PHARMACOGNOSTICAL, PHYTOCHEMICAL AND HEPATOPROTECTIVE ACTIVITY ON THE BERRIES OF *Vitex agnus-castus*

A dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY
CHENNAI - 600 032

in partial fulfilment of the requirements for the award of degree of

MASTER OF PHARMACY
IN
PHARMACOGNOSY

Submitted by
Reg. No. 261220652

Under the guidance of

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April 2014

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This is to certify that the dissertation entitled **"PHARMACOGNOSTICAL, PHYTOCHEMICAL AND HEPATOPROTECTIVE ACTIVITY ON THE BERRIES OF *Vitex agnus-castus"*** submitted by **Reg. No. 261220652**, in partial fulfilment of the requirements for the award of the degree of **Master of Pharmacy in Pharmacognosy** by The Tamil Nadu Dr. M.G.R. Medical University, Chennai, is a bonafide record of work done by her during the academic year 2013 – 2014 under the guidance of **Dr. N. Jayshree, M.Pharm., Ph.D.,** Professor & Head, Department of Pharmacognosy, Madras Medical College, Chennai – 600003.

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Dr. N. JAYSHREE

Place: Chennai

Date:

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Authentication Certificate

Certified that the plant material submitted by **J.Jennifer Margaret** was

Carefully Examined and botanically identified as **Vitex agnus-castus Linn.**

Of the family **Verbenaceae.**

Date: 7-10-2013

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TITLE:

"PHARMACOGNOSTICAL, PHYTOCHEMICAL AND HEPATOPROTECTIVE ACTIVITY ON THE BERRIES OF *Vitex agnus-castus*"

The Animal Ethical Clearance Committee experts screened her proposal Vide 8/243/CPCSEA and have given clearance in the meeting held on 22.11.13 at Dean's Chamber in Madras Medical College.

17.2.14

Signature

SPECIAL [illegible]
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1. INTRODUCTION

Ever since the birth of mankind there has been a relationship between life, disease and plants. Primitive men started studying diseases and treatments and there is no record that they used synthetic medicines for ailments. They started using plants and this knowledge of herbal remedies was transferred to generations as folk medicine. So the history of herbal medicine is as old as human history.

The traditional medicine refers to a broad range of ancient and natural health care practices including tribal practices as well as Ayurveda, Siddha and Unani. It is estimated that more than 7500 plants are used mostly in rural and tribal villages of India.[1] The turnover of herbal medicine "over the counter" ethical and classical formulations and home remedies of Ayurveda, Siddha and Unani systems of medicine is about $1 billion and the export of herbal crude extracts is about $80 million.[2]

The WHO has defined traditional medicine (including herbal drugs) as "comprising therapeutic practices that have been in existence, often for hundreds of years before development and spread of modern medicine and are still in use today."[2] The traditional preparations comprise of medicinal plants, minerals, organic matter, etc. herbal drugs constitute only those traditional medicines which primarily use medicinal plant preparations for therapy.

Herbal medicines are in great demand in the developed world due to their efficacy, safety and lesser side effects. These drugs are made from renewable resources of raw materials by eco-friendly and bio-friendly processes and bring economic prosperity. The chemical constituents present in them are a part of the physiological functions of living flora and hence they are believed to have better compatibility with the human body. Ancient literature also mentions herbal medicines for age several diseases namely **memory loss, osteoporosis, diabetic wounds, immune and liver disorders.** [3]

Along with the major health complications such as cancer, cardiovascular, respiratory diseases, the incidence of **liver disease** is also on the rise along with the growing population. It is a major cause of death still increasing every year and it is the **fifth big killer** in countries such as England and Wales. The survey report says that 16,087 people in the UK died from liver disease in 2008, a 4.5% increase since 2007.[4] Liver disease deaths have increased by 12% in just three years, since 2005.[5] According to the latest WHO data published in April

2011, in India deaths associated with liver disease reached 208,185 or 2.31% of total deaths. The age adjusted death rate is 23.59 per 100,000 of population and India ranks #27 in the world. Worldwide, the estimated annual incidence rate of drug-induced liver injury is 13.9 - 24 per 100,000 inhabitants.

The major functions of the body such as metabolism and excretion of xenobiotics are carried out by liver. Hence, the liver is vulnerable to a wide variety of metabolic, circulatory and neoplastic insults. The primary diseases of liver are viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease and hepato-cellular carcinoma. Hepatic damage also occurs secondary to some of the most common diseases in humans, such as cardiac decomposition, disseminated cancer and extrahepatic infections.[6]

Causes of liver injury [6,7]

- **Chemical induced:** Carbon tetrachloride, alcohol consumption, aflatoxins, 1,1,2,2-tetrachloroethane, carbon tetrabromide, acetylene tetrabromide, dimethyl formamide, ethylene dichloride.
- **Drug-induced:** More than 900 drugs have been implicated in causing liver injury and it is the most common reason for a drug to be withdrawn from the market. Drug induced liver injury is responsible for 5% of hospitalised persons and 50% of all acute liver failures. Some of them are Acetaminophen overdose, anti-tubercular drugs, chemotherapeutic agents, thioacetamide.
- **Virus induced**: Hepatitis A, B, C, D, E.
- **Other causes:** Non-alcoholic fatty liver, malnutrition, extrahepatic infections, ingestion of poisonous wild mushrooms, haemochromatosis (an inherited disorder that causes body to absorb and store iron too much).

Mechanism of liver damage:[7,8]

75% of blood coming to the liver arrives directly from gastrointestinal organs and then spleen via portal veins that bring drugs and xenobiotics in near-undiluted form. Several mechanisms are responsible for either inducing hepatic injury or worsening the damage process. Many chemicals damage mitochondria, an intracellular organelle, that produces

energy. Its dysfunction releases excessive amount of oxidants that, in turn, injure hepatic cells. Activation of enzymes in the cytochrome P-450 system such as CYP2E1 also leads to oxidative stress. Injury to hepatocyte and bile duct cells lead to accumulation of bile acid inside the liver. This promotes further liver damage. Non-parenchymal cells such as Kupffer cells, fat storing stellate cells and leukocytes (neutrophils and monocyte) also have a role in mechanism.

