7 gm basic bismuth nitrate and 20 gm tartaric acid are dissolved in 80 ml of water. This was further mixed in 16 gm potassium iodide and 40 ml of water.

Fehling's solution A: Approximately 34.64 gm of copper sulphate was dissolved in a 0.5 ml of sulphuric acid and made up to500 ml with water.

Fehling's solution B: Approximately a weighed quantity of 176 gm of sodium potassium tartarate were dissolved in 77 gm of NaoH and the volume

was made to 500 ml. At the time of use, the above solutions were equally mixed.

Benedict's reagent: Cupric sulphate and sodium citrate each weighing approximately 1.73 gm, 10 gm of anhydrous sodium carbonate were dissolved in water.The volume of the above solution was made to 100 ml.

Molish's reagent: To about 25 ml of ethanol, 2.5 gm of pure α-naphthol was added [125].

5.7.2 Test for Alkaloids

To a little quantity of the extract a few drops of dil. HCl was added and the solution was filtered.

- Dragedroff's reagent was added to the filtrate. An orange brown precipitate was formed. This confirmed the presence of alkaloids.
- A creamy precipitate indicated the presence of alkaloids on addition of Mayer's reagent to the filtrate.
- When extract was mixed with Wagner's reagent it formed a reddish brown precipitate.
- When extract was mixed with Hagner's reagent it formed a yellowish precipitate.

- **Tests for flavonoids**
- A few drops of concentrated sulphuric acid were added to a part of the extract. The extract became yellow in colour and disappeared after some time.

- A yellow coloration obtained on addition of few drops of 1% $AlCl_3$ to the extract confirmed the existence of flavonoids**.**

5.7.4 Test for glyco sides

Brick red coloration was observed on addition of equal volumes of Fehling's solution A and B to 1ml of the extract and heated, which confirmed the occurrence of glycosides.

5.7.5 Test for carbohydrates

- Brick red color precipitate was obtained with Fehling's solution.
- Formation of brick red precipitate was obtained with Benedict's solution confirmed the existence of reduced sugars.

5.7.5 Test for steroids

- **Libermann-Burchard's test**

Reddish violet color formation was observed when the extract underwent Libermann-Burchard's test which exhibits the occurrence of steroids.

- **Salkowski test**

A reddish blue colour was formed when the extract was tested which implicated the presence of steroids.

5.7.7 Test for saponins

About 5 ml of distilled water was added to 10 ml of the extract and was shaked briskly to form froth. An emulsion was obtained when on addition of 3 drops of olive oil.

5.7.8 Test for terpenoids

A reddish brown coloration of the interface was obtained on addition of 5 ml of the extract added along with 2 ml of chloroform and few drops of concentrated sulphuric acid which signifies the existence of terpenoids.

5.7.9 Test for amino acids

The confirmative reaction for amino acids was verified when purple color was observed on addition of ninhydrin to the extract with pH range from 4-8.

5.7.10 Test for tannins and phenolics

- Presence of tannins was confirmed when 5 ml of the extract was added with 1ml of 5% ferric chloride solution. The extract turned greenish black in colour.
- A yellowish brown precipitate was formed on addition of 5 ml of the extract alongwith 1 ml of 10% aqueous potassium dichromate solution. This confirmed the existence of tannins.

In vitro hepatoprotective activity

Liver slice culture *in vitro*

The procedure was done based on the model developed by Wormser[60] *et al.*, (1990). The liver lobes of the rat were removed and placed in pre-warmed KRH medium. They were then cut into thin slices and washed with the same medium and pre-incubated for about 60 minutes at 37° C. After replacing with 2 ml of KRH medium in potassium phosphate buffer (100mM, pH 7.8) in ice. The homogenates were centrifuged for 10 min at 10,000 rpm for the determination of lactate dehydrogenase (LDH) with the resultant supernatant for the estimation of cytotoxicity marker.

Petroleum ether, chloroform, ethyl acetate and ethanol extracts of 2 different plants were subjected to five different experimental conditions.

Group I - Control (KRH medium alone)

Group II - Paracetamol (15mM) alone

Group III A - Petroleum ether extract of *Alpinia speciosa* (10µg/ml) was present for 2 hrs with paracetamol

Group III B - Chloroform extract of *Alpinia speciosa* (10µg/ml) was present for 2 hrs with paracetamol

Group III C - Ethyl acetate extract of *Alpinia speciosa* (10µg/ml) was present for 2 hours with paracetamol

Group III D - Ethanol extract of *Alpinia speciosa* (10µg/ml) was present for 2 hrs with paracetamol

Group IV A - Petroleum ether extract of *Valeriana wallichii* (10µg/ml) was present for 2 hrs with paracetamol

Group IV B - Chloroform extract of *Valeriana wallichii* (10µg/ml) was present for 2 hrs with paracetamol

Group IV C - Ethyl acetate extract of *Valeriana wallichii* (10µg/ml) was present for 2 hrs with paracetamol

Group IV D - Ethanol extract of *Valeriana wallichii* (10µg/ml) was present for 2 hrs with paracetamol

Group V - Silymarin (100 µg/ml) was present for 2 hours with paracetamol.

5.8.3 Estimation of lactate dehydrogenase

Principle

The lactate in the presence of NAD reacts with lactate dehydrogenase and forms pyruvate. This forms pyruvate phenyl hydrazone with 2, 4 dinitrophenyl hydrazine. The colour formed was read using a spectrophotometer at 440 nm.

Chemicals

- NAD
- Dinitrophenyl hydrazine
- Sodium hydroxide

Procedure

About 1 ml of buffer substrate along with 0.1 ml supernatant and 0.2 ml water were added to the blank. About 0.2 ml of NAD was added to the test. After 15 minutes of incubation at 37° C, 1.0 ml of dinitrophenyl hydrazine was added, to this after 15 minutes 10 ml of 0.4N sodium hydroxide was added and read at 440 nm.

5.9 Acute toxicity studies

Acute toxicity studies were done by following the fixed dose procedure using the OECD guideline No. 420. Healthy adult female *Wistar* rats were the commonly used laboratory strains. Usually female rats were used because of their sensitivity. Female rats selected were nulliparous and non-pregnant. Animals should be in the age group between 8-12 weeks and weight should not differ from ± 20%. The animals were starved for 3-4 hour prior to the testing with drinking water *ad libitum*. In the sighting study one animal was administered 5 mg/kg dose in order to confirm toxic symptoms if any. If mortality was detected for 5 mg/kg dose the study is terminated and assigned

the substance to globally harmonized classification system (GHS) category 1. When no toxicity is observed the second animal were administered with 50 mg/kg and similarly as mentioned above the animals were monitored for toxic symptoms if any and if not toxic effect observed then the third animal were administered with 300 mg/kg. The procedure was continued if no toxic symptoms were observed for previous dose and the fourth rat was administered with 2000 mg/kg dose of chloroform extract of *Alpinia speciosa*. In main study, a single animal was administered with chloroform extract of 2000 mg/kg. The same procedure was carried out for the chloroform extract of *Valeriana wallichii*[126].

The rats treated with plant extracts were observed for 24 hours for 14 days. Close observations were done and changes were observed in rats throughout their body.

5.9.1 Selection of dose for *in vivo* activity

Acute toxicity was performed as per OECD guidelines (420) using fixed dose procedure for fixing the dose for biological activity. For acute toxicity studies, ten female *Wistar* rats weighing 150-200g were taken and they were fasted overnight before the experimental day. Overnight fasted rats were weighed and body weight determined for dose calculation and the test samples were administered orally starting with a lower dose of 5 mg/kg and the dose was gradually increased in a sequence of 50, 300 and 2000 mg/kg. For lower dose group 2 animals were used and for higher doses 3 animals were used. The changes like body weight, food and water consumption in the rats were monitored two times a day for the entire period.

6. In vivo hepatoprotective activity

6.1. Paracetamol-induced hepatotoxicity model

Male *Wistar* rats were distributed into 7 groups, consisting of 6 each.

Group – I : Control - Carboxy methyl cellulose

Group – II : Negative control - Paracetamol

Group –III : Chloroform extract of *Alpinia speciosa*

Group –IV : Chloroform extract of *Alpinia speciosa*

Group –V : Chloroform extract of *Valeriana wallichii*

Group –VI : Chloroform extract of *Valeriana wallichii*

Group -VII : Positive control - Silymarin

The animals in groups I and II were administered with 0.5% CMC for a period of 7 days. The animals in groups III to VII were administered with respective standard drug or extract suspended in 0.5% CMC throughout the 7 days treatment.

6.2 D-Galactosamine-induced hepatotoxicity

Male *Wistar* rats were distributed into 7 groups, consisting of 6 each.

Group –I : Control - Carboxy methyl cellulose

Group – II : Negative control – D-Galactosamine

Group – III : Chloroform extract of *Alpinia speciosa*

Group –IV : Chloroform extract of *Alpinia speciosa*

Group – V : Chloroform extract of *Valeriana wallichii*

Group –VI : Chloroform extract of *Valeriana wallichii*

Group –VII : Positive control - Silymarin

The animals in groups I and II were administered with 0.5% CMC for a period of 11 days. The animals in groups III to VII were administered with respective standard drug or extract suspended in 0.5% CMC throughout the 11 days treatment[9].

6.3 Ethanol-induced hepatotoxicity model

Male *Wistar* rats were distributed into 7 groups, consisting of 6 each. .

Group –I : Control – Carboxy methyl cellulose (CMC)

Group – II : Negative control – Ethanol

Group – III : Chloroform extract of *Alpinia speciosa*

Group – IV : Chloroform extract of *Alpinia speciosa*

Group–V : Chloroform extract of *Valeriana wallichii*

Group –VI : Chloroform extract of *Valeriana wallichii*

Group –VII : Positive control - Silymarin

The animals in groups I and II were administered with 0.5% CMC for a period of 21 days. The animals in group III to VII were administered with respective standard drug or extract suspended in 0.5% CMC throughout the 21 days treatment. Ethanol was administered orally throughout for 21 days for II to VII groups[82].

6.4 Estimation of serum biochemical parameters for all the models

In all the above three models, after 24 hours of final drug administration, blood was collected by retro orbital puncture under mild ether anesthesia. The serum was used for the assay of marker enzymes viz; alanine amino transferase, aspartate amino transferase, acid phosphatase, alkaline phosphatase and bilirubin.

6.4.1 Estimation of Alanine aminotransferase / Serum glutamate pyruvate transaminase (ALT/SGPT)

Reagents

Working reagent: Composition:

Reagent 1 (R1)

Tris buffer (pH 7.5)

L-Alanine

LDH

Reagent 2 (R2)

NADH

α – keto glutarate

Preparation

20 ml of R2 is mixed with 20 ml of R1.

Procedure

The mixture of 100 µl of serum and 1 ml of the solution was incubated at 37°C and the absorbance was read at 1 min. The change in optical density per min (ΔOD/min) was measured at 340 nm during 3 min. The activity of aspartate aminotransferase was determined by using the following formula:

ALT activity (U/L) = Δ OD/min x 1745

6.4.2 Estimation of Aspartate aminotransferase / Serum glutamate oxaloacetate transaminase (AST/SGOT)

Chemicals and reagents

Working reagent: Composition:

Reagent 1 (R1)

Tris buffer (pH 7.8)

L-Aspartate

LDH

MDH

Reagent 2 (R2)

NADH

α – keto glutarate

Preparation

20 ml of R2 is mixed with 20 ml of R1.

Procedure

About 100 µl of serum was mixed with 1 ml of the solution and the absorbance was read at 1 min. The change in optical density per min (ΔOD/min) was measured at 340 nm during 3 min. The activity of aspartate aminotransferase was determined by using the following formula:

AST activity (U/L) = ΔOD/min x 1745

6.4.3 Estimation of acid phosphatase (ACP)

Chemicals and reagents

Working reagent: Composition:

Reagent 1 (R1)

Citrate buffer (pH 5.2)

Reagent 2 (R2)

Tablets

α- naphtyl phosphate

Fast red TR

Preparation

1 tablet (R2) is dissolved in 2 ml of R1 and waited for 10 min for complete dissolution.

Procedure

About 100 µl of serum was mixed with 1 ml of the solution and the absorbance was read at 1 min. The change in optical density per min (Δ OD/min) was measured at 405 nm during 3 min. The activity of acid phosphatase was determined by using the following formula:

Acid phosphatase activity (U/L) = 750 x (Δ OD/min) of total

6.4.4 Estimation of alkaline phosphatase (ALP)

Procedure

About 100 µl of serum was mixed with 1 ml of the solution and the absorbance was read at 1 min. The change in optical density per min (ΔOD/min) was measured at 405 nm during 3 min. The activity of aspartate aminotransferase was determined by using the following formula:

ALP activity (U/L) = (ΔOD/min) x 2750

6.4.5 Estimation of bilirubin

Procedure

To the sample tube, 50 µl of serum, 1 ml of total bilirubin reagent and 20 µl of total bilirubin activator were added and mixed. To the standard tube, 50µl of standard bilirubin (10mg/ml), 1ml of total bilirubin reagent and 20 µl of total bilirubin activator were added. The change in optical density per min (ΔOD/min) was measured to 546 nm during 5 min. The concentration of total bilirubin was determined by using the following formula:

$$\text{Total bilirubin} = \frac{\text{OD of sample test - OD of sample blank}}{\text{OD of standard}} \text{ X } 100$$

6.4.6 Preparation of liver homogenate

The blood of the animals were collected and then they were sacrificed by the process of cervical dislocation. The liver was excised. The liver homogenates (10% w/v) were done in in cold 50 mM phosphate buffer of pH 7.4. The resulting supernatant after centrifugation at 5000 rpm for 10 min, n cold 50 mM phosphate buffer of pH 7.4, lipid peroxidation and enzymatic and non – enzymatic antioxidants were estimated.

6.4.7 Estimation of protein content

Chemicals and reagents

- Alkaline copper tartarate solution
- Phenol reagent
- Bovine serum albumin

Procedure

This method was estimated by Lowry *et al*., 1951. The standard used was bovine serum albumin. 0.1 ml of tissue homogenate was added with 4.0 ml of alkaline copper tartarate alongwith 0.4 ml of phenol reagent. From the standard curve the protein content was calculated [127].

6.4.8 Estimation of lipid peroxidation assays for all models

6.4.8.Estimation of malondialdehyde

Chemicals and reagents

TBA-TCA-HCl reagent

Procedure

About 0.1 ml of tissue homogenate was added with 2 ml of (1:1:1) TBA-TCA-HCl reagent and centrifuged at room temperature for 10 min at 100 rpm. The change in optical density per min (ΔOD/min) was measured[128].

6.4.9 Estimation of lipid hydroperoxides (LH)

Procedure

The method was estimated by Jiang *et al*., 1992. About 0.1 ml of tissue homogenate was treated with 0.9 ml of the abovesaid reagent and incubated at 37°C for 30 min. The color was read at 560 nm using a colorimeter. The values are expressed as nmoles of LH formed /min/mg tissue protein[129].

6.4.10 Antioxidant assays

Estimation of superoxide dismutase (SOD)

Chemicals and reagents

Carbonate buffer

Epinephrine

Procedure

This method was assayed by Kakkar 1984. The reaction mixture contained 150 µl of liver homogenate alongwith 1.8 ml of carbonate buffer (30mM, pH 10.2), 0.7 ml of distilled water and 400 µl of epinephrine (45mM). The presence of liver homogenate was measured at 480nm. SOD activity is expressed as nmoles/min/mg protein[130].

6.4.11 Estimation of catalase (CAT)

Procedure

About 0.1 ml of tissue homogenate was added to 1 ml of 0.01 m phosphate buffer of pH 7.0 and was incubated at 37°C for 15 min. At the starting of the reaction 0.4 ml of H_2O_2 was added and followed by addition of 2 ml of dichromate-acetic acid reagent and assayed calorimetrically at 620 nm.

6.4.12 Estimation of glutathione reductase (GSSH)

Procedure

The activity of glutathione reductase in the tissues was determined by the method of Racker 1955. Around 2.1 ml of 0.25 mM potassium phosphate buffer of pH 7.6 and 0.1 ml of bovine serum albumin (10 mg/ml), was added with 0.2 ml of tissue homogenate and was read at 340 nm.

6.4.13 Estimation of peroxidase (Px)

Procedure

Around 0.2 ml of 15 mM O-dianisidine, 0.1ml of tissue homogenate and 2.5 ml of 0.1 m potassium phosphate buffer of pH 5.0 was added with 0.2 ml of hydrogen peroxide. The absorbance was read at 460nm[133].

6.4.14 Estimation of glutathione peroxidase (GPx)

Procedure

Approximately 0.2 ml of 0.4 N Tris-HCl buffer, 0.1 ml of sodium azide, 0.1 ml of hydrogen peroxide, 0.2 ml of glutathione and 0.2 ml of supernatant was incubated at 37°C for 10 min after the addition of 10% trichloro acetic acid and the reading was read at 340nm.

6.4.15 Determination of non-enzymatic antioxidant assay for all models

Estimation of reduced glutathione (GSH)

Procedure

About 1 ml of supernatant alongwith 0.5 ml of Ellman's reagent and 3 ml of 0.2 M phosphate buffer of pH 8.0 and the reading was found to be at 412 nm using a spectrophotometer. The activity of GSH was expressed as nmoles/min/mg protein[135,81].

7. Histopathological evaluation of the extracts against various hepatoxicants (Paracetamol, D-Galactosamine and ethanol) induced hepatotoxicity for all models

Preparation of the tissue for histology[136]

Fixation

Procedure

Liver tissues were collected, rinsed and fixed after sacrificing the rats, using the fixative for 24 h. Then the tissues were washed thoroughly in tap water followed by rinsing in distilled water.

Dehydration

This is a preliminary but an essential process for removing the water present in tissues after fixation to ensure proper penetration of paraffin during embedding. Dehydration was effected by increasing concentrations of alcohol.

Procedure

The tissues were placed in specimen jars and passed through ascending grades of ethyl alcohol as given below.

50% alcohol
70% alcohol (overnight)
80% alcohol (30 minutes)
90% and 95% (2 hours)
Absolute alcohol (1 hour)

Clearing

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

Embedding

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

Procedure

The tissues were transferred from xylene to molten paraffin and kept in the paraffin bath for 3-4 h in the embedding oven.

Block preparation

The tissues in molten paraffin were poured into moulds of suitable dimensions and the tissues were set properly within the paraffin. To remove any air bubble in the paraffin block the tip of the forceps was gently warmed, inserted and passed around the specimen taking care to avoid touching the tissue. This is important as presence of air bubbles in the block hinders proper sectioning. The moulds were then gently immersed in cold water to cool the paraffin rapidly. This prevents crystallization of paraffin. When the blocks were solidified, they were removed from water and detached from the mould with a scapel. The tissues are now ready for sectioning.

Section cutting

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

Procedure

The excess paraffin at the sides of the block was trimmed to have a block of suitable size and the block was fixed on the block holder of the microtome. The block holder was heated on a flame and pressed directly to the base of the block. The surface of the tissue was brought just by using coarse feed adjustment. The feed mechanism was adjusted to the desired setting of 5 μ. The microtome was operated and sections were cut out. After getting an 8" ribbon, it was detached with the help of a scalpel and floated on warm water. Small portions of this ribbon with serial sections were placed on clear glass slides and gently stretched using forceps or needles. Water on the slides was drained off and the slides were allowed to dry for 12 h.

Removal of paraffin

As paraffin is poorly permeable to stain, slides were dipped in xylene (two changes each of 2-5 min) for the complete removal of paraffin.

Removal of xylene

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

Rehydration

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

Staining with haematoxylin

After washing in water, slides were treated with haematoxylin for 2-3 min. The slides were then washed in tap water for 8-10 min.

Staining with eosin

Eosin is soluble in alcoholic medium but not in aqueous medium. The haematoxylin stained slide was again passed through increasing concentrations

of alcohol, with two final changes of 3 min each in absolute alcohol. The slides were transferred from absolute alcohol. The slides were transferred from absolute alcohol to eosin solution and kept for 1-2 min.

Treatment with absolute alcohol

Three changes each of 1 min were given for the slides in absolute alcohol.

Clearing

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

Mounting

After treatment with xylene a very small amount of DPX mountant (a mixture of distyene, a plasticizer and xylene) was placed over the section. Over this, a clean cover slip was placed and pressed gently so as to avoid the formation of bubbles.

8. Statistical analysis

Quantitative data were expressed as mean ± S.E.M and all statistical comparisons were made by means of one-way analysis of variance (ANOVA) followed by Tukey's test. $P<0.05$ was considered as statistically significant.

9. Isolation of possible active constituents from chloroform extract of *Alpinia speciosa* rhizomes and *Valeriana wallichii roots*

Extraction procedure of *Alpinia speciosa*

The air dried powder (500g) was extracted with commercial grade chloroform using Soxhlet apparatus. The resulting extract was tested for phytochemical analysis and thin layer chromatographic studies. The crude extract (10g) was adsorbed with silica gel and loaded on column chromatography for isolation of active constituents[137,138].

Extraction procedure of *Valeriana wallichii*

The air dried powder (500g) was extracted with commercial grade chloroform using Soxhlet apparatus. The resulting extract was concentrated using rotary evaporator and dried in the vacuum desiccator. The residue was tested for phytochemical analysis and thin layer chromatographic studies. The crude extract (8g) was adsorbed with silica gel (100 – 200 mesh) and loaded on column chromatography for isolation of active constituents.

9.1.1 Procedure for thin layer chromatographic study of both the plant extracts:

Each plant extract was subjected to thin layer chromatography as per conventional method using silica gel 60F254, 5x3 cm were cut using TLC cutter. Plate markings were made with soft pencil. Glass capillary tubes were used to spot the extract in TLC plates. Different solvent systems ranging from lower to higher polarities were tested for the separation of bioactive components. In the TLC chamber the solvent system viz hexane: ethyl acetate (9:1); hexane: ethyl acetate (1:1) and chloroform: methanol (9:1) were used. After pre-saturation with mobile phase for 30 min, the plates were kept inside the chamber and the elution was performed using above mentioned solvent system. After completion of the elution the plates were dried and subjected to visualized under UV chamber and sprayed using different spray reagents. R_f values determined.

The compounds were eluted by column chromatography using hexane: ethyl acetate; ethyl acetate: chloroform and chloroform: methanol solvents and the isolated compounds were submitted for FT-IR, mass and ^{1}H-NMR analysis.