Patterns of liver injury[6]

The liver has a limited cellular and tissue responses to injury, regardless of the cause. They are:

- ✓ Hepatocyte degeneration and intracellular accumulations
- ✓ Hepatocyte necrosis and apoptosis
- ✓ Inflammation
- ✓ Regeneration
- ✓ Fibrosis

Diagnosis

Liver disease is an insidious process in which clinical detection and symptoms of hepatic damage may occur weeks or months after the onset of injury. Hence it is detectable only by **abnormal laboratory tests.[6]**

Treatment

Importance of Herbs in treating liver disorders [9]

Modern medicine offers limited success in providing effective cure and there is a need to develop new drugs capable of healing toxic liver damages. The available synthetic drugs to treat liver disorders also cause further damage to the liver. The use of the herbal medicines in hepatic disorders has been known in Indian since the Vedic times. About 170 phyto-constituents isolated from 110 plants belonging to 55 families have been reported so far to possess liver protective activities. It is estimated that about 6000 commercial herbal drugs are used world over as hepatoprotective drugs.

However, the following four herbal medicines have been found to be most promising in the treatment of liver disorders and viral hepatitis.

- ✓ Silymarin obtained from the seeds of *Silibum marianum*
- ✓ Extracts of *Picrrorhiza kurroa* known as kutaki
- ✓ Extracts of *Phyllanthus niruri* and *Phyllanthus amarus*
- ✓ Glycyrrhizin from *Glycyrrhiza glabra*

The genus Vitex contains 270 species distributed throughout the world. It has a proven potential of important pharmacological interest.

The species *Vitex agnus-castus* (Verbenaceae) generally called as the women's herb, has a folklore claim of treating liver disorders. Hence the present study was carried out to evaluate the potential effect of *Vitex agnus-castus* in treating liver disorders.

2. RATIONALE FOR SELECTION OF THE PLANT

- Herbalism has a long term tradition of use outside of conventional medicine. It is becoming more main stream as improvements in analysis and quality control along with advances in clinical research have enhanced the value of herbal medicine in treating and preventing diseases. There are numerous plants and traditional formulations available for the treatment of liver diseases.
- There are no specific synthetic drugs used as hepatoprotective. Many herbs contain biomolecules which are biodegradable and can be broken down into basic elements. Hence herbal drugs are preferred more for treating liver damage over synthetic drugs.
- There is a long tradition for the use of different preparations of drugs of *Vitex agnus castus* (VAC) belonging to the family Verbenaceae as complementary medicine in Europe.
- VAC is also reported to possess antioxidant activity and antioxidants are known to possess hepatoprotective activity. Plants containing flavonoids are known to enhance the hepatoprotective activity.
- This plant has been traditionally used for treating enlarged liver and no scientific studies have been reported so far. Hence the present work is intended to be carried out to evaluate the hepatoprotective activity on the berries of *Vitex agnus-castus.*

3. REVIEW OF LITERATURE

The literature review of the plant *Vitex agnus-castus* reveals the following:

- E. Svecova *et al* (2013) reported the antifungal activity of the methanolic seed extract of *Vitex agnus-castus* against *Pythium ultimum* in tomato. The 0.2% extract delayed the mycelial growth of the fungus and showed significant antifungal activity against *P. ultimum* on tomato seedlings with an efficacy comparable to that of the synthetic fungicide.[19]

- Ghannadi A *et al* (2012) reported the antibacterial activity and composition of essential oils from aerial parts of *Pelargonium graveolens L'Her and* seeds extract of *Vitex agnus-castus L.* Inhibition zones showed that the essential oils of the two plants were active against bacteria. The susceptibility of the strains changed with the dilution of essential oils in DMSO.[20]

- M. Ramezani *et al* (2010) reported the antinociceptive and anti-inflammatory effects of hydroalcoholic extract of *Vitex agnus castus* Fruit. The inflammation was caused by xylene induced ear oedema. The results indicated the Vitex extracts remarkably inhibited inflammation and second phase of nociception.[21]

- Anita Rani Shiksharthi *et al* (2012) reported the anti-anxiety and CNS modulatory activities of *Vitex agnus-castus* fruits *Linn.* The methanolic and water extract exhibited significant anti-anxiety activity at the dose of 200mg/kg with respect to the control as well as standard (diazepam 2mg/kg). [22]

- Jenive Stella *et al* (2011) reported the hypoglycemic effect on the methanolic extract of leaves of *Vitex Agnus Castus* in Streptozotocin induced diabetic rats. Streptozotocin (40mg/kg body weight) was used to induce diabetes mellitus. Treatment with *Vitex agnus-castus extract* at three doses (50, 100, 200mg/kg bw) showed a significant increase in serum insulin and significant decrease in blood glucose levels.[23]

- Shao-Nong Chen *et al* (2011) reported the phytochemical investigation of *Vitex agnus-castus fruits.*[24]

- He Zhong *et al* (2009) have carried out a prospective, randomnized multicentre placebo controlled study in China to study the effect of *Vitex agnus-castus* for the treatment of premenstrual syndrome. The study concluded that the dry extracts of *Vitex agnus-castus* fruits is an effective and well tolerated treatment for the relief of symptoms of premenstrual syndrome.[25]

- Ayse *et al* (2008) reported the antioxidant potency of flavonoids from methanolic extract of flowering stems of *Vitex agnus-castus L.* The antioxidant activity of compounds on DPPH were found to be very high.[26]

- Saberi Mehdi *et al* (2008) reported the antiepileptic activity of *Vitex agnus castus* fruit extract on amygdalin kindled seizures in male rats. The results indicate that Vitex can reduce or prevent epileptic activity as demonstrated by reduction of ADD(after discharge duration) and S5D (length of convulsion) in a dose dependent manner.[27]

- Males *et al* (1998) have studied and reported the content of the polyphenols in leaves, flowers and fruits of *Vitex agnus-castus L.*[28]

Hepatoprotective activity:

- Amol Nimba More *et al* (2013) Evaluation of activity of whole stem extracts of *Oroxylum indicum* against paracetamol induced hepatotoxicity. This study indicated that aqueous and ethanolic extracts showed significant hepatoprotective activity by preventing hepatic damage at the dose of 250mg/kg.[29]

- Sabeena Hussain Syed *et al* (2013) reported the hepatoprotective activity on the methanolic extract of the leaves of *Erythroxylum monogynum* Roxb. by paracetamol induced toxicity. It showed a dose dependent activity which is evident from the decreased level of serum enzymes and total bilirubin at dose of 400mg/kg compared to 100mg/kg and 200mg/kg.[30]

- Manoj Sony *et al* (2011) reported the Hepatoprotective activity of fruits of *Prunnus domestica* against paracetamol and CCl_4 induced hepatotoxicity. Extract of *Prunnus domestica* fruits (150mg/kg and 300mg/kg) has brought back the altered levels of biochemical markers to the near normal levels in a dose dependant manner.[31]

- Jagdish R. Bahetl *et al* (2011) reported that the methanolic extract of *Ficus bengalensis* barks exhibited antihepatotoxic effect against paracetamol and CCl_4 induced hepatic damage at the dose level of 100mg/kg and 250mg/kg.[32]
- Mohammed Ibrahim *et al* (2011) reported the hepatoprotective activity of barks *Boswellia serrata* against paracetamol induced hepatotoxicity. The chloroform bark extract was found to be hepatoprotective at the dose of 250mg.kg and 500mg/kg.[33]
- Satyaranjan Mishra *et al* (2013) Evaluation of the anti-hepatotoxic activity of methanol-dichloromethane (MDM) extract of whole plant of *Oroxylum indicum* on carbon tetrachloride induced hepatotoxicity. The MDM extract of *Oroxylum indicum* whole plant (200mg/kg) exhibited a significant reduction in biochemical parameters thus reversing hepatotoxicity causing significant liver recovery. [34]
- M. Balakrishnan *et al* (2012) reported the Hepatoprotective activity on the root bark of *Azima tetracantha* Lam. against carbon tetrachloride induced hepatotoxicity. The regeneration of damaged liver cells was obtained by the ethanolic extract of the plant at the doses 40, 80, 120mg/kg. [35]
- Manjunatha BK *et al* (2012) reported the Hepatoprotective potency on the ethanolic seed extracts of *Achyranthus aspera* which exhibited recovery against the toxic effects of CCl_4 at the dose of 100mg/kg.[36]
- Veena Rani I *et al* (2011) reported the hepatoprotective activity different extracts of *Bauhinia purpurea* against CCl_4 induced toxicity. The maximum protection was seen with alcoholic and chloroform extracts at the dose of 150mg/kg.[37]
- Rakhamaji D. Chandane *et al* (2013) reported the hepatoprotective activity of honey on anti-tubercular drugs (isoniazid, rifampicin, pyrazinamide) induced hepatotoxicity. Honey inhibits lipid peroxidation and by increasing antioxidant defence mechanism has a significant hepatoprotective action. [38]

From the above models it is known that hepatotoxicity may be caused by various methods. Drug related hepatotoxicity is the one which is life threatening and /or requires hospitalisation in serious cases. Among the drugs, paracetamol was found to be one of the common analgesic antipyretic used by the population and found to cause hepatotoxicity and liver damage at high doses. Hence paracetamol induced toxicity model was selected for this study.

4. ETHNOBOTANICAL SURVEY

PLANT PROFILE [10,11]

Plant name : *Vitex agnus-castus*

Family : Verbanaceae

VERNACULAR NAMES

Tamil : Vennochi

English : Hemp tree, Monk's pepper

PLANT TAXONOMY

Kingdom : Plantae

Division : Tracheophyta

Class : Magnoliopsida

Order : Lamiales

Family : Verbanaceae

Genus : *Vitex*

Species : *agnus-castus*

DESCRIPTION: [12]

Habit: Deciduous tree or large shrub, growing to a height of 7 metres.

Habitat: Native to western Asia and Mediterranean region.

BOTANY[12]

Leaves: Opposite, decussate, rarely whorled or alternate, exstipulate, simple, rarely compound.

Flowers: zygomorphic, bisexual and hypogynus.

Fruits: schizocarpic or drupaceous with hard endocarp, indehiscent or dehiscent.

Parts used: berries, leaves, roots

Fig. 1. ***Vitex agnus-castus*** **tree**

CHEMICAL CONSTITUENTS: [13,14]

- ✓ Flavonoids - vitexin, casticin, kampferol, quercetagetin
- ✓ Iridoid glycosides - agnoside, aucubin
- ✓ Alkaloids - viticin
- ✓ Volatile oil - 1,8-cineol, linalool, terpinyl acetate, alpha pinene, beta pinene
- ✓ Progestins - progesterone, hydroxyl progesterone
- ✓ Essential fatty acids - palmitic acid, oleic acid, linoleic acid

ETHNOBOTANICAL USES:

- ✓ Decoction of berries – Used as stimulant, diuretic and in the treatment of dropsy, liver disorders, premenstrual syndrome and cancer.[10,11]
- ✓ Leaves – Used in premenstrual syndrome, mastalgia, hormonal disorders, menstrual cramps, liver disorders. [15]

- ✓ It has other uses such as: [16,17,18]
- Antioxidant
- Antimicrobial
- Anti-Inflammatory
- Spasmolytic
- Antiviral
- Antithyroid
- Anti-tumor
- Anti-acne
- Cytotoxic

5. AIM AND OBJECTIVE

The aim of the present study is

- To standardize the berries of *Vitex agnus-castus* by carrying out the pharmacognostical, physicochemical and phytochemical parameters.