RESULTS AND ANALYSIS

6.1 Phytochemical screening of plant extracts

The preliminary phytochemical screening of active constituents was carried out for petroleum ether, chloroform, ethyl acetate and ethanol extracts of *Alpinia speciosa* and *Valeriana wallichii.*

Table 1: Phytochemical screening of *Alpinia speciosa*

Phytoconstitutents	PEAS	CEAS	EAAS	EAS
Alkaloids				
a. Dragendroff's test	-	-	-	-
b. Hagner's test	-	-	-	-
c. Wagner's test	-	-	-	-
d. Mayer's test	-	-	-	-
Flavonoids				
a. Conc. Sulphuric acid test	-	+	-	-
b. 10% lead acetate test	-	+	-	-
Reducing sugar				
a. Benedict's test	-	-	-	-
b. Fehling's test	-	-	-	-
Steroids				
Salkowsky test	-	-	-	-
Saponins				
a. Foam test	-	-	-	-
b. Distilled water	-	-	-	-

Terpenoids a. Conc. Sulphuric acid b. 10% Sodium hydroxide	+ -	+ +	- -	- -
Tannins and Phenolics a. Ferric chloride test b. Lead acetate c. Gelatin test d. Phlabotannins	+ + - -	+ + + -	+ + + -	+ - - -
Amino acids a. Ninhydrin test b. Conc. Nitric acid	- -	- -	- -	- -
Aromatic amino acid	-	-	-	-
Coumarins NaOH	+	-	-	-

6.1.1 Phytochemical screening of *Alpinia speciosa*

Flavonoids, terpenoids, tannins and phenolics were revealed in the extracts and the presence of tannins and phenolics was revealed in ethanol extract of *Alpinia speciosa* (Table: 1).

Table 2: Phytochemical screening of *Valeriana wallichii*

Phytoconstitutents	PEAS	CEAS	EAAS	EAS
Alkaloids				
a. Dragendroff's test	-	-	-	-
b. Hagner's test	-	-	-	-
c. Wagner's test	-	-	-	-
d. Mayer's test	-	-	-	-
Flavonoids				
a. Conc. Sulphuric acid test	+	-	-	+
b. 10% lead acetate test	+	-	-	+
Reducing sugar/carbohydrates				
a. Benedict's test	-	-	-	+
b. Fehling's test	-	-	-	+
Steroids				
Salkowsky test	-	-	-	-
Saponins				
a. Foam test	-	-	-	-
b. Distilled water	-	-	-	-
Terpenoids				
a. Conc. Sulphuric acid	+	+	+	+
b. 10% Sodium hydroxide	_	+	+	+
Tannins and Phenolics				

a. Ferric chloride test	+	+	+	+
b. Lead acetate	+	+	+	+
c. Gelatin test	-	+	+	+
d. Phlabotannins	-	-	-	-
Amino acids				
a. Ninhydrin test	-	-	-	-
b. Conc. Nitric acid	-		-	-
Aromatic amino acid	-	-	-	-

6.1.2 Phytochemical screening of *Valeriana wallichii*

Flavonoids and terpenoids were present in petroleum ether extract. In chloroform extract flavonoids, terpenoids, tannins and phenolics were revealed. Terpenoids, tannins and phenolics were present in ethyl acetate extract. In ethanol extract presence of flavonoids, carbohydrate, terpenoids, tannins and phenolics were revealed (Table: 2).

Table 3: *In vitro* hepatoprotective activity of various extracts of *Alpinia speciosa* and *Valeriana wallichii*

Group	Treatment at 2 hrs	Concentration Release of LDH
I	Control	0.0301 ± 0.003
II	Paracetamol	0.0634 ± 0.001[a]
Group – III – (AS) – A	Paracetamol + pet. ether extract	0.0238 ± 0.009[c]
B	Paracetamol + chloroform extract	0.0224 ± 0.005[b]
C	Paracetamol + ethyl acetate extract	0.0259 ± 0.003[c]
D	Paracetamol + ethanol extract	0.0273 ± 0.002[c]
Group – IV – (VW) - A	Paracetamol + pet. ether extract	0.0259 ± 0.004[c]
B	Paracetamol + chloroform extract	0.0217 ± 0.003[b]
C	Paracetamol + ethyl acetate extract	0.0241 ± 0.007[c]
D	Paracetamol + ethanol extract	0.0263 ± 0.002[c]
Group – V	Silymarin	0.0418 ± 0.006[c]

6.2 Assessment of paracetamol hepatotoxicity by estimation of LDH

The marker used in the study of hepatotoxicity by paracetamol, in this method was LDH leakage. The observations found in this case were that the liver slices treated with paracetamol for 2 hrs, showed the twice the amount of LDH release in comparison with untreated liver slices.

At a concentration of 10µg/ml, the chloroform extracts of *Alpinia speciosa* and *Valeriana wallichii* were non-toxic to the hepatic cells. There was no difference in the release of LDH in both the plant extract and the untreated slice. More LDH were released by the liver slices than the control in the medium in the presence of paracetamol. When extract was present along with paracetamol during incubation for 2 hrs, the LDH released was significantly ($P<0.001$) decreased in the chloroform extract of the plants when compared to other extracts, viz., petroleum ether, ethyl acetate and ethanol. This result confirmed that treatment with chloroform extract of both the plants for 2 hrs offer better protection against paracetamol-induced hepatotoxicity by liver slice model.

Based on the *in vitro* results of various extracts of *Alpinia speciosa* and *Valeriana wallichii,* it was confirmed that the chloroform extracts of both the plants possessed significant ($P<0.001$) activity when compared to other extracts. Hence the chloroform extract of *Alpinia speciosa* (CEAS) and *Valeriana wallichii* (CEVW) were subjected to *in vivo* hepatoprotective activity aganist various hepatotoxicant models.

6.3 Acute toxicity studies

Table 4: Live phase observation for *Alpinia speciosa* and *Valeriana wallichii*

Live phase animals (every day)	Observations
Body weight	Normal
Food consumption	Normal
Water consumption	Normal
Home cage	Normal

Table 5: Acute oral toxicity study of *CEAS* and *CEVW*

Parameters observed		0 hr	0.5hr	1 hr	2 hr	4 hr	Day 2&3	Day 4&5	Day 6&7	Day 8&9	Day 10&11	Day 12&13	Day 14
Respiratory	Dyspnea	-	-	-	-	-	-	-	-	-	-	-	-
	Apnea	-	-	-	-	-	-	-	-	-	-	-	-
	Nostril discharges	-	-	-	-	-	-	-	-	-	-	-	-
Motor activity	Tremor	-	-	-	-	-	-	-	-	-	-	-	-
	Hyper activity	-	-	-	-	-	-	-	-	-	-	-	-
	Hypo activity	-	-	-	-	-	-	-	-	-	-	-	-
	Ataxia	-	-	-	-	-	-	-	-	-	-	-	-
	Jumping	-	-	-	-	-	-	-	-	-	-	-	-
	Catalepsy	-	-	-	-	-	-	-	-	-	-	-	-
	Locomotor activity		-	-	-	-	-	-	-	-	-	-	-
Reflexes	Corneal reflex	-	-	-	-	-	-	-	-	-	-	-	-
	Pinna reflex	-	-	-	-	-	-	-	-	-	-	-	-
	Righting reflex	-	-	-	-	-	-	-	-	-	-	-	-
Convulsion	Tonic and clonic convulsion	-	-	-	-	-	-	-	-	-	-	-	-
Muscle tone	Hypertonia	-	-	-	-	-	-	-	-	-	-	-	-
	Hypotonia	-	-	-	-	-	-	-	-	-	-	-	-

Table 6: Acute oral toxicity study of *CEAS* and *CEVW*

Parameters observed		0 hr	0.5hr	1 hr	2 hr	4 hr	Day 2&3	Day 4&5	Day 6&7	Day 8&9	Day 10&11	Day 12&13	Day 14
Ocular sign	Lacrimation	-	-	-	-	-	-	-	-	-	-	-	-
	Miosis	-	-	-	-	-	-	-	-	-	-	-	-
	Mydriasis	-	-	-	-	-	-	-	-	-	-	-	-
	Ptosis	-	-	-	-	-	-	-	-	-	-	-	-
Skin	Edema	-	-	-	-	-	-	-	-	-	-	-	-
	Skin and fur	-	-	-	-	-	-	-	-	-	-	-	-
	Erythema		-	-	-	-	-	-	-	-	-	-	-
Cardiovascular signs	Bradycardia	-	-	-	-	-	-	-	-	-	-	-	-
	Tachycardia	-	-	-	-	-	-	-	-	-	-	-	-
Piloerection	Contraction of erectile tissue of hair	-	-	-	-	-	-	-	-	-	-	-	-
Gastro intestinal signs	Diarrhea	-	-	-	-	-	-	-	-	-	-	-	-

Table 7: Mortality record of *CEAS* - Acute oral toxicity study

Group	**Group 1 (5 mg/kg)**		**Group 2 (50 mg/kg)**		**Group 3 (300 mg/kg)**			**Group 4 (2000 mg/kg)**		
No. of animals	1	2	1	2	1	2	3	1	2	3
Body weight (g)	150	155	180	183	180	182	180	200	200	195
Sex	Female	Female	Female	Female	Female	Female	Female	Female	Female	Female
30 min	-	-	-	-	-	-	-	-	-	-
1 hr	-	-	-	-	-	-	-	-	-	-
2 hr	-	-	-	-	-	-	-	-	-	-
3 hr	-	-	-	-	-	-	-	-	-	-
4 hr	-	-	-	-	-	-	-	-	-	-
Day 1	-	-	-	-	-	-	-	-	-	-
Day 2	-	-	-	-	-	-	-	-	-	-
Day 3	-	-	-	-	-	-	-	-	-	-
Day 4	-	-	-	-	-	-	-	-	-	-
Day 5	-	-	-	-	-	-	-	-	-	-
Day 6	-	-	-	-	-	-	-	-	-	-
Day 7	-	-	-	-	-	-	-	-	-	-
Day 8	-	-	-	-	-	-	-	-	-	-
Day 9	-	-	-	-	-	-	-	-	-	-

Table 8: Mortality record of *CE VW* - Acute oral toxicity study

Group	**Group 1 (5 mg/kg)**		**Group 2 (50 mg/kg)**		**Group 3 (300 mg/kg)**			**Group 4 (2000 mg/kg)**		
No. of animals	1	2	1	2	1	2	3	1	2	3
Body weight (g)	150	155	180	183	180	182	180	200	200	195
Sex	Female	Female	Female	Female	Female	Female	Female	Female	Female	Female
30 min	-	-	-	-	-	-	-	-	-	-
1 hr	-	-	-	-	-	-	-	-	-	-
2 hr	-	-	-	-	-	-	-	-	-	-
3 hr	-	-	-	-	-	-	-	-	-	-
4 hr	-	-	-	-	-	-	-	-	-	-
Day 1	-	-	-	-	-	-	-	-	-	-
Day 2	-	-	-	-	-	-	-	-	-	-
Day 3	-	-	-	-	-	-	-	-	-	-
Day 4	-	-	-	-	-	-	-	-	-	-
Day 5	-	-	-	-	-	-	-	-	-	-
Day 6	-	-	-	-	-	-	-	-	-	-
Day 7	-	-	-	-	-	-	-	-	-	-
Day 8	-	-	-	-	-	-	-	-	-	-
Day 9	-	-	-	-	-	-	-	-	-	-

Table 9: Mortality record of *CEAS* - Acute oral toxicity study

Group	Group 1 (5 mg/kg)		Group 2 (50 mg/kg)		Group 3 (300 mg/kg)			Group 4 (2000 mg/kg)		
No. of animals	1	2	1	2	1	2	3	1	2	3
Body weight (g)	150	155	180	183	180	182	180	200	200	195
Sex	Female	Female	Female	Female	Female	Female	Female	Female	Female	Female
Day 10	-	-	-	-	-	-	-	-	-	-
Day 11	-	-	-	-	-	-	-	-	-	-
Day 12	-	-	-	-	-	-	-	-	-	-
Day 13	-	-	-	-	-	-	-	-	-	-
Day 14	-	-	-	-	-	-	-	-	-	-
Mortality	**0/2**		**0/2**		**0/3**			**0/3**		

Table 10: Mortality record of *CEVW* - Acute oral toxicity study

Group	**Group 1 (5 mg/kg)**		**Group 2 (50 mg/kg)**		**Group 3 (300 mg/kg)**			**Group 4 (2000 mg/kg)**		
No. of animals	1	2	1	2	1	2	3	1	2	3
Body weight (g)	150	155	180	183	180	182	180	200	200	195
Sex	Female	Female	Female	Female	Female	Female	Female	Female	Female	Female
Day 10	-	-	-	-	-	-	-	-	-	-
Day 11	-	-	-	-	-	-	-	-	-	-
Day 12	-	-	-	-	-	-	-	-	-	-
Day 13	-	-	-	-	-	-	-	-	-	-
Day 14	-	-	-	-	-	-	-	-	-	-
Mortality	**0/2**		**0/2**		**0/3**			**0/3**		

6.3.1 Selection of dose for *in vivo* studies

As per the OECD 420 guidelines *Alpinia speciosa and Valeriana wallichii* can be included in the category 5 or unclassified category of globally harmonized classification system (GHS). Hence, based on these results the *Alpinia speciosa and Valeriana wallichii* is considered non-toxic and $1/10^{th}$ and $1/20^{th}$ dose was used for the biological evaluation and the studies were conducted at 100 and 200 mg/kg doses.

***In vivo* hepatoprotective activity**

Paracetamol-induced hepatotoxicity model

Table 11: Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on serum biochemical parameters

GROUP	ALT	AST	ALP	ACP	BILIRUBIN
Control (CMC) (0.5%)	51.30 ± 0.46	71.08 ± 0.12	97.08 ± 0.07	44.89 ± 0.30	0.26 ± 0.00
Paracetamol (100 mg/kg b.w, p.o)	291.36 ± 0.51	302.95 ± 0.16	176.25 ± 0.086	95.14 ± 1.11	0.58 ± 0.006
CEAS (250mg/kg)	185.56± 0.50	197.84 ± 0.175	121.31± 1 0.161	72.74 ± 1.018	0.53 ± 0.006
CEAS (500mg/kg)	68.23 ± 1.16	87.55 0.674	113.64 ±0.99	63.21 ±1.14	0.47 ± 7 0.00
CEVW (200mg/kg)	128.62 ± 0.39	174.76 ± 0.277	120.15±6 0.11	73.22 ±1.06	0.50 ± 60.005
CEVW (400mg/kg)	65.90 ± 21.16	82.85 ± 1.29	105.64 ± 0.817	60.94 ± 1.36	0.41± 0.00
Silymarin (100 mg/kg b.w, p.o)	68.28 ± 1.107	77.94 ± 0.20	104.01 ± 0.07	51.60 ± 0.320	0.30 ± 0.005

6.4 Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on serum biochemical parameters

The rhizomes of CEAS and CEVW were evaluated for serum biochemical parameters in control and experimental animals. The serum enzymes levels (viz. ALT, AST, ACP, ALP and bilirubin) were significantly ($P<0.001$) increased in the paracetamol treated group when compared to control. There was a dose dependent decrease in the enzymes and bilirubin levels. The activity produced by the extract was similar to that of the standard silymarin treated group (Table 11).

Table 12: Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on tissue protein

Group	Protein	MDA	LH
Control (CMC) (0.5%)	77.98 ± 0.041	1.105 ± 0.029	1.895 ± 0.022
Paracetamol (100 mg/kg b.w, p.o)	50.088 ± 0.086	8.568 ± 0.117	8.196 ± 0.041
CEAS (250mg/kg)	52.396 ± 1.186	7.096 ± 0.051	5.588 ± 0.037
CEAS (500mg/kg)	58.97 ± 0.057	5.228 ± 0.023	3.195 ± 0.062
CEVW (200mg/kg)	56.013 ± 0.065	6.103 ± 0.029	4.328 ± 0.067
CEVW (400mg/kg)	63.55 ± 1.008	4.523 ± 0.040	2.23 ± 0.026
Silymarin (100 mg/kg., p.o)	65.038 ± 1.111	3.168 ± 0.044	2.02 ± 0.046

6.4.1 Effect of *Alpinia speciosa* and *Valeriana wallichii* on tissue protein

Total protein level was significantly ($P<0.001$) reduced in the paracetamol treated group when compared to control. The protein level was significantly ($P<0.001$) elevated when the animals were pretreated with the CEAS at higher dose (500 mg/kg) and at both the doses (250 and 500 mg/kg) of CEVW for 8 days when comparable to paracetamol control. The effect produced by the CEAS of lower dose (250 mg/kg) was not significant ($P>0.05$) when compared to paracetamol control (Table 12).

Table 13: Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on enzymatic and non-enzymatic antioxidants

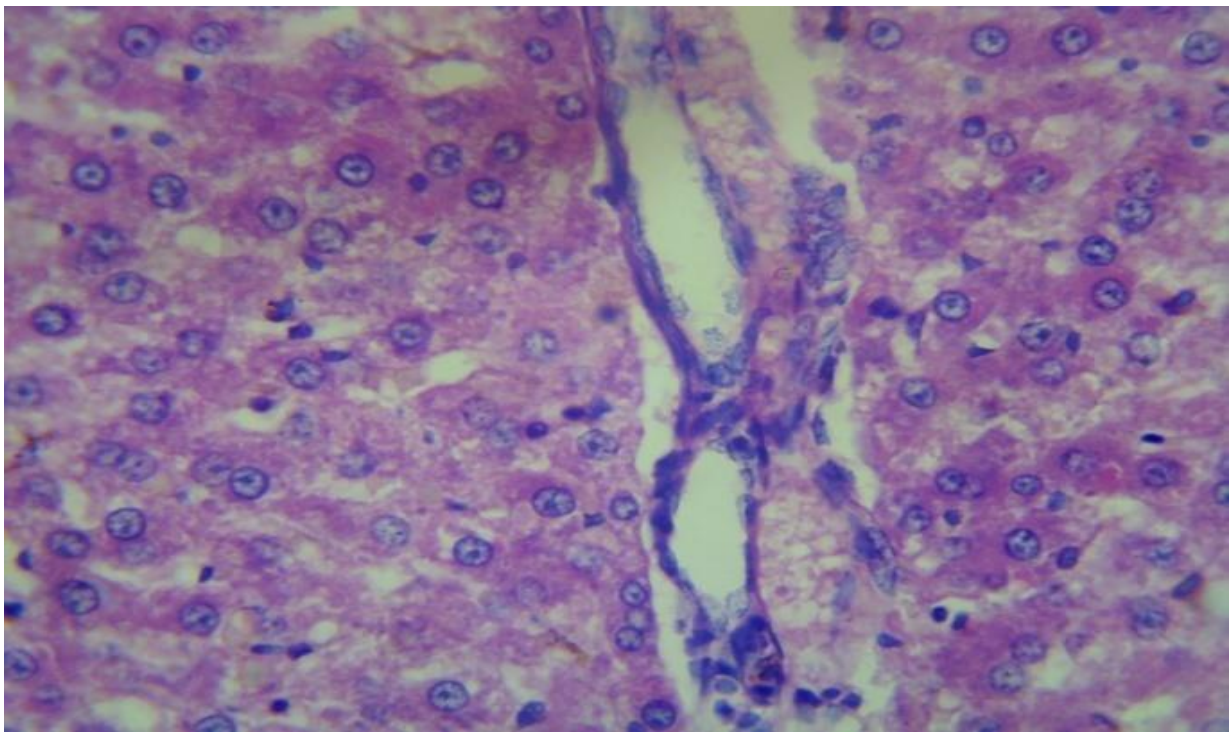
GROUP	CAT	Px	SOD	GSSH	GPx	GSH
Control (CMC, 0.5%)	4.171 ± 0.113	4.151 ± 0.054	6.325 ± 0.073	4.513 ± 0.111	6.108 ± 0.020	5.756 ± 0.047
Paracetamol (100 mg/kg b.w, p.o)	1.246 ± 0.082	2.995 ± 0.092	2.11 ± 0.034	1.638 ± 0.024	2.705 ± 0.046	1.151 ± 0.013
CEAS (250mg/kg)	2.006 ± 0.040	2.985 ± 0.255	3.008 ± 0.037	2.095 ± 0.073	3.11 ± 0.029	3.79 ± 0.28
CEAS (500mg/kg)	2.291 ± 0.067	2.595 ± 0.237	4.813 ± 0.043	2.931 ± 0.038	4.728 ± 0.032	4.385 ± 0.036
CEVW (200mg/kg)	2.115 ± 0.047	3.213 ± 0.047	3.718 ± 0.082	2.121 ± 0.022	3.801 ± 0.032	3.915 ± 0.036
CEVW (400mg/kg)	2.823 ± 0.051	3.04 ± 0.050	5.108 ± 0.082	3.12 ± 0.023	4.855 ± 0.03	4.618 ± 0.032
Silymarin (100 mg/kg., p.o)	3.746 ± 0.051	3.986 ± 0.024	5.543 ± 0.046	3.376 ± 0.026	0.571 ± 0.041	4.846 ± 0.034

6.4.2 Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on enzymatic and non-enzymatic antioxidants

The levels of tissue enzymatic antioxidants and the non enzymatic antioxidant GSH in paracetamol treated group was found to be significantly ($P<0.001$) lower when compared to the control. The levels of tissue enzymatic and non enzymatic antioxidants were significantly ($P<0.001$) increased in the groups treated with chloroform extract of both the plants for 8 days when compared to paracetamol control. The effect produced by the CEAS at a dose of 250 mg/kg on the above parameters was not significant ($P>0.05$) when compared to paracetamol control (Table 13).