- To evaluate the antioxidant activity of the various extracts of the berries of *Vitex agnus-castus.*

- To evaluate the *in vitro* Hepatoprotective activity of the various extracts of the berries of *Vitex agnus-castus* by MTT assay using normal Chang liver cell line

- Evaluation of *in vivo* Hepatoprotective activity of the berries of *Vitex agnus-castus* by using Paracetamol induced toxicity in Wistar albino rats.

6. PLAN OF WORK

1. PHARMACOGNOSICAL STUDIES

- Collection of plant material
- Authentication
- Macroscopical studies
- Microscopical studies
- Powder microscopy
- Physicochemical constants
    - Ash values
    - Extractive values
    - Loss on drying
    - Foaming index
    - Swelling index
- Inorganic and Heavy Metal Analysis

2. PHYTOCHEMICAL STUDIES

- Preparation of extracts
- Preliminary phytochemical screening of powder and extracts
- Quantitative estimation of Phytoconstituents
- Fluorescence analysis of powder and extracts
- Thin layer chromatography of extracts
- HPTLC

3. PHARMACOLOGICAL SCREENING

- Antioxidant activity
    - Hydrogen peroxide scavenging assay
    - Reducing power ability assay
- *In vitro* evaluation by MTT assay using Chang liver cell line
- *In vivo* hepatoprotective activity using paracetamol induced hepatotoxicity model.

7. METHODOLOGY

7.1. PHARMACOGNOSTICAL STUDIES

Evaluation of a drug means confirmation of its identity and to determine its quality and purity and detection of nature of adulteration. Evaluation of a crude drug can be attempted by different methods which include morphological and microscopical studies of the crude drugs or their physical, chemical and biological behaviour. Systematic identification of crude drugs and their quality assurance gives an integral part of drug description.

Pharmacognostical studies basically deals with the identification, authentication and standardization of herbal medicinal plants through organoleptic character, histological character, powder microscopy, quantitative microscopy, linear measurement, histochemical analysis and physico-chemical observations as prescribed by an authoritative source such as World Health Organization (WHO).

7.1.1. PLANT COLLECTION AND AUTHENTICATION

The fresh berries of the plant *Vitex agnus-castus* was collected from Rajavallipuram, Thirunelveli district, Tamil Nadu, India and it was botanically identified and authenticated by Dr. V. Chelladurai, Research Officer- Botany (Scientist – C), Central Council for Research in Ayurveda and Siddha, Government of India.

7.1.2 MACROSCOPY [39]

Macroscopical character which includes organoleptic characters and morphological features of various parts of the plant was studied.

7.1.3. MICROSCOPY

Staining Method

Fixation of plant organ: Berries were cut fixed in FAA solution (Formalin 5ml + Acetic acid + 90ml of 70% Ethanol). After 24 hours of fixing, the specimens were dehydrated with graded series of tertiary butyl alcohol, as per standard method.[40]

Infiltration of the specimen: It was carried out by gradual addition of 58 - 60˚C of melting pointed paraffin wax until TBA solution attained super saturation. The specimens were cast into paraffin blocks.[41]

Sectioning [42]

The paraffin embedded specimens were sectioned with the help of rotary microtome. The thickness of the sections was 10 – 12µg. Dewaxing of the sections was done by customary procedures. The sections were stained with saffranin, haematoxylin and eosin.

The dye rendered blue colour to the cellulose walls, pink colour to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. wherever necessary sections were also stained with saffranin, fast green and iodine for starch.

Photomicrographs

Microscopic description of tissues was supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon lab photo 2 microscopic units. For normal observations bright fields was used.

For the study of crystals and lignified cells, polarized light was employed. Since, these structures have birefringent property under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale – bars. Descriptive terms of the anatomical features are as given in the standard anatomy books.

7.1.4. POWDER MICROSCOPY [43]

The shade dried, powdered plant material was used for powder microscopic analysis. The organoleptic characters were observed and to identify the different characteristic features, various staining reagents were used. Powder was stained with 1% Phloroglucinol in 90% ethanol, concentrated hydrochloric acid and glycerin and observed through microscope. All the lignified cells stained pink colour. Calcium oxalate crystals were observed under the polarized light microscope.

7. 1.5. PHYSICOCHEMICAL CONSTANTS [44-47]

Shade dried powdered plant material of berries of *Vitex agnus-castus*, was used for the determination of physicochemical constants in accordance with WHO (World Health Organization) guidelines.

7.1.5.1. Determination of ash values

Ash values are helpful in determining the quality and purity of a crude drug in the powdered form. The residue remaining after incineration is the ash content of the drug, which simply represents inorganic salts, naturally occurring in drug or adhering to it or deliberately added to it, as a form of adulteration.

- **Total ash**

Total ash is designed to measure the total amount of material remaining after ignition. This includes both physiological ash which is derived from plant tissue itself and non-physiological ash which is the residue of the extraneous matter adhering to the plant surface.

Procedure

Silica crucible was heated to red hot for 30 minutes and it was allowed to cool in a dessicator. About 2-3g of powdered sample was weighed accurately and evenly distributed in the crucible dried at 100 - 105˚C for 1 hour and ignited to constant weight in a muffle furnace at 600±25˚C. The crucible was allowed to cool in dessicator and ignited to constant weight. The percentage of ash with reference to the air dried substance was then calculated.

- **Water soluble ash**

The total ash was boiled for 5 minutes with 25ml of water. The insoluble matter was then collected in ash less filter paper. It was washed with hot water and ignited for 15 minutes at a temperature not exceeding 450˚C. The weight of the insoluble matter was subtracted from the weight of ash and then the percentage of water soluble ash with reference to the air dried substance was calculated.

- **Acid insoluble ash**

In a crucible containing total ash, 15ml of water and 10ml of hydrochloric acid were added. It was boiled for 10 minutes and filtered on an ashless filter paper. The filter paper was washed with hot water until the filtrate was neutral. The filter paper with the contents was ignited to dull redness, cooled in a dessicator and weighed. The percentage of acid insoluble ash was calculated with reference to the air dried substance.

- **Sulphated ash**

2-3g of air dried substance was ignited gently at first in a crucible, until the substance was thoroughly charred. Then the residue was cooled, moistened with 1ml of sulphuric acid, heated gently until the white fumes were no longer evolved and ignited at 800±25˚C, until all the black particles disappeared. The crucible was allowed to cool, a few drops of sulphuric acid was added and heated. Then it was ignited as before, cooled and weighed. The percentage sulphated ash with reference to the air-dried substance was then calculated.

7.1.5.2. Determination of solvent extractive values

This method determines the amount of active constituents in a given amount of medicinal plant material when extracted with a particular solvent. The extraction of any crude drug with a particular solvent yields a solution containing different Phytoconstituents. The composition of these Phytoconstituents in that particular solvent depends upon the nature of the drug and solvent used. The use of a single solvent can be the means of providing preliminary information on the quality of a particular drug.

- **Water soluble extractive value**

Procedure

5g of the air-dried drug, coarsely powdered was macerated with 100ml of water in a closed flask for 24 hours, shaking frequently during first 6 hours and allowed to stand for 18 hours. Thereafter, filter rapidly taking precautions against loss of water, evaporate 25ml of the filtrate to dryness in a tared, flat-bottomed shallow dish, dry at 105˚C and weigh. The percentage of water-soluble extractive with reference to the air dried drug was calculated.

- **Alcohol soluble extractive value**

The alcohol soluble extractive value is also indicative for the same purpose as water soluble extractive value. The solvent strength of alcohol varies from 20 – 95 % v/v. The solvent strength has to be chosen depending on the nature of drugs to be extracted.

Procedure

5g of the air dried and coarsely powdered drug was macerated with 100ml of ethanol of 50ml in a closed flask for 24 hours, shaking frequently during the first 6 hours and allowed to stand for 18 hours. Thereafter, the solution was filtered rapidly taking precaution against loss of ethanol. Evaporate 25ml of the filtrate to dryness in a tared flat-bottomed shallow dish, dry at 105˚C and weigh. The percentage of ethanol-soluble extractive with reference to the air-dried drug was calculated.

- **Non-volatile ether soluble extractive value (Fixed oil content)**

A suitably weighed quantity of the crushed air dried drug was transferred to an extraction thimble and extracted with solvent ether or petroleum ether (boiling point 40-60˚C) in a soxhlet for 6 hours. The extract was filtered into a tarred evaporating dish, evaporated and dried at 105˚C to constant weight. The percentage of non-volatile ether soluble extractive value with reference to the air dried drug was calculated.

- **Volatile ether soluble extractive value**

About 2g of dried powdered drug was accurately weighed and extracted with anhydrous ethyl ether in a continuous extractive apparatus for 20 hours. The ether solution was transferred to a tared porcelain dish and evaporated spontaneously. Then it was dried over phosphorous pentoxide for 18 hours and the total ether extract was weighed. The extract was heated gradually and dried at 105˚C to constant weight. The loss in weight represents the volatile portion of the extract.

7.1.5.3. Loss on drying

Specified quantity of substance was transferred to a previously ignited and cooled silica crucible and the substance was evenly distributed by gentle sidewise shaking. The crucible with the contents and the lid were weighed accurately. Then the loaded uncovered

crucible and the lid were placed in a drying chamber (105˚C). The substance was heated for sufficient period of time until constant weight was obtained. The crucible was covered with the lid and allowed to cool in a dessicator at room temperature before weighing. Finally the crucible was weighed to calculate the loss on drying with reference to the air dried substance.

7.1.5.4. Determination of foaming index

1g of the coarsely powdered drug was weighed and transferred to 500ml conical flask containing 100ml boiling water. The flask was maintained at moderate boiling point at 80-90˚C for about 30 minutes. Then it was cooled and filtered into a volumetric flask and sufficient water was added through the filter to make up the volume to 100ml (V_1).

The decoction was poured into 10 stoppered test tubes (height 16cm, diameter 16mm) in successive portions of 1ml, 2ml, 3ml, etc., upto 10ml, and the volume was adjusted in each tube to 10ml water. Stopper the tubes and shake them in a lengthwise motion for 15 seconds, two shakes per second. Allow to stand for 15 minutes and measure the height of the foam. The results are accessed as follows.

If the height of the foam in every tube is less than 1cm, the foaming index is less than 100. If a height of 1cm is measured in any tube, the volume of the plant material decoction in the tube (a) is used to determine the index. If this tube is the first or second tube in a series, prepare an intermediate dilution in a similar manner to obtain a more precise result.

If the height of the foam is more than 1cm in every tube, the foaming index is over 1000. In this case repeat the determination using a new series of dilutions of the decoction in order to obtain a result.

Calculate the foaming index using the following formula,

Foaming index = 1000/a

where a = the volume in ml of the decoction

7.1.5.5. Determination of swelling index

The swelling index is the volume in ml occupied by the swelling of 1g of plant material under specified conditions. A specified quantity of the plant material previously reduced to the required fineness and accurately weighed, into a 25ml glass stopper measuring

cylinder. The internal diameter of the cylinder should be about 16mm, the length of the graduated portion about 125mm, marked in 0.2ml divisions from 0 to 25ml of water and shake the mixture thoroughly every 10 minutes for 1 hour. Allow to stand for 3 hours at room temperature, or as specified. The volume in ml occupied by the plant material was measured including any sticky mucilage. The mean value of the individual determination, related to 1g of plant material was calculated.