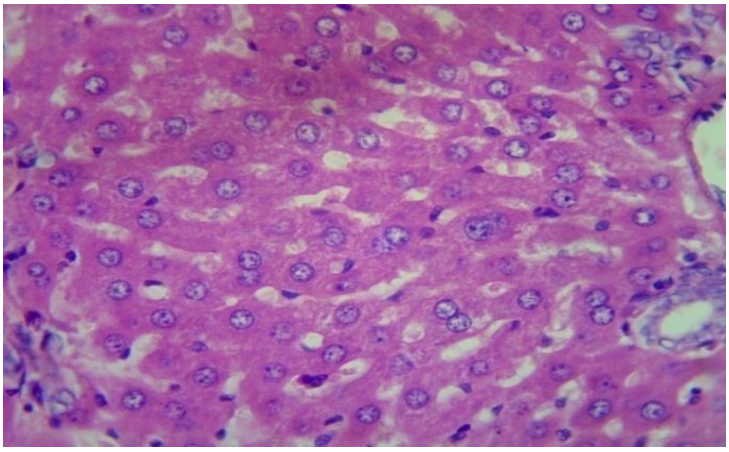
Histopathological report of paracetamol-induced hepatotoxicity in the liver

Figure 3: Section of rat liver treated with vehicle control



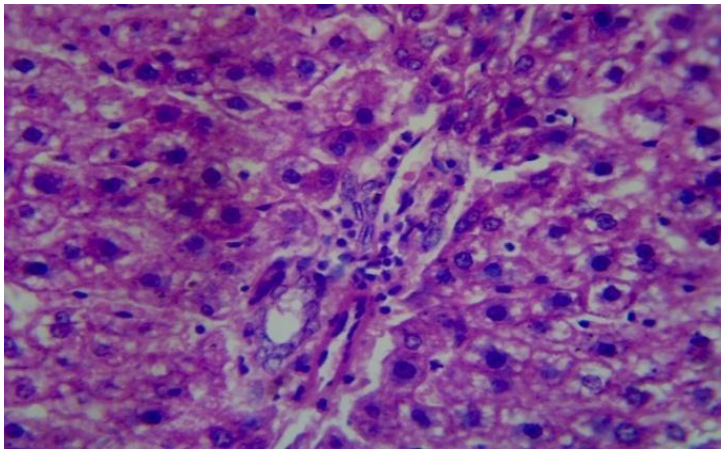
Control-Section studied from the liver showed normal lobular architecture. Individual hepatocytes are normal. The portal traid shows no significant pathology. The sinusoids show mild dilatation.

Figure 4: Section of rat liver treated with negative control – paracetamol



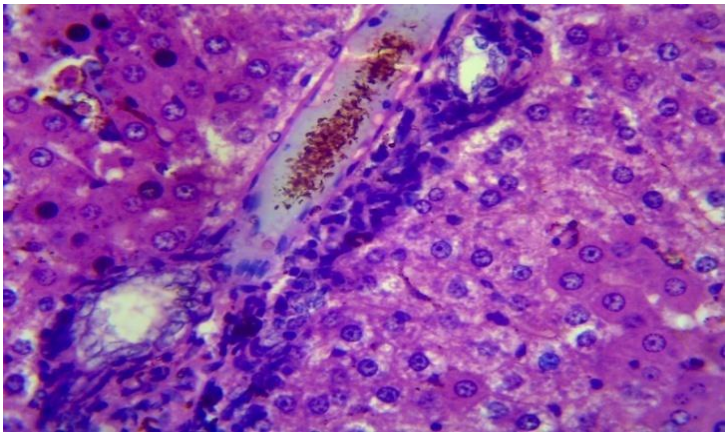
Negative control – Paracetamol - Section studied from the liver showed distorted architecture.

Figure 5: Section of rat liver treated with standard silymarin



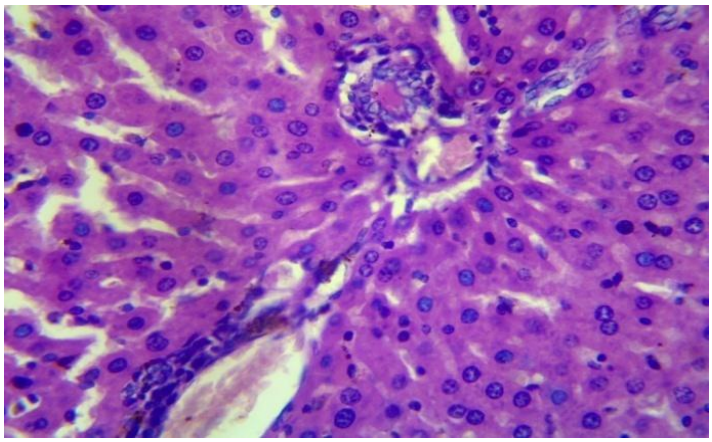
Standard - Silymarin -Section studied from the liver showed maintained lobular architecture. Individual hepatocytes showed cytoplasmic vacuolation. The central vein and sinusoids showed mild dilatation and congestion. The portal traid shows bile duct hyperplasia.

Figure 6: Section of rat liver treated with the low dose of CEAS



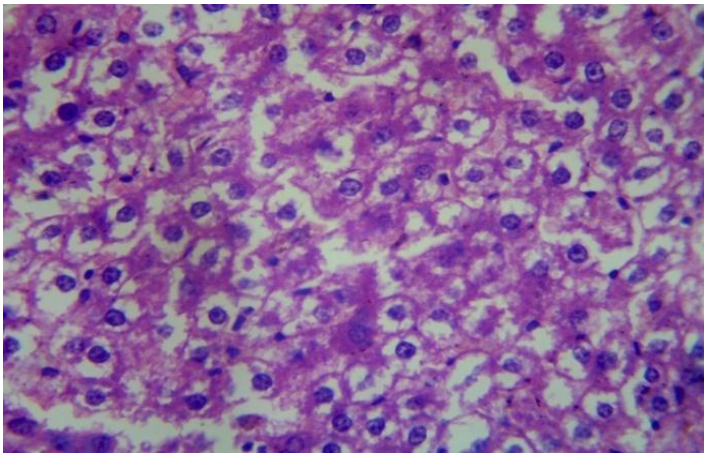
Low dose of CEAS- Section studied from the liver showed maintained lobular architecture.. Sinusoids showed mild dilatation. The portal traid shows bile dilatation. The portal traid showed bile duct hyperplasia.

Figure 7: Section of rat liver treated with high dose of CEAS



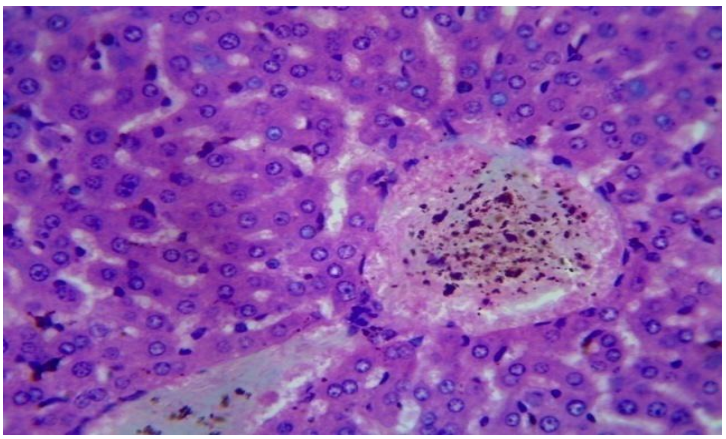
High dose of CEAS - Section studied from the liver showed normal lobular architecture. The central vein showed congestion. Sinusoids showed mild dilatation. The portal traid showed mild periportal inflammation.

Figure 8: Section of rat liver treated with low dose of CEVW



Low dose of CEVW - Section studied from the liver showed lobular architecture panlobular inflammation. The central vein showed congestion. Sinusoids showed mild dilatation. The portal traid showed bile duct hyperplasia with mild periportal inflammation.

Figure 9: Section of rat liver
treated with high dose of CEVW



High dose of CEVW - Section studied from the liver showed normal lobular architecture. Individual hepatocytes showed normal no significant pathology. The sinusoids showed mild dilatation.

D-Galactosamine-induced hepatotoxicity model

Table 14: Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on serum biochemical parameters

GROUP	ALT	AST	ALP	ACP	BILIRUBIN
Control (CMC, 0.5%)	61.606 ± 6.109	176.15 ± 7.60	262.44 ± 9.91	44.15 ± 2.78	0.62 ± 0.092
D-Galactosamine (400 mg/kg, i.p)	81.741 ± 1.413	763.12 ± 9.72	675.89 ± 5.45	68.29 ± 1.10	1.44 ± 0.080
CEAS (250mg/kg)	76.47 ± 1.27	346.21 ± 11.52	4.66.95 ± 0.80	61.68 ± 1.18	1.153 ± 0.018
CEAS (500mg/kg)	72.26 ± 0.988	194.26 ± 11.52	377.78 ± 1.74	58.95 ± 1.51	0.966 ± 0.023
CEVW (200mg/kg)	74.106 ± 1.117	344.87 ± 9.73	450.63 ± 1.51	59.78 ± 1.43	1.051 ± 0.010
CEVW (400mg/kg)	66.89 ± 1.083	193.96 ± 1.45	374.72 ± 1.15	56.66 ± 0.98	0.93 ± 0.018
Silymarin (100 mg/kg., p.o)	68.28 ± 1.107	182.45 ± 6.73	372. 67 ± 1.09	55.98 ± 1.089	0.83 ± 0.052

6.5 Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on serum biochemical parameters

The serum biochemical parameters were significantly ($P<0.001$) increased in the D-Galactosamine treated group when compared to control. The serum enzyme levels and bilirubin was significantly ($P<0.001$) decreased when the animals were pretreated with the CEAS and CEVW at higher doses. In both the plants, the effect produced by the chloroform extract at lower doses (250 and 200 mg/kg) of *Alpinia speciosa* and *Valeriana wallichii* respectively was not significant (Table: 14).

Table 15: Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on tissue protein

GROUP	PROTEIN	MDA	LH
Control (CMC, 0.5%)	7.32 ± 0.04	0.114 ± 0.002	0.276 ± 0.015
D-Galactosamine (400 mg/kg, i.p)	3.25 ± 0.03	0.596 ± 0.001	0.745 ± 0.017
CEAS (250mg/kg)	5.03 ± 0.02	0.490 ± 0.003	0.651 ± 0.013
CEAS (500mg/kg)	5.94 ± 0.05	0.014 ± 0.002	0.596 ± 0.020
CEVW (200mg/kg)	5.28 ± 0.04	0.471 ± 0.001	0.628 ± 0.020
CEVW (400mg/kg)	6.13 ± 0.012	0.153 ± 0.008	0.573 ± 0.017
Silymarin (100 mg/kg., p.o)	1.668 ± 0.034	0.141 ± 0.009	0.510 ± 0.013

6.5.1 Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on tissue protein

In the D-Galactosamine treated group the total protein level was significantly ($P<0.001$) reduced. Pretreatment of animals with chloroform extract of both the plants for 12 days significantly elevated. When the animals were pretreated with the chloroform extract of higher dose of *Alpinia speciosa* (500 mg/kg) and both the doses of *Valeriana wallichii* for 12 days, the protein level was significantly ($P<0.001$) elevated The effect produced by the chloroform extract of lower dose of *Alpinia speciosa* was not significant ($P>0.05$) to that of the D-Galactosamine treated group (Table 15).

Table 16: Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on enzymatic and non-enzymatic antioxidants

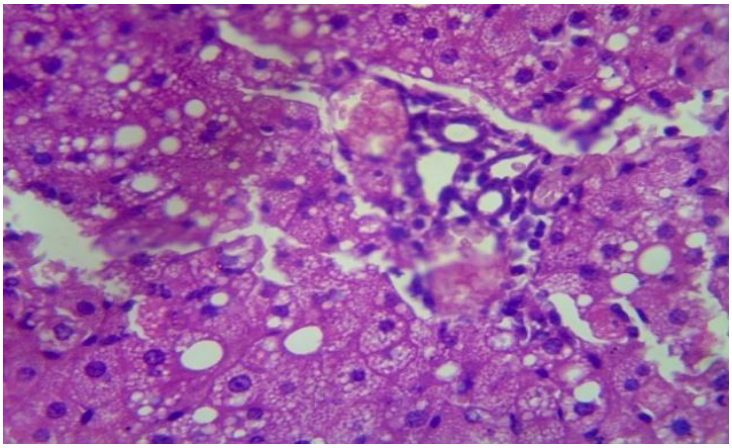
Group	CAT	Px	SOD	GSSH	GPx	GSH
Control (CMC, 0.5%)	2.435 ± 0.016	4.785 ± 0.021	1.645 ± 0.017	2.805 ± 0.017	3.436 ± 0.017	2.95 ± 0.015
D – Galactosamine (400 mg/kg, i.p)	0.803 ± 0.058	2.22 ± 0.059	0.806 ± 0.075	0.663 ± 0.017	1.451 ± 0.013	1.558 ± 0.013
CEAS (250mg/kg)	0.738 ± 0.017	3.271 ± 0.047	0.906 ± 0.015	1.465 ± 0.015	1.55 ± 0.015	1.603 ± 0.013
CEAS (500mg/kg)	1.551 ± 0.011	3.705 ± 0.075	1.083 ± 0.012	1.938 ± 0.009	1.963 ± 0.014	1.968 ± 0.017
CEVW (200mg/kg)	0.73 ± 0.01	3.021 ± 0.099	0.938 ± 0.019	1.641 ± 0.014	1.631 ± 0.016	1.655 ± 0.012
CEVW (400mg/kg)	1.57 ± 0.01	3.898 ± 0.118	1.143 ± 0.019	2.06 ± 0.014	2.068 ± 0.008	2.003 ± 0.017
Silymarin (100 mg/kg., p.o)	1.601 ± 0.023	4.061 ± 0.009	1.215 ± 0.026	2.183 ± 0.012	2.978 ± 0.028	2.118 ± 0.014

6.5.2 **Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on enzymatic and non-enzymatic antioxidants**

The rhizomes of CEAS and roots of CEVW were evaluated for enzymatic (SOD, CAT, GPX, GSSH and the non enzymatic antioxidants GSH). The levels of tissue enzymatic and non-enzymatic antioxidant in D-Galactosamine treated group was found to be significantly reduced. Pretreatment of animals with chloroform extract of both the plants for 12 days significantly ($P<0.001$) increased the enzymatic and non enzymatic antioxidant levels compared to D-Galactosamine control. The activity produced by the extract was similar to that of standard silymarin treated group. The lower doses of CEAS and CEVW respectively was not significant ($P>0.05$) when compared to the D-Galactosamine control (Table 16).

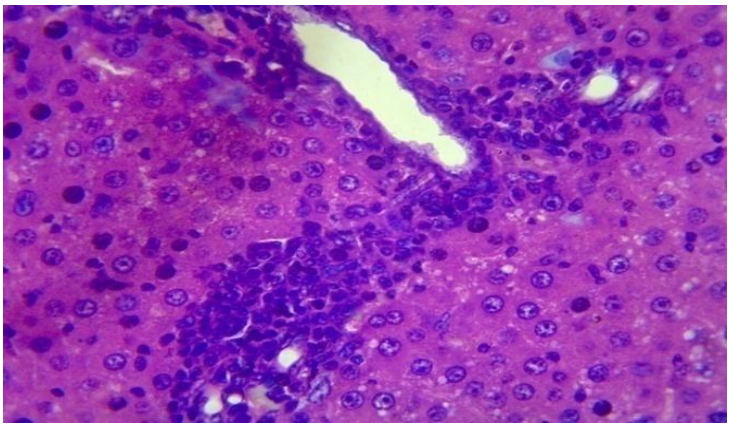
Histopathological report of D-Galactosamine-induced hepatotoxicity in the liver

Figure 10: Section of rat liver treated with D-Galactosamine



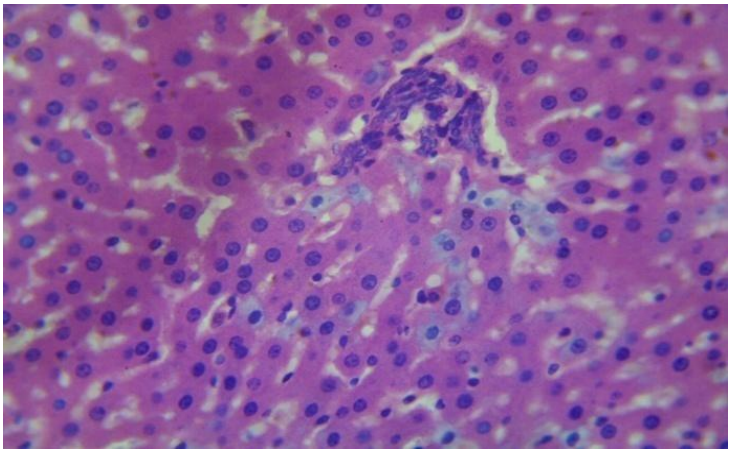
Negative Control – D-Galactosamine - Individual hepatocytes showed both micro and macro vesicular steatosis and the sinusoids are normal.

Figure 11: Section of rat liver treated with standard



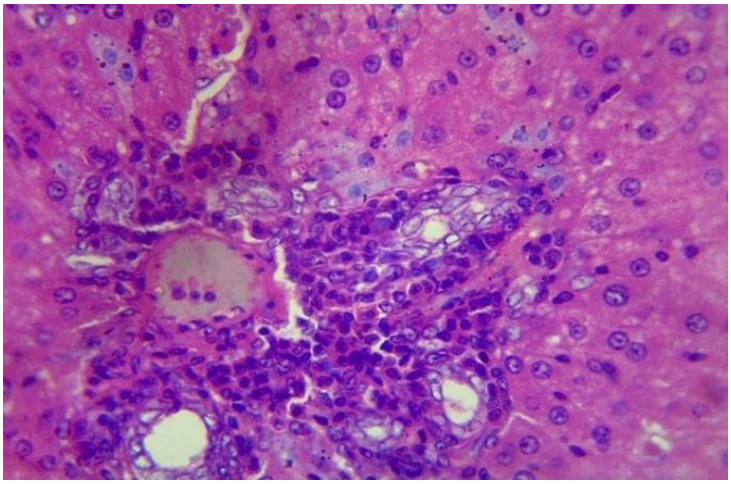
Standard - section studied from the liver showed maintained lobular architecture. The portal tract showed dense periportal inflammation.

Figure 12: Section of rat liver treated with low dose of CEAS



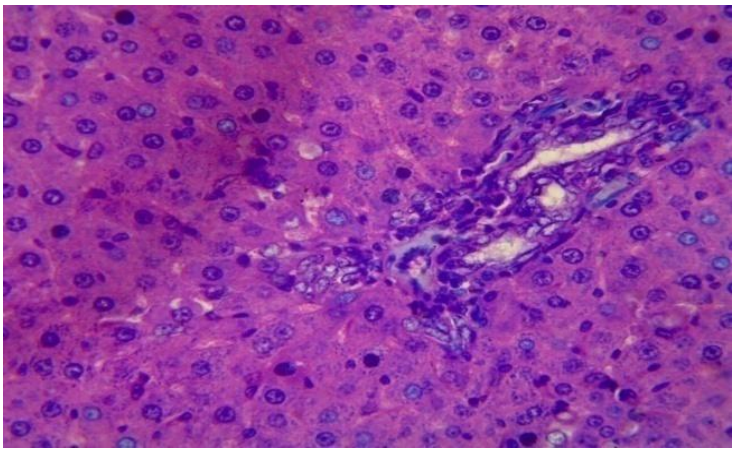
Low dose of CEAS - section studied from the liver showed normal lobular architecture. The central vein and sinusoids showed dilatation and congestion. The portal traid shows mild periportal inflammation.

Figure 13: Section of rat liver treated with high dose of CEAS



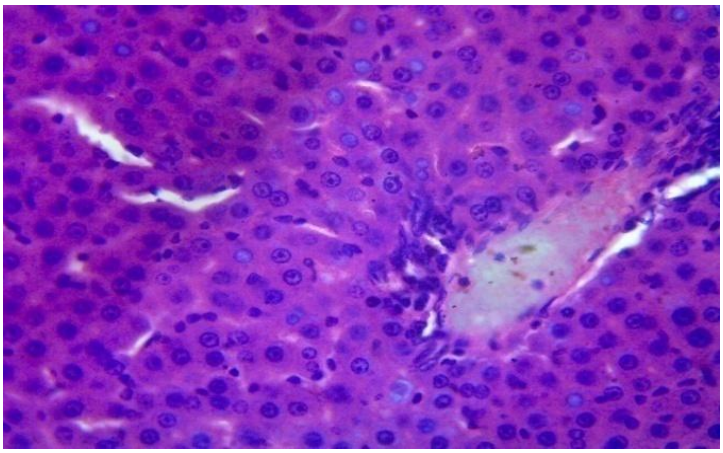
High dose of CEAS - Section studied from the liver showed normal lobular architecture.

Figure 14: Section of rat liver treated with low dose of CEVW



Low dose of CEAS - The central vein and sinusoids showed dilatation and congestion. The portal traid showed periportal inflammation.

Figure 15: Section of rat liver treated with high dose of CEVW



High dose of VW - section studied from the liver showed normal lobular architecture. The central vein showed congestion. The portal traid showed mild periportal inflammation.

Ethanol-induced hepatotoxicity model

Table 17: Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on serum biochemical parameters

Group	ALT	AST	ALP	ACP	BILIRUBIN
Control (CMC, 0.5%)	59.883 ± 1.179	153.645 ± 0.958	244.276 ± 0.836	44.88 ± 0.751	0.321 ± 0.010
Ethanol (5 g/kg of 25%w/v alcohol)	114.28 ± 1.003	220.275 ± 0. 922	278.808 ± 1.164	64.991 ± 0.760	0.725 ± 0.011
CEAS (250mg/kg)	72.78 ± 0.910	184.061 ± 1.126	269.561± 1.068	54.136 ± 0.790	0.606 ± 0.012
CEAS (500mg/kg)	67.28 ± 1.016	167.425 ± 0.934	263.201 ± 0.728	52.905 ± 0.478	0.533 ± 0.018
CEVW (200mg/kg)	70.835 ± 0.882	181.661 ± 1.313	266.655 ± 0.873	51.433 ± 0.863	0.605 ± 0.011
CEVW (400mg/kg)	64.956 ± 0.953	164.641 ± 1.085	261.68 ± 0.627	50.771 ± 0.713	0.513 ± 0.012
Silymarin (100 mg/kg., p.o)	62.47 ± 0.847	163.616 ± 0.892	258.121 ± 0.579	49.261± 1.077	0.491 ± 0.010

6.6 Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on serum biochemical parameters

The serum biochemical parameters were significantly ($P<0.001$) increased in the ethanol treated group when compared to control. The serum enzyme levels (viz. ALT, AST, ACP, ALP and bilirubin) were significantly ($P<0.001$) reduced when the animals were pretreated with the chloroform extract for both the plants for 21 days when compared to ethanol control. There was a decrease in the enzyme and bilirubin levels (Table 17).