7.1.6. QUALITATIVE AND QUANTITATIVE ESTIMATION OF HEAVY METALS AND INORGANIC ELEMENTS [48]

Plant minerals play a vital role in metabolism and osmolity. Presence of elements vary with the soil, climate conditions etc. There are essential and non-essential elements which may be beneficial or harmful to living things. Non-essential elements like lead, arsenic, cyanide, chromium, cadmium, aluminium and silver bring about toxic effects resulting in intoxication. Hence, qualitative and quantitative estimation of inorganic elements in the plant *Vitex agnus-castus* were carried out.

7.1.6.1. Qualitative analysis of inorganic elements and Heavy metals

To the ash of the drug material 50% $^{v}/_{v}$ HCl was added and kept for 1 hour. It was filtered and the filtrate was used for the following tests.

Aluminium: White gelatinous precipitate of aluminium hydroxide [$Al(OH)_3$] is formed on addition of ammonia solution. It is slightly soluble in excess of the reagent. It dissolves readily in strong acid and base.

Arsenic: Arsenious salts in neutral solution react with solution of copper sulphate to form green precipitate (Scheele's green) which on boiling gives a red precipitate of cupric oxide.

Borate: The mixture obtained by the addition of sulphuric acid and alcohol (95%) to a borate, when ignited burns with flame tinged with green.

Calcium: Solution of calcium salts, when treated with ammonium carbonate solution yields a white precipitate. The mixture after boiling and cooling is insoluble in ammonium chloride solution.

Carbonate: Carbonate, when treated with dilute acid produces effervescence liberating CO_2 which is colourless and produces a white precipitate in calcium hydroxide solution.

Chlorides: Chlorides, when treated with silver nitrate solution yield a curdy white precipitate which is insoluble in nitric acid, but soluble after being well washed with water.

Copper: An excess of ammonia solution added to a solution of a cupric salt, produces first a bluish precipitate and then a deep blue coloured solution.

Iron: Solution of ferric salts, when treated with potassium ferrocyanide solution, yields an intense blue precipitate which is insoluble in dilute HCl.

Lead: Strong solution of lead salts, when treated with HCl, yield a white precipitate which is soluble in boiling water and is re-deposited as crystals when the solution is cooled.

Magnesium: Solution of magnesium salts, when boiled with ammonium carbonate solution yields white precipitate, but no precipitate is produced with ammonium chloride solution.

Mercury: Solution of mercuric salts, when treated with sodium hydroxide solution, yields a yellow precipitate.

Nitrate: With solution of ferrous sulphate no brown colour was observed but if sulphuric acid is added (slowly along the sides of the test tube), a brown colour is produced at the junction of two liquids, indicating the presence of nitrates.

Phosphate: Solution of phosphate when treated with silver nitrate solution yield yellow precipitate of normal silver orthophosphate Ag_3PO_4 (distinction from meta and pyrophosphate) solution in dilute ammonia solution and in dilute nitric acid.

Potassium: Moderately strong solution of potassium salts, which have been previously ignited to remove ammonium salts, when treated with perchloric acid (60%) yield a white crystalline precipitate.

Silver: Solution of silver salts, when treated with potassium iodide solution yield a cream coloured precipitate which is insoluble in dilute ammonia solution and nitric acid.

Sulphate: Solution of sulphate, when treated with lead acetate solution yields a white precipitate which is insoluble in ammonium acetate solution and sodium hydroxide solution.

7.1.6.2. Quantitative estimation of inorganic elements:

Instrumental parameters:

Instrument Name : Inductively Coupled Plasma Optical Emission Spectrometry

Instrument Model : PE Optima 5300 DV ICP-OES

Optical system Dual View – axial or radial

Detector system **:** Charge coupled detector, (UV-VISIBLE detector which is maintaining at - 40˚C) to detect the intensity of the emission line wavelength range from 165 to 782nm.

Torch (Light source) : Positioned horizontally in the sample compartment along the central axis of the spectrometer optics. Changing from axial to radial viewing in a simple software command and is accomplished by computer control of a mirror located in the optical path. The torch assembly of this system comprises of two concentric quartz tubes.

Spray chamber **:** Scott type

Nebulizer **:** Cross flow Gem tip

Preparation of samples by acid digestion method

Weighed 50g of powdered mixture of powered drug treated with acid mixture of Sulphuric acid: Water in the ratio of 4 : 1 in the kjeldhal flask and heated continuously till the solution is colourless. The sample mixture was then transferred in a 25ml volumetric flask and made upto the volume with distilled water.

Blank solution was prepared as above without sample.

The standards of arsenic, lead, cadmium, manganese, boron, calcium, mercury were prepared as per the protocol and the calibration curve was developed for each of them.

Detection

Samples were analysed for the detection and Quantification of arsenic, lead, cadmium, manganese, boron, calcium, mercury by Inductive Coupled Plasma Optical Emission Spectrometry.

7.2. PHYTOCHEMICAL STUDIES

Phytochemical evaluation is used to determine the nature of phytoconstituents present in the plant by using suitable chemical tests. It can be done by qualitative analysis using specific reagents followed by confirmation with different chromatographic techniques like TLC and HPTLC. Therefore a complete investigation is required to characterize the phytoconstituents qualitatively and quantitatively.

MATERIALS AND METHODS

7.2.1. PREPARATION OF EXTRACTS [49]

Extraction is the preliminary step involved in the phytochemical studies. It brings out the primary and secondary metabolites into the extracting solvent depending on its polarity.

Continuous hot percolation method

The dried coarsely powdered berries of *Vitex agnus-castus* were extracted, using soxhlet apparatus, with solvents of increasing polarity such as Hexane, Ethyl acetate and Ethanol at 60-70˚C for of 18 hours. All the extracts were redistilled and concentrated under rotary vacuum evaporator and percentage yield was calculated. The extracts were tested for qualitative and quantitative analysis.

7.2.2. PRELIMINARY PHTOCHEMICAL SCREENING [50,51]

7.2.2.1. QUALITATIVE ANALYSIS

1. **Detection of Carbohydrates**

- **Molisch test**

The extracts and powder were treated with a few drops of alcoholic α-naphthol, then add few drops of concentrated sulphuric acid through the sides of the test tube. Formation of purple to violet colour ring appears at the junction of the test tubes indicating the presence of Carbohydrates.