Table 18: Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on tissue protein

GROUP	PROTEIN	MDA	LH
Control (CMC, 0.5%)	8.606 ± 0.213	0.241 ± 0.013	0.141 ± 0.001
Ethanol (5 g/kg of 25%w/v alcohol)	5.01 ± 0.036	0.72 ± 0.049	0.395 ± 0.001
CEAS (250mg/kg)	6.916 ± 0.094	0.611 ± 0.026	0.342 ± 0.001
CEAS (500mg/kg)	8.714 ± 0.059	0. 381 ± 0.024	0.224 ± 0.001
CEVW (200mg/kg)	6.818 ± 0.073	0.635 ± 0.024	0.335 ± 0.001
CEVW (400mg/kg)	8.738 ± 0.009	0.308 ± 0.014	0.216 ± 0.001
Silymarin (100 mg/kg., p.o)	9.475 ± 0.018	0.350 ± 0.001	0.203 ± 0.0009

6.6.1 Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on tissue protein

Total protein level was significantly reduced in the ethanol treated group when compared to the control. Pretreatment of animals with CEAS and CEVW for 21 days significantly ($P<0.001$) elevated the protein level when compared to the ethanol control. There was increase in the protein level and the activity produced by the extract (Table 18).

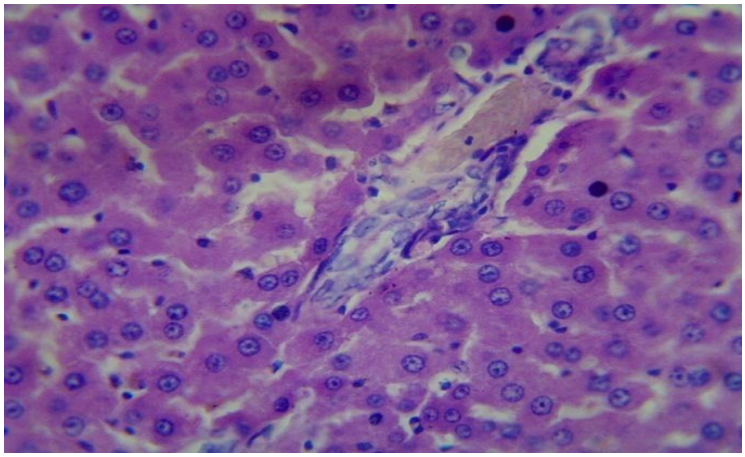
Table 19: Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on enzymatic and non-enzymatic antioxidants

Group	CAT	Px	SOD	GSSH	GPx	GSH
Control (CMC, 0.5%)	119.708 ± 0.851	6.643 ± 0.106	3.318 ± 0.177	1.851 ± 0.009	9.401 ± 0.017	74.138 ± 0.026
Ethanol (5 g/kg of 25%w/v alcohol)	87.27 ± 0.559	1.043 ± 0.008	1.57 ± 0.003	0.041 ± 0.009	5.725 ± 0.016	44.05 ± 0.092
CEAS (250mg/kg)	82.165 ± 1.11	2.75 ± 0.053	1.471 ± 0.010	0.081 ± 0.013	7.233± 0.043	51.086 ± 0.784
CEAS (500mg/kg)	109.516 ± 0.983	8.518 ± 0.106	5.378 ± 0.069	2.068 ± 0.017	10.313 ± 0.025	80.168 ± 0.013
CEVW (200mg/kg)	81.765 ± 0.948	3.228 ± 0.026	1.538 ± 0.031	0.068 ± 0.013	6.39± 0.036	50.54 ± 0.028
CEVW (400mg/kg)	111.841 ± 0.851	8.181 ± 0.015	5.121 ± 0.013	2.121± 0.017	10.788 ± 0.063	81.31 ± 0.222
Silymarin (100 mg/kg., p.o)	114.56 ± 1.107	8.36 ± 0.017	4.693 ± 0.012	2.443 ± 0.020	11.728± 0.089	84.40 ± 1.22

6.6.2 Effect of chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* on enzymatic and non-enzymatic antioxidants

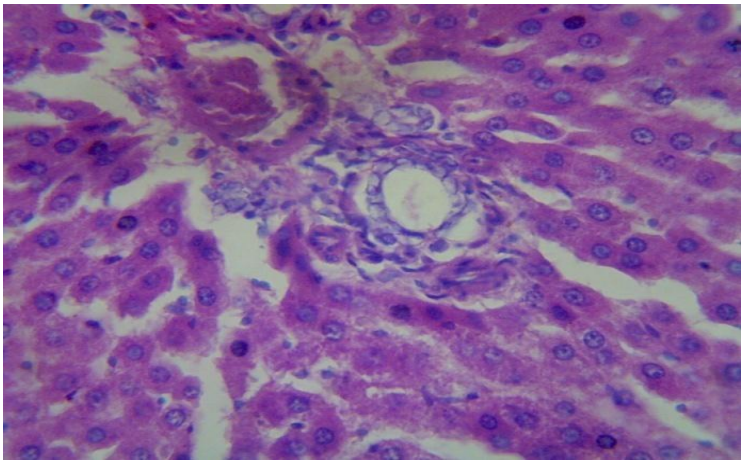
The chloroform extracts of rhizomes of *Alpinia speciosa* and roots of *Valeriana wallichii* were evaluated for enzymatic (SOD, CAT, GPX, GSSH and non enzymatic antioxidant (GSH). The levels of tissue enzymatic and the non enzymatic antioxidants were significantly increased in the groups treated with the chloroform extract of both the plants for 21 days when compared to the ethanol control. The enzymatic and the non enzymatic antioxidants were significantly elevated when the animals were pretreated with the higher doses of CEAS and CEVW respectively. The CEAS and CEVW at lower doses respectively was not significant (Table 19).

Figure 16: Section of rat liver treated with negative control - ethanol



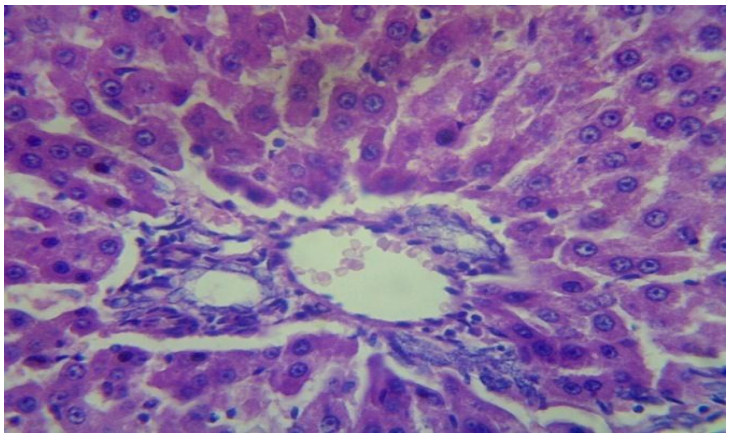
Negative Control - Section studied from the liver showed normal lobular architecture. Individual hepatocytes showed reactive hepatocytes (reversible injury).

Figure 17: Section of rat liver treated with low dose of CEAS



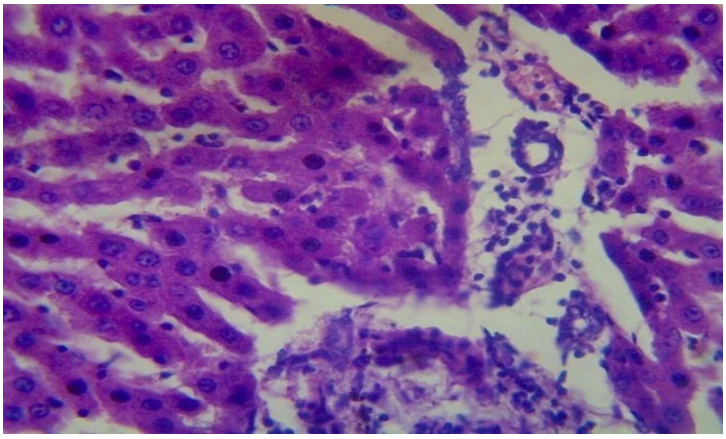
Low dose of CEAS - Section studied from the liver showed mild altered architecture with pan lobular inflammation. The central vein and sinusoids showed mild dilatation and congestion. The portal traid showed bile duct hyperplasia.

Figure 18: Section of rat liver treated with high dose of CEAS



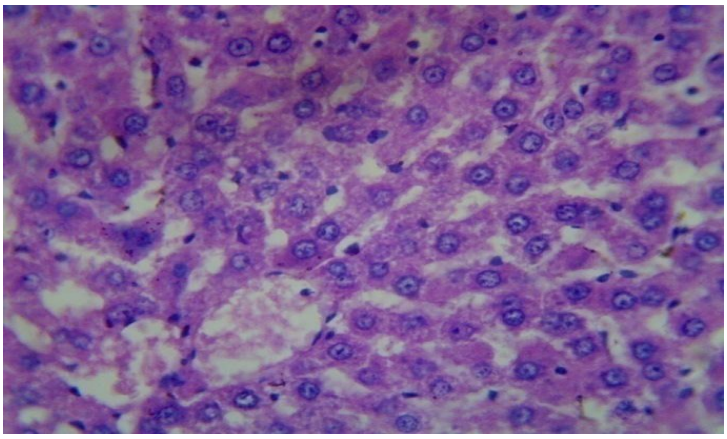
High dose of CEAS - Section studied from the liver showed normal lobular architecture. The central vein showed congestion. Sinusoids showed mild dilatation. The portal traid showed mild periportal inflammation.

Figure 19: Section of rat liver treated with low dose of CEVW



Low dose of CEVW - Section studied from the liver showed lobular architecture panlobular inflammation. The central vein showed congestion. Sinusoids showed mild dilatation. The portal traid showed bile duct hyperplasia with mild periportal inflammation.

Figure 20: Section of rat liver treated with high dose of CEVW



High dose of CEVW - Section studied from the liver showed normal lobular architecture. Individual hepatocytes showed normal no significant pathology. The sinusoids showed mild dilatation.

6.7 Isolation of possible active constituents

Chloroform extracts of *Alpinia speciosa* rhizomes and *Valeriana wallichii* roots were subjected to preliminary thin layer chromatographic studies followed by column chromatography for isolation of the possible active constituents.

TLC of various extracts of *Alpinia speciosa* and *Valeriana wallichii* – UV – 254nm

Figure 21: TLC of various extracts of *Alpinia speciosa* and *Valeriana wallichii* using hexane: ethyl acetate solvent system (9:1)

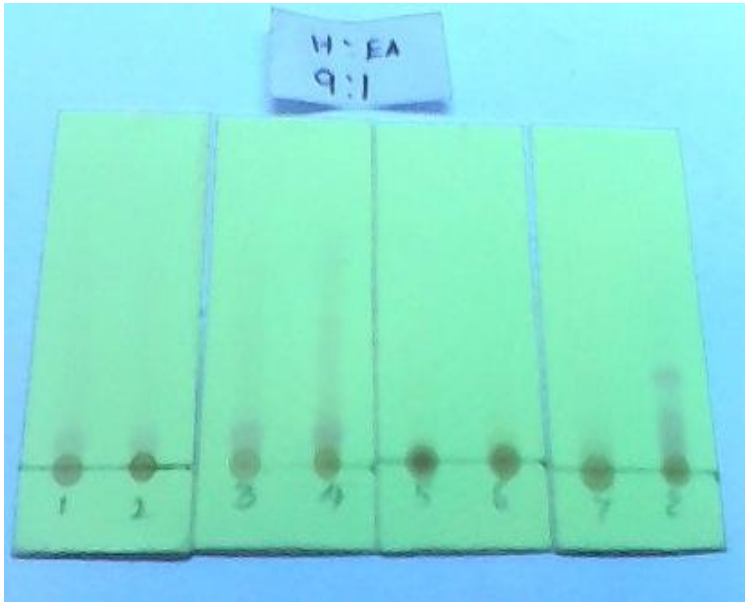


Figure 22: TLC of various extracts of *Alpinia speciosa* and *Valeriana wallichii* using hexane: ethyl acetate solvent system (1:1)

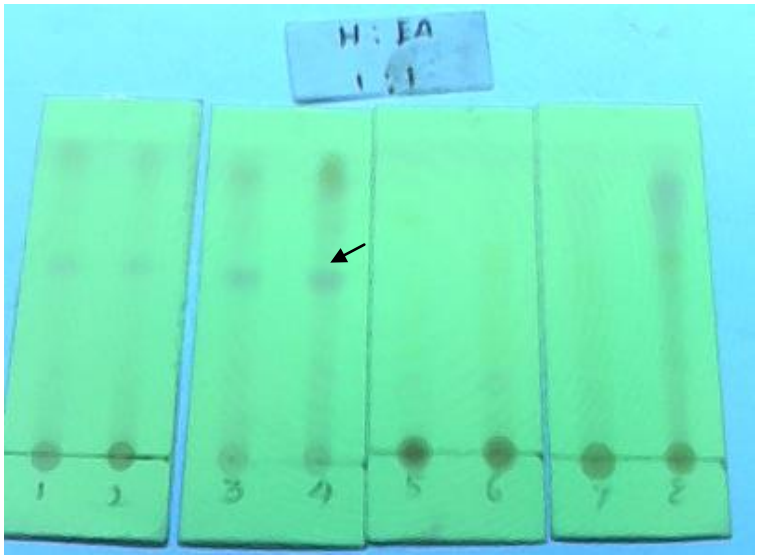


Figure 23: TLC of various extracts of *Alpinia speciosa* and *Valeriana wallichii* using chloroform: methanol solvent system (9:1)

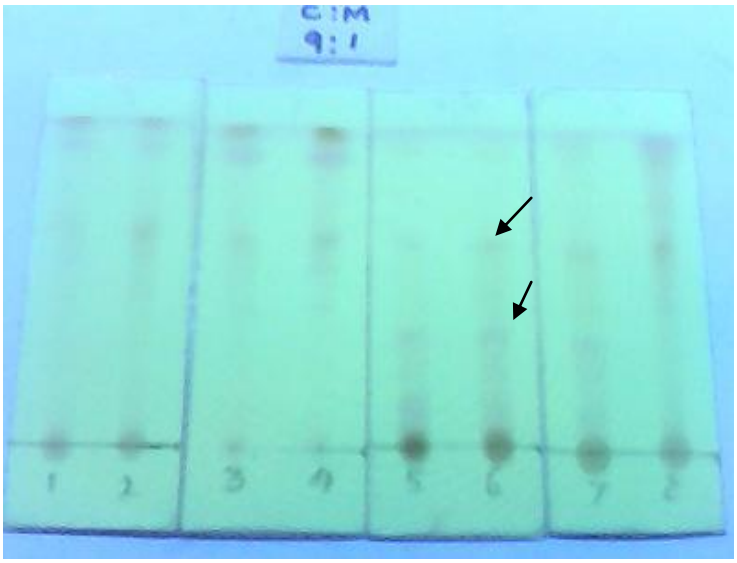


Figure 24: TLC of various extracts of *Alpinia speciosa* and *Valeriana wallichii* using chloroform: methanol solvent system (8:2)

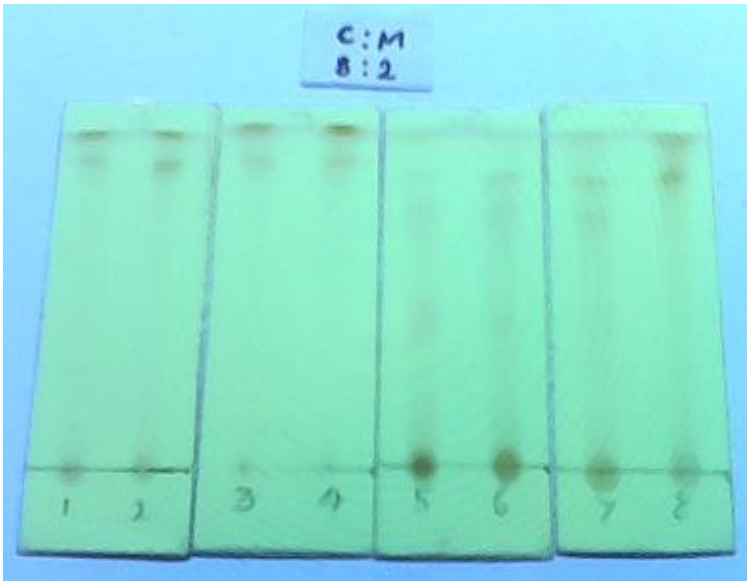


Figure 25: TLC of *Alpinia speciosa* stained with KMnO4 using hexane: ethyl acetate solvent system (1:1)

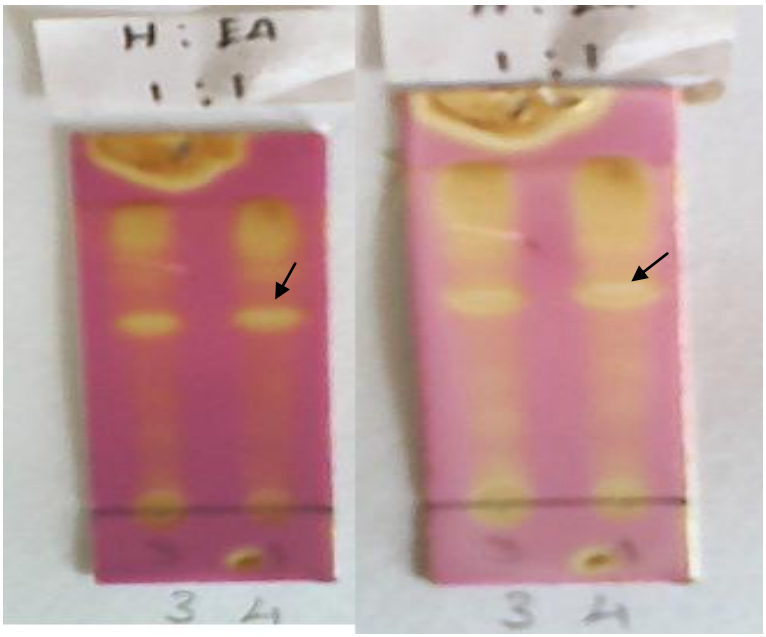


Figure 26: TLC of various extracts of *Valeriana wallichii* stained with KMnO4and visualised under UV-254 nm using hexane: ethyl acetate solvent system (1:1)

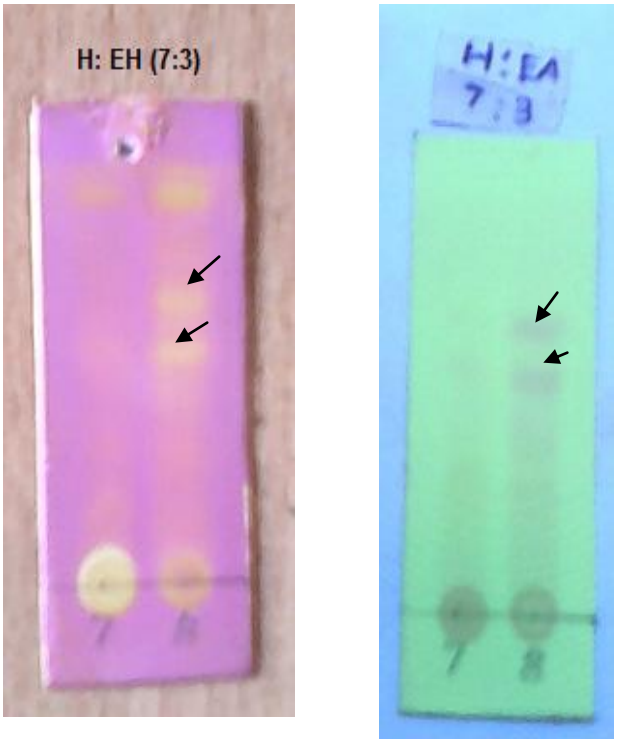


Figure 27: TLC of *Alpinia speciosa* and *Valeriana wallichii* stained with ferric chloride reagent using chloroform: methanol solvent system (8:2)

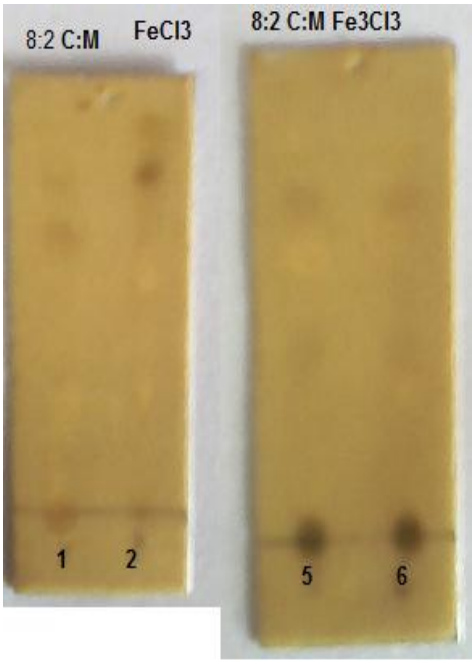


Figure 28: TLC of *Alpinia speciosa* and *Valeriana wallichii* stained with Dragendroff reagent using hexane: ethyl acetate solvent system (1:1)

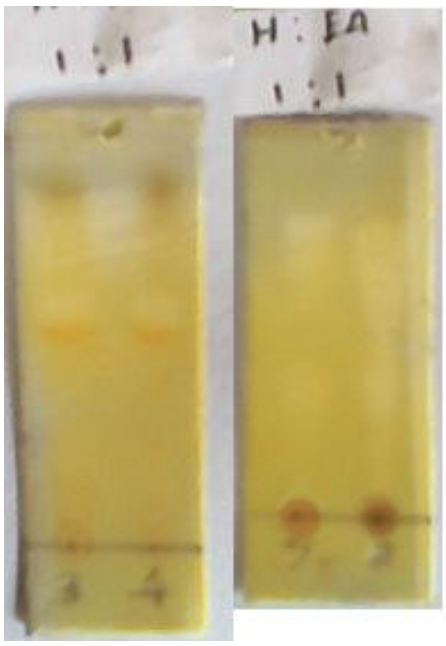


Figure 29: TLC of *Alpinia speciosa* and *Valeriana wallichii* stained with ferric chloride using chloroform: methanol solvent system (9:1)

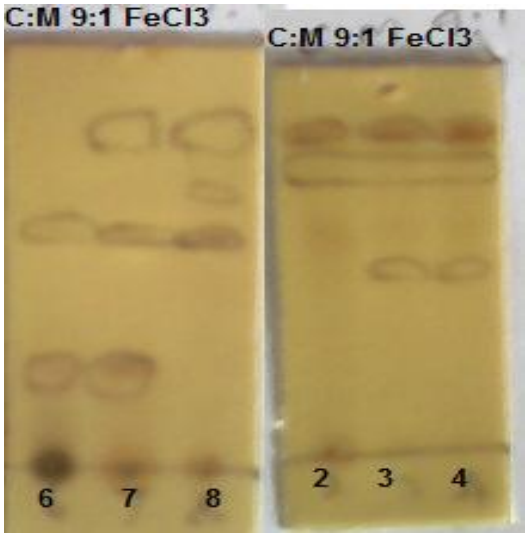
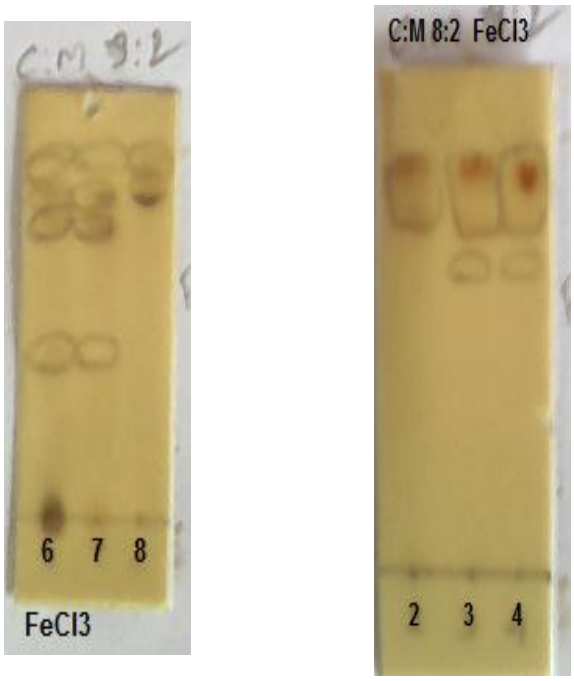


Figure 30: TLC of *Alpinia speciosa* and *Valeriana wallichii* stained with ferric chloride using chloroform: methanol solvent system (9:1)



In the present study the TLC system for analysis was shown to be flavonoids, alkaloids and sterols with the largest discriminating power. The above mentioned figures represents 1,2,3,4-ethanolic, ethyl acetate, chloroform and pet. ether extract of *Alpinia speciosa* and 5,6,7,8- ethanolic, ethyl acetate, chloroform and pet. ether extract of *Valeriana wallichii.*

TLC plates shown in the fluorescence light under UV at 254 - 365 nm wavelength and find the active spots in TLC plate with following Rf values (0.42, 0.68, 0.76) these values indicates the presence of steroids, alkaloids and flavonids glycosides.