- **Fehling's test**

The extracts and the powder were treated with Fehling's A and Fehling's B solution and heated. Formation of red colouration indicates the presence of sugar.

2. Detection of Alkaloids

- **Dragendorff's reagent**

The extracts and the powder were treated with a few drops of Dragendorff's reagent. Formation of an orange or orange red precipitate indicates the presence of Alkaloids.

- **Mayer's reagent**

The extracts and the powder were treated with a few drops of Mayer's reagent. Formation of creamy white precipitate indicates the presence of Alkaloids.

- **Wagner's reagent**

The extracts and the powder were treated with a few drops of Wagner's reagent. Formation of brown or reddish brown precipitate indicates the presence of alkaloids.

3. Detection of Flavonoids

- **Shinoda test**

The extracts and the powder were treated with a few magnesium turnings and concentrated hydrochloric acid added drop wise. Formation of pink, scarlet, crimson red or occasionally green to blue colour which appear after a few minutes, indicates the presence of flavonoids.

- **Alkaline reagent test**

The extracts and the powder were treated with a few drops of sodium hydroxide solution; formation of intense yellow colour indicates the presence of flavonoids.

4. Detection of Glycosides

- **Borntrager's test**

The extracts and the powder were boiled with 1ml of sulphuric acid for 5 minutes and filtered while hot. The filtrate was cooled and shaken with equal volume of chloroform. The lower layer of chloroform was separated and shaken with half of its volume of dilute ammonia. Formation of rose pink to red colour in the ammoniacal layer indicates the presence of glycosides.

- **Test for hydroxy-anthraquinones**

The extracts and the powder were treated with a few drops of potassium hydroxide solution. Formation of red colour indicates the presence of glycosides.

5. **Detection of Saponin**

- **Froth formation test**

The extracts and the powder were shaken well with water. Formation of stable froth indicates the presence of glycosides.

6. **Detection of Tannins (Phenolic compounds)**

- **Ferric chloride test**

The extracts and the powder were treated with a few drops of ferric chloride solution. Formation of green colour indicates the presence of tannins.

- **Gelatin test**

The extracts and the powder were treated with a few drops of 1% gelatin solution containing 10% sodium chloride. Formation of precipitate indicates the presence of tannins.

7. **Detection of Phytosterols**

- **Libermann-Burchard test**

The extracts and the powder were treated with a few drops of acetic anhydride, boiled and cooled. Then concentrated sulphuric acid was added along the sides of the test tube. A brown ring formation at the junction of two layers and upper layer turning green shows the presence of steroids and formation of deep red colour indicates the presence of triterpenoids.

8. **Detection of Proteins and Amino acids**

- **Biuret test**

The extract and the powder were treated with a few drops of Biuret reagent. Formation of violet colour indicates the presence of proteins.

- **Xanthoprotein test**

The extracts and the powder were treated with a few drops of conc. Nitric acid and boiled, yellow precipitate is formed. After cooling it, add 40% sodium hydroxide solution. Formation of orange colour indicates the presence of proteins.

- **Ninhydrin test**

The extracts and the powder were treated with a few drops of 0.25 % ninhydrin reagent and boiled for few minutes. Formation of blue colour indicates the presence of proteins.

9. Gums and mucilage

The extracts and the powder were treated with ruthenium red solution. Formation of pink colour indicates the presence of gums and mucilage.

The extracts and the powder were dissolved in 5 to 10 ml of acetic anhydride by means of gentle heat, cooled and 0.05ml of conc. Sulphuric acid was added. Formation of a bright purplish red colour indicates the presence of gums and mucilage.

10. Detection of Fixed oils and fats

- **Stain test**

Small quantities of extracts were pressed between two filter papers. An oily stain on filter paper indicates the presence of fixed oils and fats.

11. Detection of resins

- **Acetone-water test**

Extracts were treated with acetone. Small amount of water was added and shaken. Appearance of turbidity indicates the presence of resins.

7.2.2.2. QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

- **Total flavonoid content** [52]

Aluminium chloride colorimetric method was used for Flavonoids determination. The plant extracts (0.5ml of 1:10 mg/ml in methanol) were separately mixed with 1.5ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1ml of 1M sodium acetate and 2.8ml of

distilled water. It was kept at room temperature for 30 minutes and absorbance of the reaction mixture was measured at 415nm with double beam UV spectrophotometer. The calibration curve was prepared by preparing Quercetin solutions at concentrations 10, 20, 30, 50 to 100µg/ml in methanol.

- **Total phenol content** [53]

Standard Gallic acid (10mg) was dissolved in 100ml distilled water in a volumetric flask (100µg/ml of stock solution). From the above stock solution, 0.5 to 2.5ml of aliquots was pipetted out into 25ml volumetric flasks. Then 10ml of distilled water and 1.5ml of Folin-Ciocalteu reagent, diluted according to the label specification were added to each of the above volumetric flasks. After 5 minutes, 4ml of 1M sodium carbonate was added and volume was made upto 25ml with distilled water. At the same time, the plant extracts (0.5ml of 1:10mg/ml) in methanol were separately mixed with above reagents. After 30 minutes, absorbance at 765nm was recorded and calibration curve for standard was plotted as absorbance Vs concentration. From this graph the amount of phenolic content was determined.

7.2.3. FLUORESCENCE ANALYSIS [54,55]

Fluorescence analysis was carried out according to the method of Chase and Pratt (1949) and Kokoshi et al. (1958) in day light and in UV light. The plant powders and extracts were treated with different solvents and the fluorescence was observed in day light and in near far UV light and results were tabulated.

7.2.4. CHROMATOGRAPHY [56,57]

Chromatographic technique is an important analytical tool in the separation, identification and estimation of different phytoconstituent presents in the plant extract.