Isolation of active constituents of *Alpinia speciosa*

Chloroform fraction was subjected to gradient elution with hexane: ethyl acetate (100:0, 80:20, 70:30, 60:40, 50:50, 30:70; 0:100; each 200 ml) as solvent system to give the 28 (F1-F45). The Two compounds from *Alpinia speciosa* and one compound from *Valeriana wallichii* were isolated and identified by FT-IR, mass spectroscopy, ^{1}H-NMR analysis.

Figure 31: Column chromatography of *Alpinia speciosa*

Figure 32: Fractions collected from column chromatography of *Alpinia speciosa*

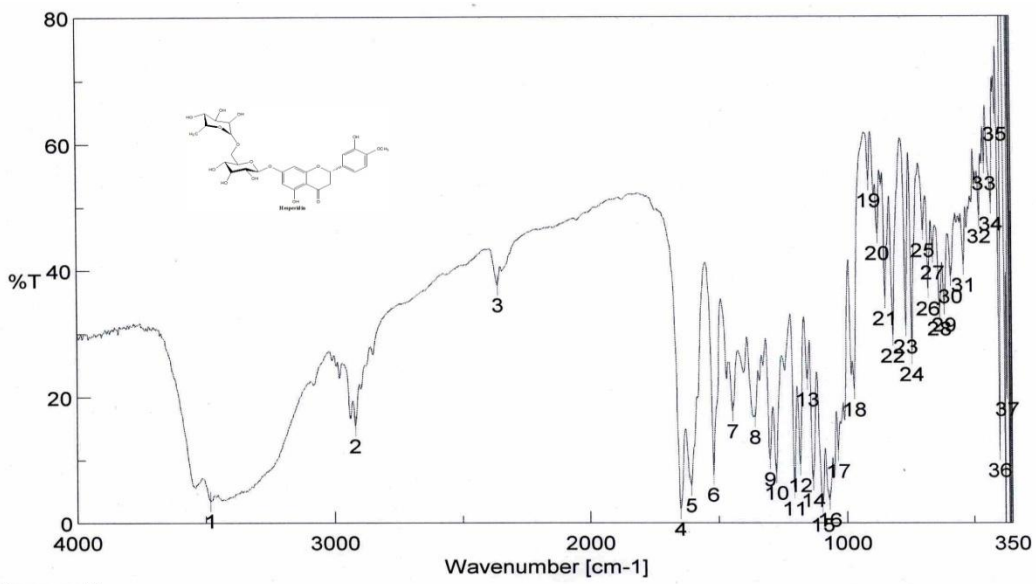
Isolation of active constituents of *Valeriana wallichii*

The chloroform extract was dissolved in methanol and adsorbed in silica gel 60 – 120. After evaporation of the solvent it was loaded into silica gel column (100 – 200), prepared in hexane. The column was eluted with hexane followed by slowing increasing polarity with hexane: ethyl acetate (90:10; 80:20; 70:30; 50:50; 60:40; 20:80) and finally with 100% ethyl acetate. The column further eluted with chloroform: methanol (98:2; 94:2; 92:8; 90:10; 85:15; 80:20). Fractions were monitored under TLC and similar fractions were observed in fractions number 18-24 and 32-46. The resulting crude materials were purified by using activated charcoal in hot chloroform and the fractions are kept for crystallization. The obtained solid was submitted for FT-IR, mass and ^{1}H-NMR spectroscopy.

Figure 33: Column chromatography of *Valeriana wallichii*

Figure 34: Fractions collected from column chromatography of *Valeriana wallichii*

Figure 35: IR of Hesperidin



[Comment]

Sample Name	AL-1
Comment	CONSULTANCY
User	GOPINATHAN & ANANDBABU
Division	CONSULTANCY
Company	SRMC

[Data Information]

Creation Date	01-Jan-02 1:31 AM
Data array type	Linear data array
Horizontal	Wavenumber [cm-1]
Vertical	%T
Start	349.053 cm-1
End	4000.6 cm-1
Data pitch	0.964233 cm-1
Data points	3788

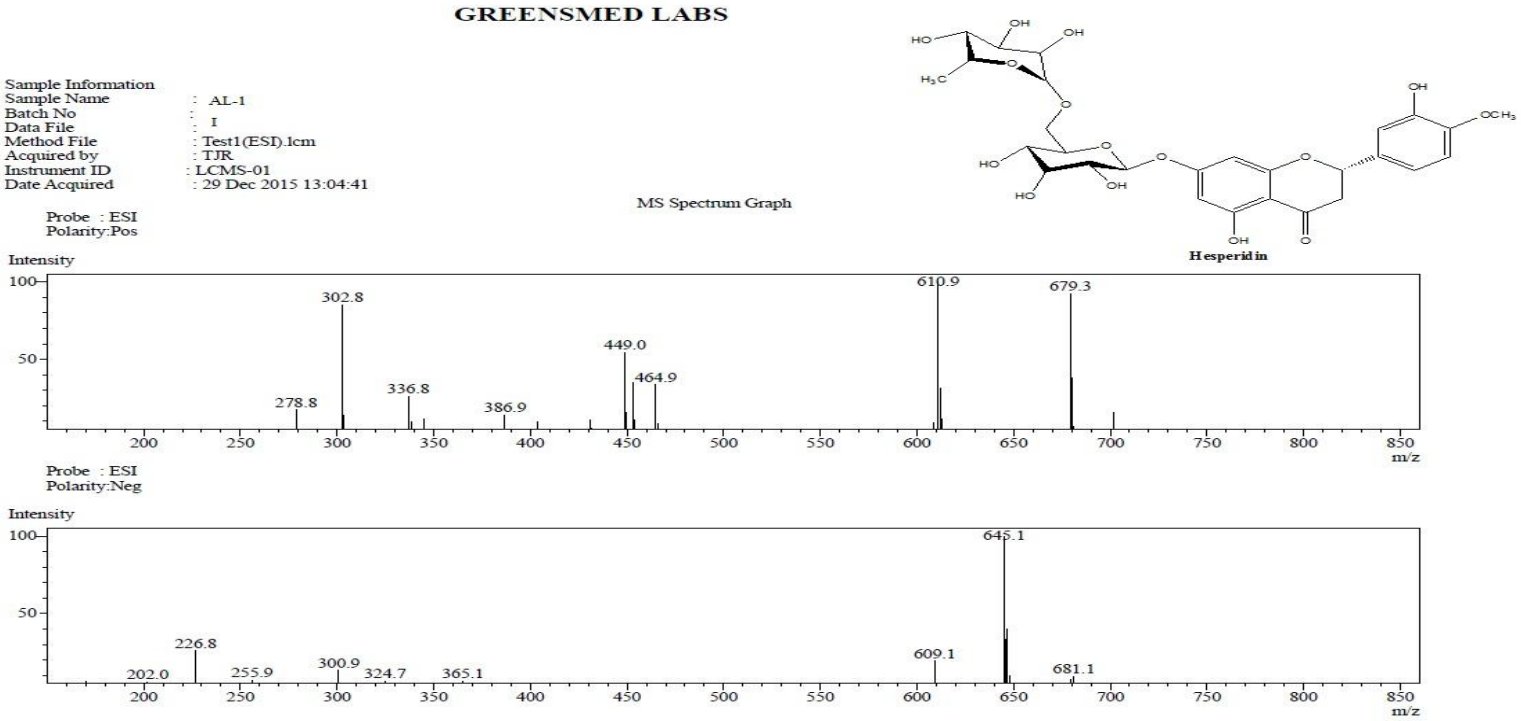
[Measurement Information]

Model Name	FT/IR-4100typeA
Serial Number	B101061016
Light Source	Standard
Detector	TGS
Accumulation	250
Resolution	4 cm-1
Zero Filling	On
Apodization	Cosine
Gain	Auto (16)
Aperture	Auto (7.1 mm)
Scanning Speed	Auto (2 mm/sec)
Filter	Auto (30000 Hz)

[Result of Peak Picking]

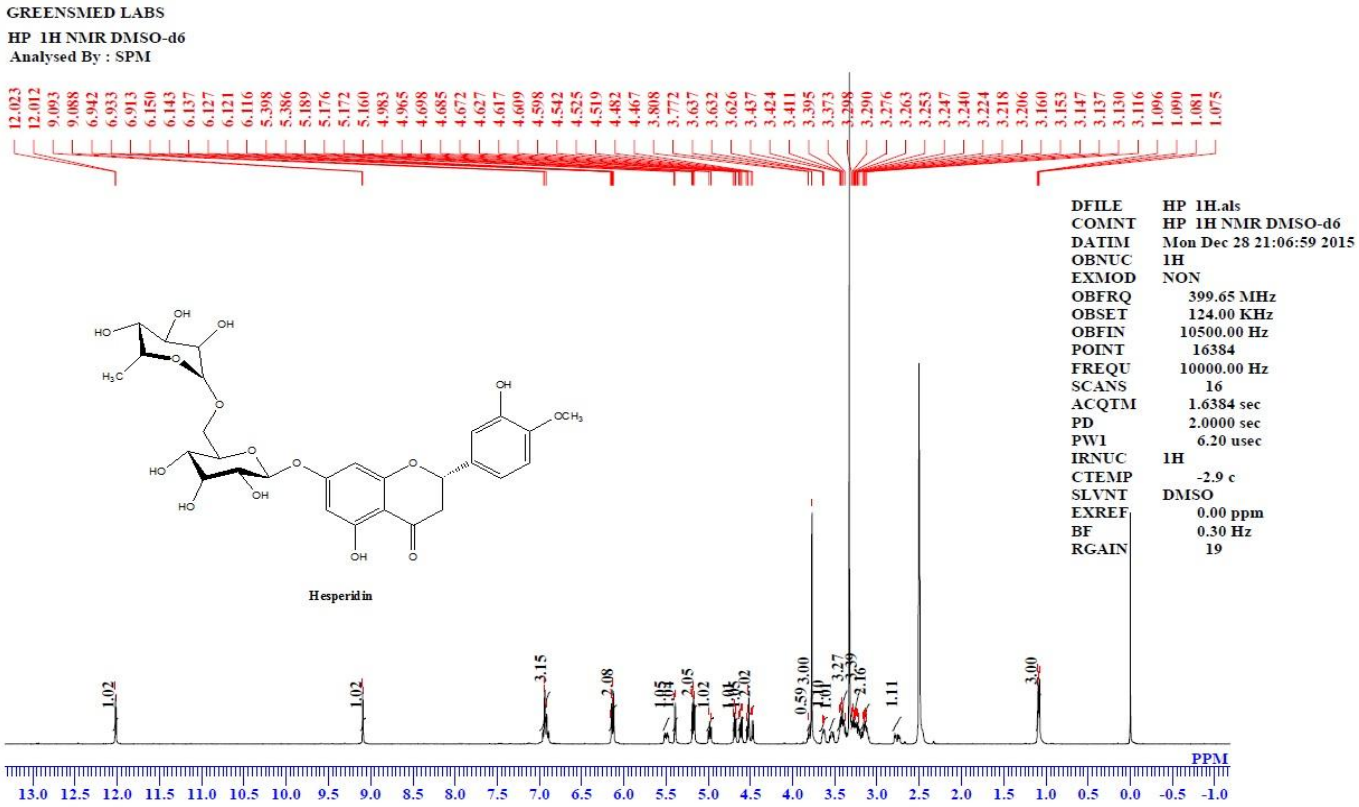
No.	Position	Intensity	No.	Position	Intensity
1	3482.81	3.45113	2	2918.73	15.3611
3	2359.48	37.6127	4	1648.84	2.16872
5	1606.41	5.95168	6	1519.63	7.65475

Figure 36: MS of Hesperidin



EI-MS m/z: 610.9

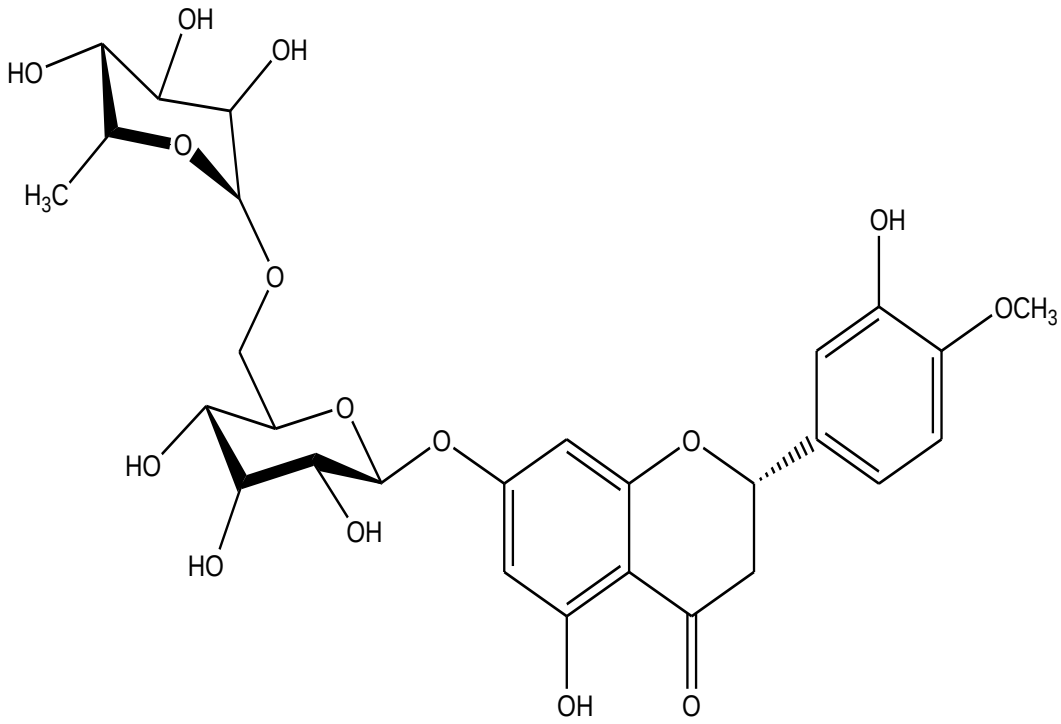
Figure 37: NMR of Hesperidin



^{1}H-NMR (DMSO-*d6*): δ ppm: 1.07-1.09 (3H,m, H-6’’’); 2.73-2.79 (1H, m, H-3a); 3.09-3.18 (2H, m, H-3b); 3.24-3.29 (3H,m, H -2, H-3); 3.35-3.43 (3H, m, H-4); 3.51 -3.53 (1H, d, H-5); 3.62- 3.64 (1H, m, H-6); 3.77 (3H, S, -OCH_3); 4.46-4.48 (2H, d, H-1); 4.59-4.62(1H, m, H-1’’’); 4.67-4.69(1H, m, -OH glucose); 4.96 -5.00 (1H, t, -OH glucose); 5.16 -5.18 (2H, m, -OH glucose); 5.38 -5.39 (1H, d, -OH glucose); 5.47-5.52 (1H, m, H-2); 6.11-6.15 (2H, m, H-6, H-8); 6.89 – 6.96 (3H, m, H-5’, H-6’ & H-2’); 9.08 -9.09 (1H, d, 3-OH); 12.01- 12.02 (1H, d, 5-OH).

Figure 38: Structure of Hesperidin

This pattern of the spectrum was identical with that of compound Hesperidin The structure of **compound-1** as shown below.

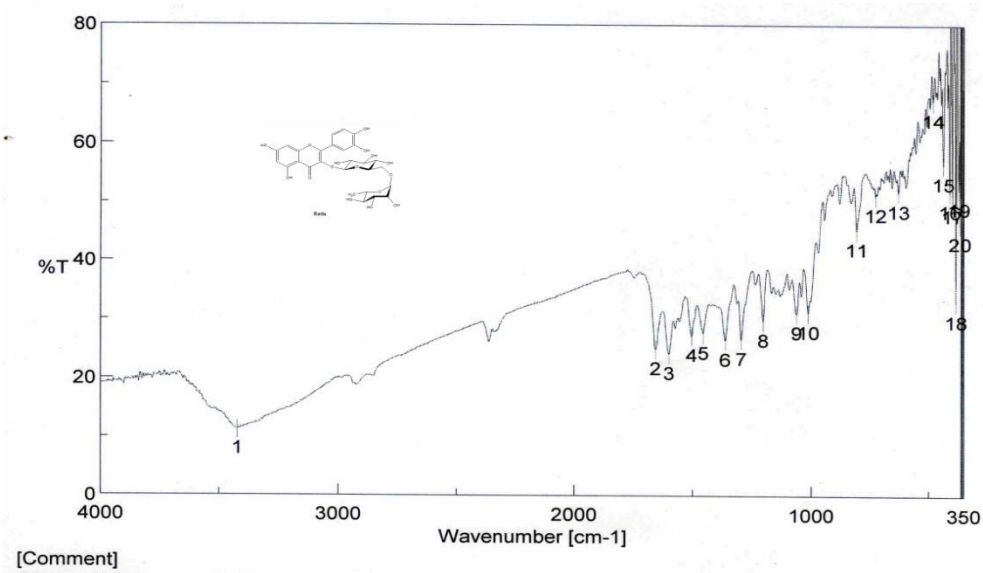


Hesperidin

Figure 39: FT-IR of Rutin

Compound-2:

Pale yellow colour powder, m.p 245 - 246 °C; $C_{27}H_{30}O_{16}$



[Comment]	
Sample Name	AL-2
Comment	CONSULTANCY
User	ANANDBABU & GOPINATHAN
Division	CONSULTANCY
Company	SRMC

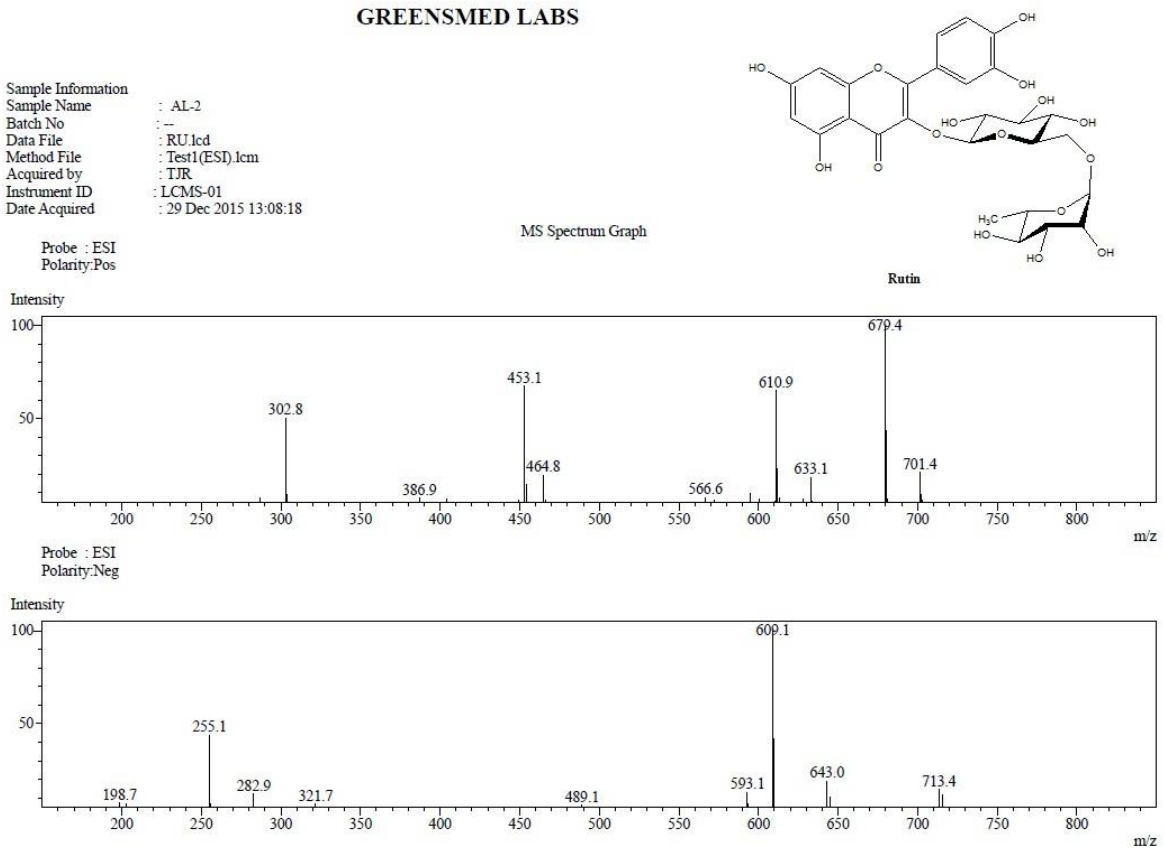
[Data Information]		[Measurement Information]	
Creation Date	01-Jan-02 1:07 AM	Model Name	FT/IR-4100typeA
Data array type	Linear data array	Serial Number	B101061016
Horizontal	Wavenumber [cm-1]		
Vertical	%T	Light Source	Standard
Start	349.053 cm-1	Detector	TGS
End	4000.6 cm-1	Accumulation	250
Data pitch	0.964233 cm-1	Resolution	4 cm-1
Data points	3788	Zero Filling	On
		Apodization	Cosine
		Gain	Auto (16)
		Aperture	Auto (7.1 mm)
		Scanning Speed	Auto (2 mm/sec)
		Filter	Auto (30000 Hz)

[Result of Peak Picking]

No.	Position	Intensity	No.	Position	Intensity
1	3423.99	11.1914	2	1655.59	24.882
3	1599.66	24.0857	4	1504.2	27.1479
5	1455.99	27.5286	6	1362.46	26.4122

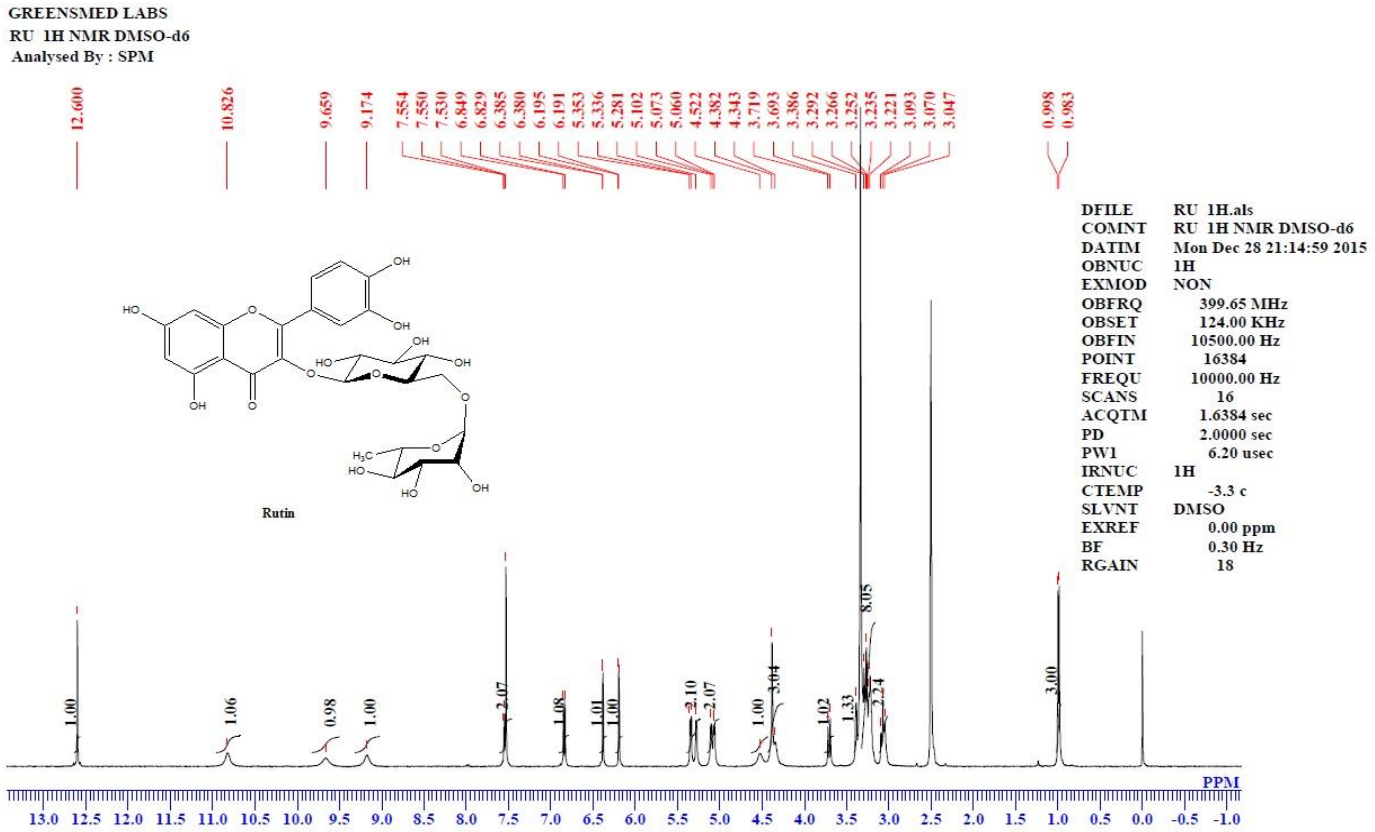
FT-IR (KBr cm-1): 3423 cm^{-1} (-OH stretch); 2934 cm^{-1} & 2912 cm^{-1} (-CH stretch); 1655 cm^{-1} (-C=O); 1504 cm^{-1} (-C=C); 1455 cm^{-1} (-CH bend); 1362 cm^{-1} (C-OH bend).

Figure 40: Mass Spectra of Rutin



EI-MS m/z: 610.9

Figure 41: NMR of Rutin

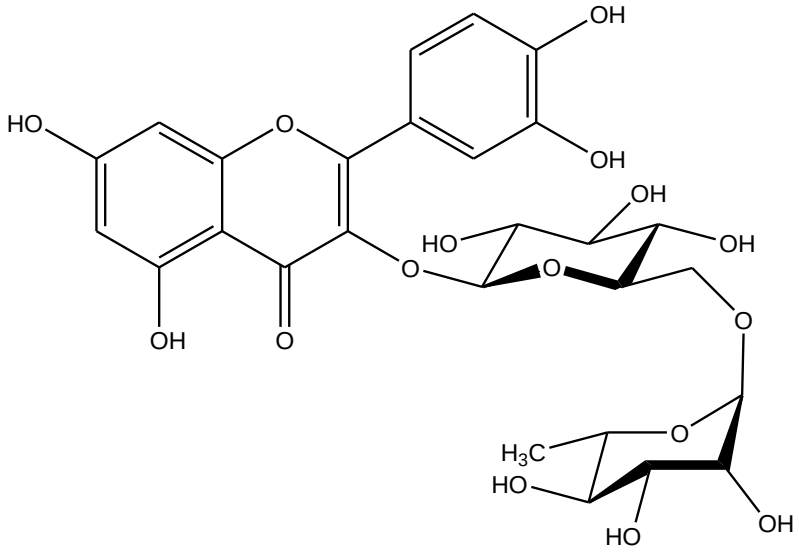


^{1}H-NMR (DMSO-*d6*): δ ppm: 0.98-0.99 (3H,m, H-6´´); 3.04-3.09 (2H, t, H-3´´, H-5´´); 3.22-3.29 (6H**,** m, H -2´´, H-3´´, H -4´´, H-5´´, H-6´´, H-4´´); 3.38 (1H, m, H-2´´); 3.69- 3.71 (1H, d, H-6´´); 4.34 (1H, s, H-1´´); 4.38 (1H, S, 2´´-OH); 4.52 (1H, S, 4´´-OH); 5.06-5.10 (2H, m, 2´´´- 3´´´-OH); 5.28 (1H, d, 4´´´-OH); 5.33 -5.35 (1H, d, H-1´´); 6.19 (1H, d, H-6); 6.38 (1H, d, H-8); 6.82 – 6.84 (1H, d, H-5´); 7.53 – 7.55 (2H, d, H-6´ & H-2´); 9.17 (1H, S, 4´-OH); 9.65 (1H, S, 3´-OH); 10.82 (1H, S, 7-OH); 12.60 (1H, S, 5-OH).

Figure 42: Structure of Rutin

This pattern of the spectrum was identical with that of compound **Rutin**

The structure of **compound-2** as shown

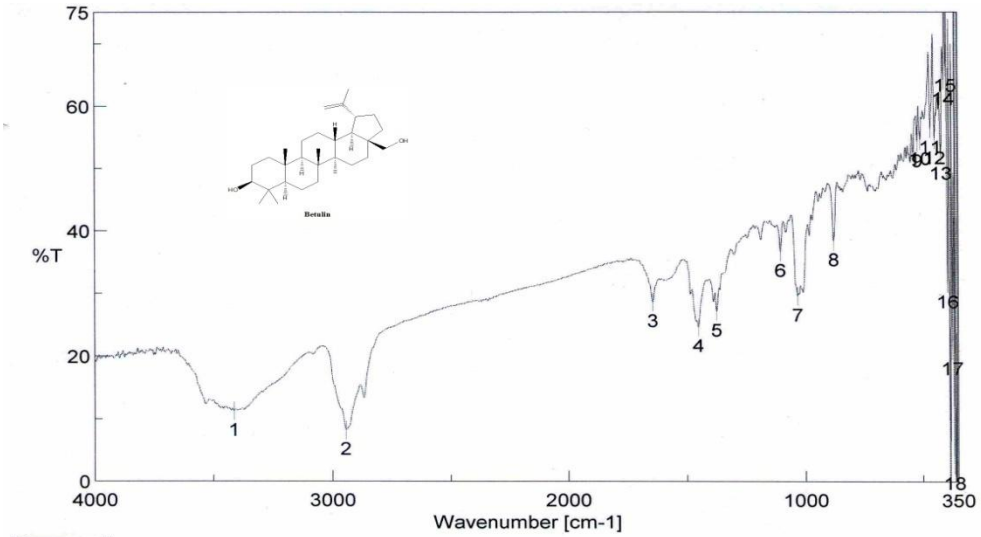


Rutin

Figure 43: FT-IR of betulin

Compound-1:

Colour less powder, m.p. 248 - 250 °C; $C_{30}H_{50}O_2$



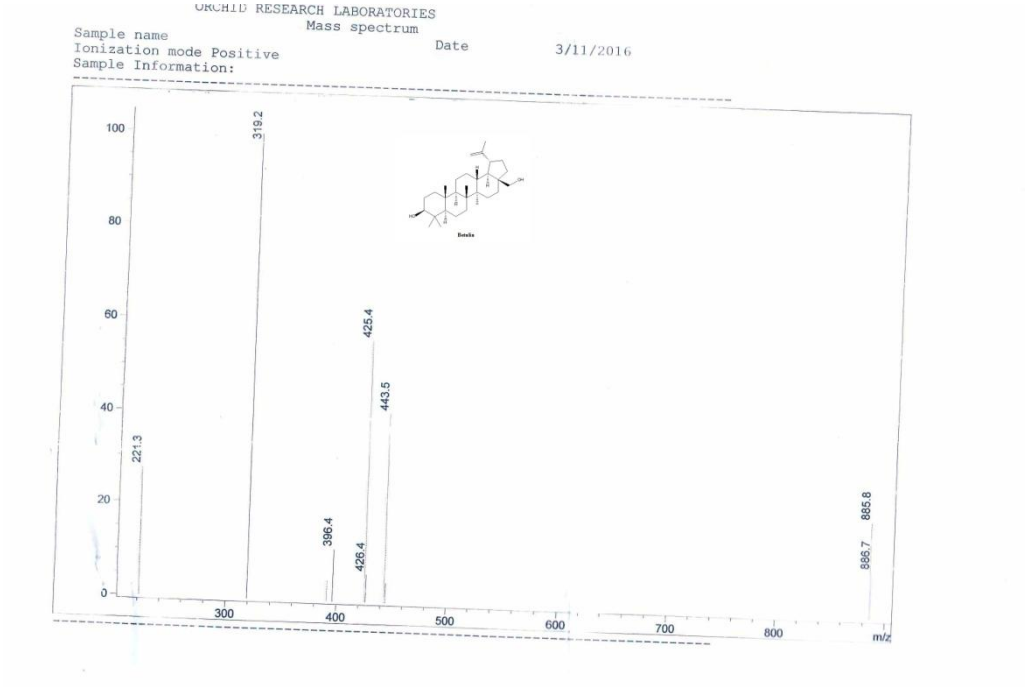
[Comment]	
Sample Name	VA-1
Comment	consultancy
User	Gopinathan & Anandbabu
Division	consultancy
Company	SRMC

[Data Information]		[Measurement Information]	
Creation Date	01-Jan-02 12:25 AM	Model Name	FT/IR-4100typeA
Data array type	Linear data array	Serial Number	B101061016
Horizontal	Wavenumber [cm-1]		
Vertical	%T	Light Source	Standard
Start	349.053 cm-1	Detector	TGS
End	4000.6 cm-1	Accumulation	250
Data pitch	0.964233 cm-1	Resolution	4 cm-1
Data points	3788	Zero Filling	On
		Apodization	Cosine
		Gain	Auto (16)
		Aperture	Auto (7.1 mm)
		Scanning Speed	Auto (2 mm/sec)
		Filter	Auto (30000 Hz)

[Result of Peak Picking]

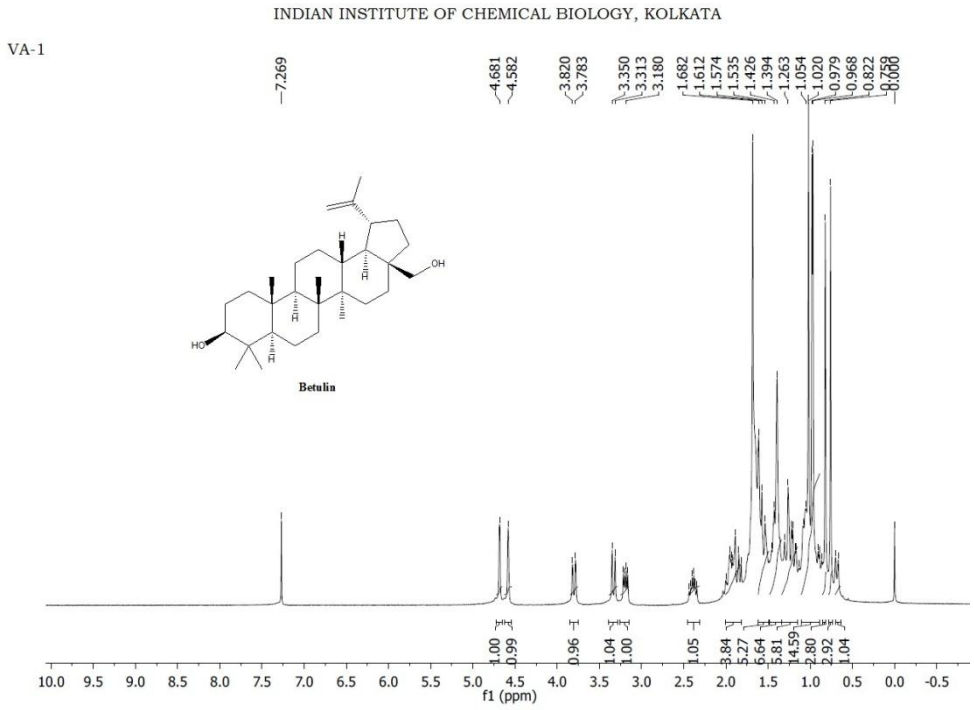
No.	Position	Intensity	No.	Position	Intensity
1	3414.35	11.2145	2	2943.8	8.20052
3	1644.02	28.6009	4	1452.14	24.6387
5	1375	27.0892	6	1105.98	36.6929

Figure 44: Mass spectra of Betulin



EI-MS m/z: 443.1

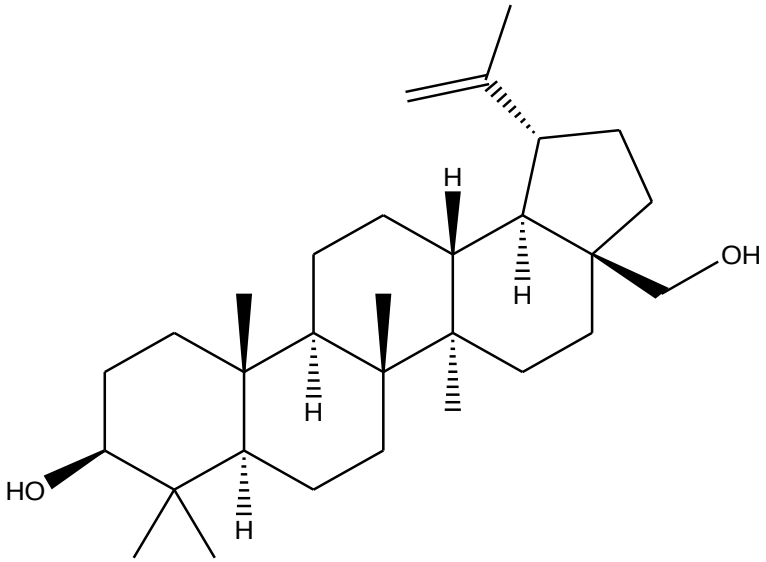
Figure 45: NMR of Betulin



^{1}H-NMR ($CDCl_3$): δ ppm: 0.66-0.70 (1H, m,19); 0.75 (3H, m, H-24); 0.82 (3H, m, H-25); 0.89 - 1.08 (14H, m,H-5,H-6,H-8,H-9) ; 1.16 -1.30 (6H, m,H-13, H-29); 1.39- 1.45 (7H, m,H-26); 1.53 -1.61 (5H, m, H-30); 1.81-1.20 (4H, m, H-18); 2.38-2.43 (1H, m,H-15); 3.22-3.24 (1H, m, 16a); 3.34-3.38 (1H, m, H-28a); 3.78-3.82 (1H, m, 28b); 4.58 (1H, d, H-29a); 4.68 (1H, d,29b).

Figure 46: Structure of Betulin

This pattern of the spectrum was identical with that of compound **Betulin** The structure of **compound-1** as shown below.



Betulin

Table 20: Comparison of hesperidin with the standard

Parameters	Reported as	Standard
Powder	Pale yellow powder	Pale yellow powder
m.p	252 - 254° C	252 - 256° C
Rf	0.54 – 0.80	-
Mol. formula	-	$C_{28}H_{34}O_{15}$
EI-MS	-	610.90
H-NMR	DMSO – D6	DMSO – D6
IR – spectrum, KBr disk	3425, 1295	3482
C=O stretching	Present	at 1648
Aromatic alcohol stretching -OH	Present	-
C=C stretching	Present	at 1606
Aliphatic -CH	-	C=H at 2918, 2359

Table 21: Comparison of rutin with the standard

Parameters	Reported as	Standard
Powder	Pale greenish white powder	Pale yellow powder
m.p	242 - 243° C	245 - 246° C
Mol. formula	-	$C_{27}H_{3}O_{16}$
EI-MS	611	610.90
H-NMR (Proton & carbon NMR)	30 protons & 27 carbons	DMSO – D6
FT-IR – spectrum, KBr disk	-	3423 cm-1
OH stretching	3423	at 1648
CH	2920 & 2912	2912
C=O	1662	at 1655
δ ppm	-	3423

Table 22: Comparison of betulin with the standard

Parameters	Obtained as	Standard
Color	Yellowish white	Colorless powder
State	solid	solid
Solubility	Soluble in chloroform and ethyl acetate	-
m.p	242-246° C	248-250° C
Rf value	0.4705	-
EI-MS	443.43	443.1
^{1}H-NMR, δ value	1.0-1.1	0.66 – 0.70
FT-IR	C-H 2943 C=C1644 CH bending -1375	C-H 2943 C=C 1644 OH – 3414 CH bending -1375

DISCUSSION

7.1 Phytochemical screening of plant extracts

A number of terpenoids exhibit cytotoxicity against a variety of tumour cells. One such classification is the monoterpenoids. Terpenoids behave as potential chemo preventive and therapeutic agents in liver cancer[149].

In our study, the preliminary phytochemical screening of active constituents was carried out for various extracts of *Alpinia speciosa* and *Valeriana wallichii.* In petroleum ether extract of *Valeriana wallichii* flavonoids and terpenoids were present. In chloroform extract flavonoids, terpenoids, tannins and phenolics were revealed. Terpenoids, tannins and phenolics were present in ethyl acetate extract. In ethanol extract, presence of flavonoids, carbohydrate, terpenoids, tannins and phenolics were revealed.

There are three methods to study *in vitro* hepatotoxicity of plant extracts. They are liver slice culture, microsomes and isolated hepatocytes. Of these, *in vitro* liver slice culture model provides constructive approaches for the plant extracts for their hepatoprotective activity and elucidation of possible mechanism of action. To examine the experimental analysis of hepatotoxic events, this is the most suitable model[63].

7.2 In vitro hepatoprotective activity

The advantages of *in vitro* liver slice model are lobular structures are preserved, selective intralobular effects are detected, studies on human liver are possible and also studies on several compounds at different concentrations are possible. Limitations of liver slice model are viability is between 6 hr to 2 days, no bile collection is possible, and not all the cells are similarly preserved[150].

The extent of damage caused to the cell is proportional to the amount of enzyme released[63]. The measurement of release of cytosolic enzyme, LDH

into the medium provides the analysis of hepatotoxic events. Thus *in vitro* model offers many advantages than the *in vivo* model. In the present study *Alpinia speciosa* and *Valeriana wallichii* at a dose of 10µl for each plant were used for the evaluation of *in vitro* hepatoprotective activity.

Among the various extracts, the chloroform extract showed a reduction in the release of LDH than the other extracts. Therefore, chloroform extract is selected for further *in vivo* hepatoprotective activity.

Based on the above literatures, the present study was carried out to evaluate the hepatoprotective activity of two doses (lower and higher) of chloroform extract of rhizomes of *Alpinia speciosa* and roots of *Valeriana wallichii* in rats.

7.3 Paracetamol-induced hepatotoxicity model

In the experimental animals, the hepatic injury is caused by overdosing of drug which is called drug induced hepatotoxicity. Paracetamol at higher doses causes severe hepatotoxicity, in both humans and experimental animals. So paracetamol induced hepatotoxicity was selected as one of the experimental model of liver damage.

Paracetamol (acetaminophen), the active metabolite of phenacetin belongs to a class of a-ine analgesics[73]. It was discovered at the end of 19th century in Germany[152]. It is a commonly used analgesic and antipyretic drug[153]. Paracetamol is the classic experimental model to study the therapeutic agents (plants and drugs) having hepatoprotective activity[154,155]. The most widely used model for liver injury in rats for the experimental intoxication is induced by paracetamol (640 mg/kg body weight)[157].

At therapeutic doses is metabolized in the liver, principally through conjugation with sulphate and glucuronide and then excreted by the kidneys.

Only a small portion (5-10%) is oxidized by hepatic cytochrome P_{450} enzyme system (specifically CYP_2E_1 and CYP_1A_2) to generate highly reactive and cytotoxic intermediate N- acetyl - ρ - benzoquinoneimine (NAPQI) which is quickly conjugated to a harmless water-soluble product, mercapturic acid[158,159]. Paracetamol toxicity in hepatocytes initiates a sequence of events leading to cell death. Toxic doses of paracetamol will deplete the levels of hepatic glutathione (GSH) followed by covalent binding of NAPQI to hepatic parenchymal cell proteins and DNA with resultant liver injury[160]. Evidence suggests that lipid peroxidation resulting from oxidative stress, generation of reactive oxygen and nitrogen species, mitochondrial dysfunction and disruption of calcium homeostasis are the mechanisms that may contribute to the initiation and progression of paracetamol-induced hepatotoxicity[158]. Among the causes of hepatic failure, globally paracetamol is one of the foremost causes of hepatic diseases.

Alaline transaminase (ALT) is present in hepatic and biliary cells.. It is routinely assessed to monitor the functional status of the liver[2]. It has been known that the serum enzyme levels such as ALT, AST, ALP, ACP and bilirubin are elevated[101]. To assess the liver damage caused by hepatotoxicants determination of serum enzymes levels are widely employed.

AST is found in hepatocytes of mitochondria that are released from heart, liver, skeletal muscle and kidney. Cell necrosis of the liver releases AST levels in serum producing severe viral hepatitis and acute cholestasis and this level can be measured in the serum.

ALP is a marker enzyme for the plasma membrane and cytoplasmic membrane. It is elevated which may be due to hepatic parencymal or disease of bile ducts such as primary biliary cirrhosis or primary sclerosing cholangitis or by hepatic parenchymal or duct cells[76].

Bilirubin which is a major breakdown product of hemoglobin rises when there is liver injury or damage[114]. Silymarin chiefly contains flavonoids, including silybin, silybinin, silydianin and silychristin and is used for liver diseases of varying origin. Silymarin is being used in the current study as a standard drug for the comparison of rhizomes of *Alpinia speciosa* and roots of *Valeriana wallichii.*

In paracetamol induced hepatotoxicity, there is a marked elevation in serum hepatic enzymes in the control and untreated group[17]. This is same with Parmar *et al*., 2013 which suggested that paracetamol causes liver damage in rats and increased the serum levels when compared to the standard silymarin which has a remarkable reduction of serum levels towards paracetamol induced hepatotoxicity.

Oral administration of both the doses of CEAS, CEVW and silymarin showed an inhibition in the elevated serum enzyme levels when compared to paracetamol. Increased level of ALP was observed after paracetamol administration and it indicates the disturbed excretory function of the liver. It was brought to normal by the higher doses of CEAS and CEVW. Oral administration of the CEAS, CEVW and silymarin decreased the elevated levels of total bilirubin in serum than the paracetamol alone treated rats in a dose dependent manner indicating the effectiveness of these extracts in normal functional status of the liver.

. The formation of altered proteins decreases the normal protein production in liver and is an indication of the liver cell injury[155]. Serum total protein and bilirubin levels reveal the functional status of the hepatic cells[163].

Pretreatment with CEVW (200 and 400 mg/kg) increased the level of serum total protein mostly by the action by hepatic cell regeneration than the paracetamol alone treated rats. Low dose of CEAS (200 mg/kg) didnot show a

significant difference with the hepatoprotective effects with that of the standard drug silymarin.

It is a complex process mediated through free radical mechanism and is implicated in many pathological conditions[67]. Under normal conditions, low concentrations of lipid peroxides are seen in cells. However, there is an increase in its concentration in pathological conditions.

Studies have demonstrated that superoxide anion and its dismutation product, hydrogen peroxide, have been generated during the formation of NAPQI by cytochrome P_{450}, leading to increased superoxide toxicity.

Catalase (CAT) is heme containing tetrameric enzyme. It is mainly localized in the mitochondria and respiratory organelles of most mammalian cells. It is acting as a catalyst in the conversion of hydrogen peroxide and molecular oxygen. The main function of catalase is to reduce the formation of hydroxyl radicals from hydrogen peroxide via Fenton reaction [80,111, 155]

It was observed that treatment with both the extracts and silymarin caused an increase in hepatic SOD and CAT activities which were decreased in paracetamol control. The effect produced by the CEAS at a dose of 250 mg/kg on the catalase showed a non significant difference when compared to paracetamol control.

7.4 D-Galactosamine-induced hepatotoxicity model

D-GalN is an amino sugar, is found in acetylated form in certain structural polysaccharides[83]. D-GalN is a well established experimental hepatotoxicant. It is widely used model with a single administration resulting in dose dependent hepatic damage with focal necrosis and periportal in lammation both in its morphologic and functional characteristics[83,87,88,103]. Therefore it is considered as a very useful inducing agent for evaluation of hepatoprotection. D-GalN induces a decrease in liver uracil nucleotides which rapidly inhibits

both mRNA and protein synthesis. These changes induce cellular damage of the hepatocytes and subsequent development of acute hepatitis with disseminated hepatocellular necrosis and infiltration of poly morphonuclear leukocytes (PMNL). The important mechanism to explain the mode of development of D-GalN hepatitis has also been documented. In recent years, the primary cause in D-GalN-induced liver damage is may be due to the ROS[169]. Evidences support in the fact that the release of ROS and cytokines such as TNF-α and Interleukin-1 by Kupffer cells (KC) in the liver may also contribute towards the hepatocyte damage in D-GalN hepatotoxicity[82].

Pre-treatment with CEAS and CEVW of higher doses attenuated the increased serum levels of hepatic enzymes. Silymarin as reported by Swarnalatha *et al* exhibited the effect in decreasing elevated levels of serum transaminases and alkaline phosphatase in D-Galactosamine induced damage. The extract-mediated suppression of the increased bilirubin level suggested the possibility of the higher doses (500mg/kg and 400mg/kg) of CEAS and CEVW being able to stabilize biliary dysfunction. It was found that the protein level of the lower dose of the CEAS exhibited a non significant difference when compared to D-GalN treated control.

The levels of MDA and LH were increased and reduced GSH levels in the animals treated with toxicant D-GalN in our study. This indicated elevated lipid peroxidation since it is an indicator of lipid peroxidation and was found to be significantly increased in liver homogenates of D-GalN treated rats as reported.

The antioxidant enzyme levels are reported to be decreased in liver of rats treated with hepatotoxicant D-GalN. Thereafter the standard hepatoprotective drug silymarin and the chloroform extracts of the higher doses of CEAS and CEVW increased the levels of the antioxidant enzymes. The decrease in SOD, CAT by treatment with hepatotoxicant D-GalN and

restoration to normal value with protein isolated from leaves of herb *Cajanus indicus* were reported. It was also found that the chloroform extract of lower doses of CEAS and CEVW (250 and 200 mg/kg) respectively showed a non significant difference when compared to D-GalN treated group.

7.5 Ethanol-induced hepatotoxicity model

Treatment with higher doses of CEAS and CEVW markedly elevated the total protein level which was compared with standard drug silymarin. There was a dose dependent increase in the protein level. However, on administration of CEAS and CEVW (250 and 500 mg/kg b.w), protective effect was demonstrated by attenuating GSH depletion from liver tissue.

Pretreatment with CEAS and CEVW increases the activities of catalase to protect the liver from ethanol induced toxication by preventing the accumulation of excessive free radicals.

The increase in free radical scavenging mechanism is the result of increase in level of GPx. The chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* at lower doses (250 and 200 mg/kg) respectively showed a non significant difference when compared with the ethanol treated group.

Inhibition of CAT suggests the enhanced synthesis of superoxide ions during ethanol ingestion since superoxide can be a powerful inhibitor of CAT.

Isolation of possible active constituents

In our study, the most suitable TLC system for isolation of active constituents was shown to be hexane: ethyl acetate and chloroform: methanol with the increasing polarity. These solvent systems were used in column chromatography to isolate the active constituents from the above mentioned plant extracts.

In our study, the most suitable TLC system for isolation of active constituents was shown to be hexane: ethyl acetate and chloroform: methanol with the increasing polarity. These solvent systems were used in column chromatography to isolated the active constituents from the above mentioned plant extracts.

The ^{1}H-NMR spectrum of the compound displayed a signal at δ 12.01- 12.02 (1H, d, 5-OH). Indicated the presence of a chelated hydroxyl group. Further, a signal observed at 9.08 -9.09 (1H, d, 3-OH) was due to a phenolic hydroxyl group. The singlet was observed in the range of 3.77 (3H, S, -OCH_3); assigned to the methoxy groups. The 1H NMR also demonstrated three protons multiplets at δ 6.89 – 6.96 (3H, m, H-5', H-6' & H-2'). The appearance of multiplets 4.69(1H, m, -OH glucose); 4.96 -5.00 (1H, t, -OH glucose); 5.16 -5.18 (2H, m, -OH glucose); 5.38 -5.39 (1H, d, -OH glucose) showed the presence of glucose moiety in the structure. The mass spectrum of the compound showed important mass peaks at m/z [M+H], 610.9. This pattern of the spectrum was identical with that of compound Hesperidin.

The spectral data also supported the presence of glucose and rhamnose moieties with the anomeric protons at δH 4.38 and glucose signal at δH 5.33-5.35. A doublet signals corresponding to methyl group of rhamnose was appeared at δH 0.98-0.99 (3H, H-6´´´). The other protons present in the in the sugar moiety showed between 3.22 and 3.71 ppm. In addition to this comparison of ESI-mass spectra of the isolated compound with that of rutin revealed in the literature. Hence this pattern of the spectrum was identical with that of compound Rutin.

^{1}H NMR spectrum of compound-1 showed olefinic protons at δ 4.68 and δ4.58 and oxygenated methane at 3.22-3.24. The rest of the aliphatic protons of CH, CH_2 and CH_3 groups were appeared in the region of 1.39-1.45 (7H, m, H-26); 1.53 -1.61 (5H, m, H-30); 1.81-1.20 (4H, m, H-18). This pattern of the spectrum was identical with that of compound Betulin.

Hesperidin and Rutin were the compounds that were isolated from the chloroform extract of *Alpinia speciosa* (CEAS) and betulin was isolated from the chloroform extract of *Valeriana wallichii*, (CEVW) and were identified by FT-IR, mass and NMR spectroscopy.

SUMMARY AND CONCLUSION

A number of hepatotoxins such as viruses, bacteria, chemicals, medicines and alcohol target the liver and cause liver injury. These antioxidants are rich in natural sources of drugs, especially plants.. In this aspect, plants that were chosen for the study are the rhizomes of *Alpinia speciosa* and roots of *Valeriana wallichii.* In the indigenous system of medicine *Alpinia speciosa* is used as an ornamental plant. Valeriana wallichii roots have numerous medicinal uses. Based on the facts of the two plants, the current study was undertaken.

The study investigated hepatoprotective activities of rhizomes of *Alpinia speciosa* and roots of *Valeriana wallichii*. Phytochemical screening of various plant extracts of both the plants was carried out. The chloroform extracts of both the plants had showed good activity among all the extracts. Acute toxicity studies were carried out and the animals were found to be safe *In vivo* hepatoprotective activity of chloroform extracts of both the plants at two different doses (lower dose and higher doses) was determined. CEAS and CEVW possessed significant ($P<0.001$) hepatoprotective activity. Further attempts were made to isolate and identify the possible active principles from the chloroform extracts of *Alpinia speciosa* and *Valeriana wallichii*.

The presence of active constituents (flavonoids, tannins, triterpenoids and phenolics) in these plants might be responsible for the hepatoprotective activity. Therefore, CEAS and CEVW proposed to protect the liver against the paracetamol, D-Galactosamine and ethanol induced oxidative damage The *in vivo* hepatoprotective properties of these plants can consequently propose a liver protection to the population whoever consumes it and prevent liver injuries.

The hepatoprotective activities of the plant extracts may be due to the presence of isolated compounds, Hesperidin and rutin from *Alpinia speciosa* and betulin from *Valeriana wallichii* and were identified by infra red spectroscopy, mass spectroscopy and nuclear magnetic resonanace spectroscopy. These isolated compounds had acted as an antioxidant by scavenging the free radicals that had produced by the induction of hepatotoxins (paracetamol, D-Galactosamine and ethanol) and proved as an effective hepatoprotective agent. Thus the isolated compounds from the respective plants will be key leads for producing novel bioactive constituents and may possess more significance in the treatment of hepatic diseases.

IMPACT OF THE STUDY AND RECOMMENDATION

The rhizomes of *Alpinia speciosa* and roots of *Valeriana wallichii* were selected for the study against various hepatotoxins induced liver damage. The hepatotoxins used for the study were paracetamol, D-Galactosamine and ethanol. The improvement of liver histology in the chloroform extract of *Alpinia speciosa* and *Valeriana wallichii* at higher doses remarkably exhibited the hepatoprotective effect of both the plant extracts by decreased serum levels of these enzymes. The presence of active constituents (flavonoids, tannins, triterpenoids and phenolics) in these plants might be responsible for the hepatoprotective activity. The *in vivo* hepatoprotective properties of these plants also propose a liver protection to the population whoever consumes it and prevent liver injuries. The hepatoprotective activities of the plant extracts may be due to the presence of isolated compounds, Hesperidin and rutin from *Alpinia speciosa* and betulin from *Valeriana wallichii.* The isolated compounds from the respective plants will be key leads for producing novel bioactive constituents and may possess more significance in the treatment of hepatic diseases.

RECOMMENDATION

The isolated compounds, Hesperidin and rutin from the rhizomes of *Alpinia speciosa* and betulin from the roots of *Valeriana wallichii* were proved for its hepatoprotective property to cure hepatic damage. It can be recommended that these three compounds from the respective plants will be the biomolecules for producing novel active constituents and may possess profound implication in the treatment of various hepatic diseases caused by hepatotoxins.

1. Swati S. *In vitro* antioxidant activity and hepatoprotective effects of the plant *Ocimum sanctum* Linn. Against paracetamol induced hepatotoxicity. Int J Res P*hytochem* Pharmacol. 2011;1(4):214-218.

2. Mahmood ND, Mamat SS, Kamisan FH, Yahya F *et al.,* Amelioration of paracetamol-induced hepatotoxicity in rat by the administration of methanol extract of *Muntingia calabura* L. leaves. BioMed Res Int 2014, Article ID 695678 pages http://dx.doi.org/10.1155/2014/695678.

3. Ahsan AR, Islam KM, Musaddik A and Haque E. Hepatoprotective activity of methanol extract of some medicinal plants against carbon tetrachloride induced hepatotoxicity in albino rats. Global J Pharmacol. 2009;3(3):116-122.

4. Anusha M, Venkateswarlu M, Prabhakaran V, Taj VSS, Kumara BP. Hepatoprotective activity of aqueous extract of *Portulaca olercea* in combination with lycopoene in rats. Ind J Pharmacol. 2011;43(5):563-567.

5. Sumathi AS, Vijayakumar S. Antioxidant and hepatoprotective activity of methanolic extract of *Hygrophila schulli* rats. Int Res J Pharm. 2012;3(7):148-151.

6. Padmanabhan P and Jangle SN. Hepatoprotective activity of herbal preparation (HP- 4) against alcohol induced hepatotoxicity in mice. Int J Appl Sci Biotech. 2014;2(1):50-58.

7. Adebiyi OE and Abatan MO. Protective effects of *Enantia chlorantha* stem bark extracts on acetaminophen induced liver damage in rats. Jordan J Biol Sci. 2013;6(4):284-290.

8. Yimam M, Ping Jiao P, Moore B, Hong M *et al.,* Hepatoprotective Activity of Herbal Composition SAL, a Standardize Blend Comprised of *Schisandra chinensis, Artemisia capillaris, and Aloe barbadensis* J

Nutrition Metab 2016, Article ID 3530971, 10 pages http://dx.doi.org/10.1155/2016/3530971

9. Jung-Woo Kang, Seok-Joo Kim, Hyo-Yeon Kim, Soon Hyun Cho, Kang. Protective Effects of HV-P411 complex against D-Galactosamine induced hepatotoxicity in rats. Am J Chin Med. 2016;40(3):467–480.

10. Roy SP, Kannadasan T and Gupta R. Screening of hepatoprotective activity of *Madhuca longifolia* bark on D-Galactosamine induced hepatotoxicity in rats. Biomed Res. 2015;26(2):365-369.

11. Subramanian V, Middha A. Chronic ethanol consumption–induced hepatotoxicity and protective effect of *Boswellia serrata*. Natl J Physiol Pharm Pharmacol. 2016; 6(2): 170-174.

12. Swarnalatha L, Reddy PN. Hepatoprotective activity of *Sphaeranthus amaranthoides* on D-Galactosamine induced hepatitis in albino rats. Asian Pac J Trop Biomed. 2012; 1900-1905.

13. Singh AK, Singh S, Chandel HS. Evaluation of hepatoprotective activity of *Abelmoschus moschatus* seed in paracetamol induced hepatotoxicity on rat. IOSR Journal Pharmacy. 2012;2(5):43-50.

14. Anbarasu C, Rajkapoor B, Kalpana J. Protective effect of *Pisonia aculeate* on paracetamol induced hepatotoxicity in rats. J Exp Integr Med. 2011;1(3):167-172.

15. Padmalochana K, Rajan DMS, Lalitha R, Sivasankari H. Evaluation of the antioxidant and hepatoprotective activity of *Cryptolepsis Buchanani*. J Appl Pharm Sci. 2013;3(2):99-104.

16. Kumar SC, Mohan S. Fabrication, characterization and evaluation of hepatoprotective activity drug loaded flavono nanoparticle delivery system. J Phytopharmacol. 2015; 4(2):90-96.

17. George M, Joseph L, Deshwal N, Joseph J. Hepatoprotective activity of different extracts of *Pterospermum acerifolium* against paracetamol induced hepatotoxicity in albino rats. Pharm Innov. J 2016; 5(3): 32-36.

18. Forouzandeh H, Azemi ME, Rashidi I, Goudarzi M and Kalantari H. Study of the protective effect of *Teucrium polium* L. extract on acetaminophen–induced hepatotoxicity in mice. Iran J Pharm Res. 2013;12(1):123-129.

19. Hena, Tiwari P, Srivastava M, Ghoshal S. hepatoprotective and histopathological activity of ethanol and aqueous extracts of stem of *Aloe vera* linn (ghee gangwar) against paracetamol-induced liver damage in rats. Int J Pharm Biol Sci. 2016;12(1):1-7.

20. Sasidharan S, Chen Y, Saravanan D, Sundram KM, Yoga lethal LY. Extraction, isolation and characterization of bioactive compounds from plants extracts. Afr J Tradit Complement Altern Med. 2011;8(1):1-10.

21. Chacko N, Thomas A, Sahtry CS, Shetty P. Hepatoprotective activity of *Elettaria cardamomum* against paracetamol induced hepatotoxicity. Int J Pharm Pharm Sci. 2012;4(3):611-613.

22. Sreejith G, Jayasree M, Latha PG et al. Hepatoprotective activity of *Oxalis corniculata* L. ethanolic extract against paracetamol induced hepatotoxicity in *Wistar* rats and its *in vitro* antioxidant effects. Indian J Exp Biol. 2014;52(2):147–152.

23. Murugesh MS, Yeligar VC, Maiti BC and Maity TK. Hepatoprotective and antioxidant role of *Berberis tinctoria Lesch* leaves on paracetamol induced hepatic damage in rats. Int J Pharmtech Res 2005; 4:64-69.

24. Taju G, Jayanthia M, Bashab AN, Nambib KSN, Sivaraj A. Hepatoprotective effect of Indian medicinal plant *Psidium guajava* Linn. leaf extract on paracetamol induced liver toxicity in albino rats. J Pharm Res. 2010;3(8):759-1763.

25. Mohanasundari L, Suja S. Qualitative phytochemical screening of rhizomes on *Alpinia calcarata* and *Alpinia speciosa.* J Pharmacogn Phytochem. 2015;4(2):53-56.

26. Lima RM, Caetano PH, Souza KC, Marcos Antonio Fernandes Brandao, Salgado I and Nadia Rezenda Barbosa Raposo. Assessment of different biological capacities of *Alpinia speciosa* (Pers.) B. L. and R.M. Sm. J Young Pharm. 2015;7(4):500-504.

27. Thenmozhi S, Chaturvedi M, Dwivedi S and Subasini U. Investigation of anti inflammatory, analgesic and antipyretic rhizome volatile oil of *Alpinia speciosa*. Asian J Med Pharm Res. 2013;3(3):82 – 84.

28. Chompoo J, Udpadhyay A, Gima S, Fukuta M and Tawata S. Antiatherogenic properties of acetone extract of *Alpinia zerumbet* seeds. Molecules. 2012;6237-6248.

29. Thenmozhi S, Dwivedi S, Chaturvedi M, Dwivedi A and Subasini U. Pharmacognostical, physiochemical and chromatographic estimation of rhizomes and rhizome oil of *Alpinia speciosa* Roxb. Int J Drug Dis Herbal Res. 2013;3(3):649-651.

30. Santos BA, Roman – Campos D, Carvalhom MS, Miranda FMF, Carneiro DC, Cavalcante PH, Candido EAF, Xavier Filho, Cruz JS, Gondim ANS. Cardio depressive effect elicited by the essential oil of

Alpinia speciosa is related to L- type Ca current blockade. Phytomedicine. 2011;539-543.

31. Wong LF, Lim YY and Omar M. Antioxidant and antimicrobial activities of some Alpinia species 2009; 835-851.

32. Mendonca VLM, Oliveria CLA, Craveiro AA, Rao VS, Fonteles MC. Pharmacological and toxicological evaluation of *Alpinia speciosa* 1991; 86,sup II,93-97.

33. Srivastav N, Saklani S, Juyal V, Tiwari BK. Anticonvulsant activity of root extracts of *Valeriana Jatamansii* Linn in experimental rats Int J Life Sci Scienti. Res. 2016;2 121-126.

34. Sridharan S, Kumar KM, Jeepipalli SPK and Sadras SR. *In vitro* neuroprotective effect of *Valeriana wallichii* extract against neurotoxin and endoplasmic reticulum stress induced cell death in SH-SY5Y cells. American J Phytomed Clin Ther 2014; 2(4):509-523.

35. Bhandarkar AV, Shasidhara S and Deepak. Comparative anxiolytic activity of petroleum extract of *Valeriana jatamansi* from different accessions in mice. Research and reviews: J Pharmacol Toxicol Stud. 2014;2(2):34-39.

36. Shu-Ning Shi, Jin-Li Shi, Yong Liu, Yan-Li Wang et al., the anxiolytic effects of valtrate in rats involves changes of corticosterone levels. Evid. Based Complement. Alternat. Med. 2014; doi

37. Joseph L, Rejeesh EP, Narayan RS. Supraadditive effect of hydro ethanolic extract of *Valeriana wallichii* (Indian Valerian) root and phenobarbitone against maximal electroshock seizure in mice. Int J Bioassays 2013; 22(8): 1158-161.

38. Khuda F, Iqbal Z, Zakiullah, Khan A, Nasir F. Antimicrobial and anti-inflammatory activities of leaf extracts of *Valeriana wallichii* D C. Pak J Pharm Sci. 2013;26(3): 451-4.

39. Nabi. Effect of *Valeriana wallichii* DC on learning and memory impairment in streptozotocin induced animal model of diabetes. Int J Universal Pharm Bio Sci. 2013; 2(6):476-493.

40. Sahu S, Ray K, Kumar Y, Gupta G, Kauser H, Sanjeev K, Mishra KS, Panjwani U. *Valeriana wallichii* root extract improves sleep quality and modulates brain monoamine level in rats. Phytomedicine. 2012;19:924-929.

41. Sudhanshu, Mittal NRS and Menghani E. Evaluation of antioxidant properties of *Valeriana wallichii* to scavenge free radicals. Asian J Pharm Clin Res. 2012;5(3):238-240.

42. Katoch O, Kaushik S, Sadashiv M, Kumar Y, Agrawala PK, Mishra K. Radioprotective property of an aqueous extract from *Valeriana wallichii*. J pharm bioall sci. 2012;4(4):327-332.

43. Mhaske DM, Patil DD, Wadhawa GC. Antimicrobial activity of methanolic extract from rhizome and roots of *Valeriana wallichii.* Int J Pharm Biomed Res. 2011;2(4): 107-111.

44. Sah SP, Mathela CS and Chopra K. Elucidation of possible mechanism of analgesic action of *Valeriana wallichii* DC. (Patchouli alcohol) in experimental animal models. Indian J Exp Biol. 2010;48:289-293.

45. Rehni AK, Pantlya HS, Sri R and Singh M. Effect of chlorophyll and aqueous extracts of *Baccopa monniera* and *Valeriana wallichii* on ischaemia and reperfusion – induced cerebral injury in mice. Indian J Exp Biol. 2007;45:764-769.

46. Gilani AH, Arif-ullah Khan, QaiserJabeen, Subhan F, Ghafar R. Antispasmodic and blood pressure lowering effects of *Valeriana wallichii* are mediated through K^+ channel activation. J Ethnopharmacol. 2005;100:347-352.

47. Lefebvre T, Foster BC. *In vitro* activity of commercial Valerian extracts against human cytochrome P450 3A4. J Pharm Pharmaceut sci. 2004;7(2):265-273.

48. Wormser U, Zakine BS, Stivelband E, Eizen O, Nyska A. The liver slice system: A rapid *in vitro* acute toxicity test for primary screening of hepatotoxic agents. Toxicol *In vitro*. 1990;4(6):783-9.

49. Jyothi Y, Sangeetha D and Kumar KK. *In vitro* hepatoprotective activity of *Limnanthemum indicum* (whole plant) against paracetamol induced toxicity using Brl-3a cell line. Am J Pharmtech Res. 2014;4(1):932-937.

50. Naik RS, Mujumdar AM, Ghaskadbi S. Protection of liver cells from ethanol cytotoxicity by curcumin in liver slice culture *in vitro*. J Ethnopharmacol. 2004;95:31-37.

51. Hanafy A, Aldawsari HM, Badr JM, Ibrahim AK, *et al*., Evaluation of hepatoprotective activity of *Adansonia digitata* extract on acetaminophen-induced hepatotoxicity in rats. Evid Based Comp Altern Med Volume 2016, Article ID 4579149, 7 pages

52. Hafez EM, Paulis MG, Ahmed MB, NagyFathy M, Lateff A and Algandaby. Protective and antioxidant activity of the *Euryops arabicus* against paracetamol induced hepatorenal toxicity in rats. Clin Toxicol. 2015;5(1): http://dx.doi.org/10.4172/2161-0495.1000227.

53. Fogha VJ. Tchamgoue AD, Domekouo, Tarkang PT, Agbor GA. *Morinda lucida* stem bark protects paracetamol induced liver damage. Int J Pharm Sci Rev Res. 2015;3(1): 198-204.

54. Rajasekhar U, Swamy BMV, Jayavera KN. Hepatoprotective activity of hydro-alcoholic extract of whole plant of *Sloanum dulcamara* L. and *Nephrolepis cordifolia* (L) C. presl. Against paracetamol induce hepatotoxicity in albino rats. Asian J Pharm Clin Res. 2015;8(2):364-370.

55. Santiago LA, Buccat JM, Dolmalanta MRT and Mayor ABR. Hepatoprotective activity of *Ficus Pseudopalma Blanco* against acetaminophen –induced liver toxicity in Sprague-Dawley rats. Int J Pharm Teaching Practice. 2015;6(1):1603-1608.

56. Qadir MI, Murad MSA, Ali M, Saleem M and Farooqi AA. Hepatoprotective effect of leaves of aqueous ethanol extract of *Cestrum nocturnum* against paracetamol induced hepatotoxicity. Bangladesh J Pharmacol. 2014;9:167-170.

57. Arote, Gupta SK, Lodha P, Yadhav P, Lodha M. Hepatoprotective activity of herbal formulation against paracetamol-induced hepatotoxixcity in rats. Indo American J Pharma Res. 2014;4(1):451-456.

58. Ogbonnaya EA, Uanseoje SO and Ojeaburu SI. Effect of *Gongronema Latifolium* leaves ethanolic extract on paracetamol – induced hepatotoxicity in rats. J Phys Pharmacol Adv. 2014;4(2):337-341.

59. Baig MK, Irshad I, Naseer F. Hepatoprotective activity of ethanol extract. Professional Med J. 2013; 20(3): 078-084.

60. Thummala S, Reddy GVK, Rao YN, Rao MP. Evaluation and screening of hepatoprotective and antioxidant activity of *Mussaenda*

Frondosa root against paracetamol induced hepatotoxicity in *Wistar* rats. Int J Pharm Res Sch. 2013;2(3):39-145.

61. Shrivatsava N, Agrawal P, Kare A, Rajput S, Baghel RS, Baghel SS. Hepatoprotective activity of *Scindapsus officinalis* fruit in paracetamol induced hepatotoxicity in rats. Int J Pharm Sci Res. 2013;4(4):1598-1609.

62. Seok-Joo Kim, Hyo-Sun Choi, Hong Ik Cho, Young-Woo Jin *et al*., Protective effect of Wild ginseng cambial meristematic cell on D-Galactosamine-induced hepatotoxicity in rats. J Ginseng rats. 2015;39:370-383.

63. Jayachitra J and Rubavahini V. Antioxidant effect of *Nyctanthes arbor tritis* L. on D-Galactosamine induced hepatotoxicity in rats. World J Pham Pharm Sci. 2015;4(5): 1009-1018.

64. Kadam SD, Sreedhar C and Chandrasekhar. D-Galactosamine/liposaccharide induced hepatoprotective activity of *Boswellia ovalifoliolata*. J Academicia Industrial Research. 2015;3(8):348-354.

65. Padmanabhan, Jangle SN. Hepatoprotective activity of herbal preparation (HP-4) against D-Galactosamine induced hepatotoxicity in mice. Int J Pharm Sci Drug Res 2014; 6(1): 31-37.

66. Gani AMS and John SA. Evaluation of hepatoprotective effect of *Nigella sativa* L. Int J Pharm Pharm Sci. 2013;5(4):428-430.

67. Pandian S, Badami S, Shankar M. Hepatoprotective activity of methanolic extract of *Oldenlandia herbacea* against D-Galactosamine induced in rats. Int J Appl Res Nat Prod 2013;6(1):16-19.

68. Ganesan R, M Venkatanarasimhan S Pawar, G Pramod Reddy, T Anandhan and G Masilamani. Hepatoprotective effect of *Coldenia procumbens* linn against D-Galactosamine induced acute liver damage in rats. Int J Intergr Sci InnovationTech 2013;2(2):9-11.

69. Gupta RK, RK Vaishali panda SK, Murthy PN, Panigrahi G, Swain SR, Sahoo J. Antihepatotoxic and antioxidant activity of *Nardostachys jatamansi* against D-Galactosamine-induced hepatotoxicity in experimental animals. World J Pharm Pharm Sci. 2013;2(6):6274-6287.

70. Yi-Hang Wu, Bing Jie hao, E. Shen Qien-Li Meng, Ming-Hui Hu, Hu Zhao. Protective properties of *Laggera alata* extract and its principle components against D-Galactosamine injured hepatocytes. Scientia Pharmaceutica. 2012;80:447-456.

71. Shukla R, Surana SJ, Tatiya AU, Das SK. Investigation of hepatoprotective effects of piperine and silymarin on D-Galactosamine induced hepatotoxicity in rats. Res J Pharm Biol Chem Sci. 2011;2(3):975-982.

72. Srividhya AR, Varma SK, Dhanapal SP, Vadivelan R, Vijayan P. *In vitro* and *in vivo* evaluation of hepatoprotective activity of *Gymnema sylvestre*. Int J Pharm Sci Nanotechnology 2010;2(4):768-772.

73. Jaishree V, Badami S. Antioxidant and hepatoprotective effect of Swertiamarin from *Enicostemma axillare* against D-Galactosamine induced acute liver damage in rats. J Ethnopharmacol 2010; 130 103-106.

74. Adaramoye OA and Adeyemi EO. Hepatoprotection of D-Galactosamine-induced toxicity in mice by purified fractions from *Garcinia kola* seeds. Basic Clin Pharmacol Toxicol. 2006;98 135–141.

75. Rao AS and Satyanarayana S. Hepatoprotective activity of herbal extract mixture (HEM) on ethanol induced hepatotoxicity in rats. Scholars Academic J Pharm 2015; 4(2):93-101.

76. Varalakshmi D and Abhinov T. Protective effect of aqueous extract of *Curcuma longa* on ethanol induced hepatotoxicity in rat. Indian J Res Pharm Biotech. 2015;3(1):81-84.

77. Rojin TS, Shetty S and Rajendra holla R. Hepatoprotective effect of *Mussaenda erythrophylla* and *Aegle marmelos* in ethanol induced rat hepatotoxicity model. 2015;6(3):223-228.

78. George S. An evaluation of the protective effect of the methanol extract of Thai Basil (*Ocimum basilicum* L.) on ethanol-induced hepatotoxicity in rats. European J Med Plants. 2014;4(2):158-170.

79. Amrani A, Boubekri N, Benaissa Zama D, Benayache F, Benayache S. Ethanol induced toxicity and lipid peroxidation in pregnant mice. Protective effects of butanolic extract from leaves of *Chrysanthemum fontanesii* Vitamin E and C. J Stress Physiology Biochemistry. 2014;10(2):35-43.

80. Adaramoye OA and Adeyemi EO. Hepatoprotection of D-Galactosamine – induced toxicity in mice by purified fractions from *Garcinia kola* seeds. Basic Clin Pharmacol toxicol. 2006;98135-141.

81. Hong –Ying Gao, Jian Huang, Hang-yu Wang, Xiao-wei Du, Suo-ming Cheng, Ying han, Li-fei wang, Guo-yu-Li and Jin-hui wang. Protective effect of zhuyeging liquor, a Chinese traditional health liquor, on acute alcohol-induced liver injury in mice. J Inflam. 2013; 10: 30.

82. Rekha S, Sowjanja P, Rao S, Rao SN, Govinda S, Suresh T and Giribabu N. Protective effect of *Vitis vinifera* L. seed extract against

ethanol induced oxidative damage of liver in *Wistar* rats. Int J biol Pharm Allied Sci. 2013;2(3):665-674.

83. Kumar KE, Harsha KN, Sudheer V, Giribabu N. *In vitro* antioxidant activity and *in vivo* hepatoprotective activity of aqueous extract of *Allium cepa* bulb in ethanol induced liver damage in *Wistar* rats. Food Science Human Wellness. 2013;2132-138.

84. Basu S, Das M and Data G. Protective activity of ethanolic extract of *Amorphophallus campanulatus* against ethanol induced hepatotoxicity in rats. Int J Pharm Pharm Sci. 2013;5(2):412-417.

85. Sudir N, Suhas PA. Protective effects of *Commelina Benghalensis* Lin (root) extract on ethanol induced acute hepatotoxicity in rats. Int J Sci Inventions Today. 2013;2(3):172-183.

86. Maity T, Ahmad A, Pahari N. Evaluation of hepatotherapeutic effects of *Mikania scandens* (L). Willd. On alcohol induced hepatotoxicity in rats. Int J Pharm Pharm Sci 2012;4(3):490-494.

87. Hoskeri J, Krishna V, Babu RK, Badrinath DK. Prophylatic effects of *Flaveria Trinervia* extract against ethanol induced hepatotoxicity using rats. Int J Pharm Pharm Sci. 2012;4(1):127-134.

88. Hammouda FM, El-Hawary SS, Kassem HA, Tawfik, Motaal AAA, Nazif NM and El—Shamy SS. Hepatoprotective and antioxidant activities of phenolic compounds isolated from *Alpinia zerumbet* (Pers.)B.L. grown in Egypt. Res J Pharm Biol Chem Sciences. 2015;6(2):448- 456.

89. Rajalakshmy MR, Sindhu A and Geetha G. Phytochemical evaluation and biomarker isolation from a polyherbal formulation. American J Pharmtech Research. 2014;4(3):169-180.

90. Thusoo S, Gupta S, Sudan R, Kour J, Bhagat S, Hussain R and Bhagat M. Antioxidant activity of essential oil and extracts of *Valeriana jatamansi* roots 2014; Article ID 614187, 4 pages http://dx.doi.org/10.1155/2014/614187.

91. Saklani N, Purohit VK, Andola HC, Rana M, Chauhan RS and Nautiyal AR. Biological activities of *Valeriana wallichii* DC. Medicinal plants. 2012;4(3):125-128.

92. Padalia CS. Chanotiya and Sundaresan V. Compositional variability in essential oil from different parts of *Alpinia speciosa* from India. Nat Prod Comm. 2010;5(2):279-282.

93. Ghosh S, Debnath S, Hazra S, Hartung A, Thomale K, Schultheis M, Kapkova P, Schurigt V, Moll H, Holzgrabe U, Hazra B. *Valeriana wallichii* root extract and fractions with activity against Leishmania spp. Parasitol Res. 2011;108 861- 871.

94. Kokate CK, purohit AP ang Gokhale CB. Textbook of pharmacognosy. Nirali Prakashan:Pune54:105-107.

95. Mishra G, Khosa RL, Singh P, Jha KK. Hepatoprotective activity of ethanolic extract of *Leea indica* (Burm .f.) Merr. (Leeaceae) stem bark against paracetamol induced liver toxicity in rats. Niger J Exp clin Biosci. 2014;2(1):59-63.

96. Lowry OH, Rosenbourgh NJ,Farr AL and Randall RJ. Protein measurement with folin phenol reagent. J Biochem 1951;193265-275.

97. Niehaus WG and Samuelson B. Formation of MDA from phospholipids arachidonate during microsomal lipid peroxidation. Eur J Biochem. 1986 6:126-130.

98. Jiang ZY, Hunt JV and Wolff SP. Detection of lipid hydroperoxides using the "Fox method. Analytical Biochem. 1992;202:384-389.

99. Kakkar P, Das B and Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. Indian J Biochem Biophy 1984;2:130-132.

100. Sinha AK. Colorimetric assay of catalase. Analytical Biochem 1972;47:389-394.

101. Racker E. Glutathione reductase from bakers yeast and beef liver. J BioChem 1955;217:855-866.

102. Lobarzewski J and Ginalska G. Industrial use of soluble or immobilized plant peroxidases. Plant peroxidase news letter 1995:6:3-7.

103. Paglia DE, Valentine WN. Studies on the qualitative and quantitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med. 70:158-169.

104. Ellman GL. Tissue sulphydryl groups. Archives of Biochem Biophy.1959;32:70-77.

105. Pearse AGE. Histochemistry, Vol-I, 3rd ed. Chruchill Livingstone, London,1968;pp.20-100.

106. Ghareeb MA, Shoeb HA, Madkour HMF, Refahy LA, *et al*., Radical scavenging potential and cytotoxic activity of phenolic compounds from *Tectona grandis* Linn. Global J Pharmacol. 2013;7(4):486-497.

107. Srinivasan R, Natarajan D and Shivakumar MS. Antioxidant compound quercetin-3-O-α-L-rhamnoside (1-6)-β-D-glucose (rutin) isolated from ethyl acetate leaf extracts of *Memecylon edule* Roxb (Melastamataceae). Free radicals and antioxidants. 2015;5(1):35-42.

108. Jasani KP, Kaushik M. Bhimani, Parth M. Dave and Yogesh V. Ushir. Isolation and determination of triterpenoid from roots of *Hyptis suaveolens*. Pharm tech medica 2012; 81-85.

109. Saxena J, Nema R, Singh D and Gupta A. Phytochemistry of Medicinal Plants J Pharmacogn Phytochem. 2013;1(6):168-182.

110. Lydia J and Sudarsanam D. Phytoconstitutents of *Cyperus rotundus* L. that attribute to its medicinal value and antioxidant property. Int J Pharm Sci Res. 2012;3(9):3304-3308.

111. Guillouzo A. Liver cell models in *in vitro* toxicology environmental health perspectives 1998;106:2 511 – 531.

112. Datta S, Dhar S, Nayak SS and Dinda SC. Hepatoprotective activity of *Cyperus articulates* Linn. against paracetamol induced hepatotoxicity in rats. J Chem Pharm Res. 2013;5(1):314-319.

113. Jayadeviah, Bhat IK, Joshi AB, Vijayakumar MMJ, Rawal P. Hepatoprotective activity of *Desmodium oojeinense* (Roxb)H. ohashi against paracetamol induced hepatotoxicity. Asian J Pharm Health Sci. 2012;2(2):312-315.

114. More AN, Parab PB and Apte KG. Evaluation of activity of whole stem extracts of *Oroxylum indicum* on paracetamol induced hepatotoxicity. Int J Pharm Bio Sci. 2013; 4(4):255-265.

115. Lalee A, Bhattacharya B, Das M, Mitra D, Kaity S, Bera S and Samanta A. Hepatoprotective activity of ethanolic extract of *Aerva sanguinolenta* (Amaranthaceae) against paracetamol induced liver toxicity on *Wistar* rats. NSHM Journal Pharmacy Health Care Management. 2012;3,57-65.

116. Parmar MY, Shah PA, Thakkar VT, Salim al-Rejaie, Abdulla H. Al-Assaf, Gandhi TR. Hepatoprotective and antioxidant activity of methanolic activity of *Vetiveria zizanioides* roots against paracetamol – induced liver damage in rats. Life Sci. 2013; 10(4):1184 –1190.

117. Hussain L, Ikram J, Rehman K, Tariq M. Hepatoprotective effects of *Malvia sylvestris* L. against paracetamol-induced hepatotoxicity. Turkish J Biol. 2014; 38396-402.

118. Jollow, D. J., Mitchell, J. R., Potter, W. Z., Davis, D. C., Gillette, J. R., and Brodie, B. B. (1973). Acetaminophen-induced hepatic necrosis. II. Role of covalent binding *in vivo*. J Pharmacol Exp Ther. 2006;187,195–202.

119. Hammam WO, Musa SA, Ikyambe DT, Umana UE, Adelaiye AB, Nok AJ and Ojo SA. Acute and chronic hepatotoxicity study of orally administered ethanol extract of *Carica papaya* seeds in adult *Wistar* rats. British J Pharmacol Toxicol. 2011;2(4):209-211.

120. Halder PK, Adhikari S, Bera S, Bhattacharya S, Panda SP, Kandar CC. Hepatoprotective efficacy of *Swietenia mahagoni* L. Jacq. (Meliaceae) bark against paracetamol-induced hepatic damage in rats. Ind J Pharm Edu Res. 2011;45(2):108-113.

121. Myagmar BE, Shinno E, Ichiba T, Aniya Y. Antioxidant activity of medicinal herb *Rhodococcum vitis-idaea* on D-Galactosamine-induced liver injury in rats. Phytomedicine. 2004;11 416-423.