7.2.4.1. THIN LAYER CHROMATOGRAPHY

Principle

Thin layer chromatography is a technique used for the separation, identification and estimation of single or mixture of components present in the various extracts. It is a reliable technique in which solute undergoes distribution between two phases, stationary phase and mobile phase. The separation is mainly based on the differential migration that occurs when a solvent flows along the thin layer of stationary phase. This may be achieved by partition and adsorption depending on stationary phase used.

TLC Plate preparation

The plates were prepared using TLC spreader. 40g of silica G was mixed with 85ml of water to prepare homogenous suspension and poured in a spreader. 0.25mm thickness of plates was prepared, air dried until transparency of the layer disappeared, then dried at 110˚C for 30 minutes and kept in a dessicator.

Selection of mobile phase

The solvent mixture was selected on the basis of the phytoconstituents present in each extract. Factors such as nature of components, stationary phase, mobile phase, polarity, influence the rate of separation of constituents.

Solvent system:

The extracts were run in following mobile phases:

Ethyl acetate: formic acid: glacial acetic acid: water :: 100:11:11:26

Hexane: ethyl acetate :: 6:4

Toluene: ethyl acetate: diethylamine :: 70:20:10

Detection was done under UV at 254nm and 365nm

7.2.4.2. HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY-FINGER PRINT PROFILE [58]

HPTLC is one of the versatile chromatographic method which helps in identification of compounds and thereby authentication of purity of herbal drugs. The time required in this method for the demonstration of most of the characteristic constituents of a drug is very quick and short. In addition to qualitative detection, HPTLC also provides semi-quantitative information on major active constituents of a drug, thus enabling an assessment of drug quality.

HPTLC serves as a convenient tool for finding the distribution pattern of phytoconstituents which is unique to each plant. The fingerprint obtained is suitable for monitoring the identity and purity of drugs and for detecting adulteration and substitution. HPTLC technique is helpful in order to check the identity, purity and standardize the quantity of active principles present in the herbal extract.

Instrument conditions

Sample used **:** Ethyl acetate extract

Instrument **:** CAMAG HPTLC

HPTLC Applicator **:** CAMAG LINOMAT IV

HPTLC Scanner **:** CAMAG TLC SCANNER II

Sample dilution **:** 10mg of sample dissolved in 1ml of ethyl acetate

Volume of injection **:** 20µl

Mobile phase **:** ethyl acetate: hexane:: 6:4

Lamp **:** deuterium 254nm

Stationary phase **:** TLC silicagel $60F_{254}$, Merck

Chromatographic condition

The estimation has been done using the following chromatographic conditions. Chromatography was performed on a 10×10 cm pre activated HPTLC silica gel 60 F254 plate. Samples were applied to the plate as 6mm wide band with an automatic TLC applicator Linomat IV with Nitrogen flow (CAMAG, Switzerland), 8mm from the bottom. Densitometric scanning was performed on CAMAG scanner II. The plates were pre washed.

7.3. PHARMACOLOGICAL STUDIES

7.3.1. ANTIOXIDANT ACTIVITY

7.3.1.1. Hydrogen peroxide scavenging method

The ability of the *Vitex agnus-castus* to scavenge hydrogen peroxide was determined according to the method of Ruch.[59] A solution of hydrogen peroxide (2mmol/l) was prepared in phosphate buffer (pH 7.4). *Vitex agnus-castus* (10-100µg/ml) were added to hydrogen peroxide solution (0.6ml). Absorbance of hydrogen peroxide at 230nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide.

$$\% \text{ scavenging activity } [H_2O_2] = \frac{A \text{ (control)} - A \text{ (standard)}}{A \text{ (control)}} \times 100$$

where A (control) – Absorbance of the control

A (standard) – Absorbance of the standard/extract

Based on the values, a graph was plotted.

7.3.1.2. Reducing power ability assay

The reducing power was determined according to the method of Oyaizu.[60] Various concentrations of the plant extracts (1ml) were mixed with 1ml of 200mmol/l sodium phosphate buffer (pH 6.6) and 1ml of 1% potassium ferricyanide. The mixtures were incubated at 50˚C for 20 minutes. 1ml of 10% trichloroacetic acid (w/v) was added, the mixture was centrifuged at 2000rpm for 10 minutes. The upper layer solution (2.5ml) was mixed with 2.5ml of deionised water and 0.5ml of fresh ferric chloride (0.1%). The absorbance was measured at 700nm.

7.3.2. *IN-VITRO* HEPATOPROTECTIVE ACTIVITY BY MTT ASSAY USING NORMAL CHANG LIVER CELL LINE

Materials and methods

Plant extract

- Hexane
- Ethyl acetate
- Ethanol

Reagents

- MEM – (Minimal Essential Media) purchased from Hi Media Laboratories
- FBS – (Fetal bovine serum) purchased from Cistron laboratories
- Trypsin, MTT – [3-(4,5-Dimethyl thiazol -2-yl) -2,5-diphenyl tetrazolium bromide] and DMSO (Dimethyl sulfoxide) were purchased from Sisco research laboratory chemicals Mumbai.

Cell line and culture

- Normal chang liver cell lines were obtained from National Centre for Cell Sciences, Pune (NCCS). The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100µg/ml) in a humidified atmosphere of 50µg/ml CO_2 at 37˚C.
- Maintenance of cultures was passaged weekly and the culture medium was changed twice a week.

Preparation of solutions

Toxicants

100mg of paracetamol were dissolved in 10ml of DMSO and diluted to 100ml with minimum essential medium. 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8 µg/ml solutions were prepared by diluting with distilled water.

Standard drug

100 mg of Silymarin were dissolved in 10ml of DMSO and diluted to 100ml with minimum essential medium. 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8 µg/ml solutions were prepared by diluting with distilled water.

Sample solutions

100mg of various extracts of test samples such as Hexane, Ethyl acetate and Ethanol were dissolved in 10ml of DMSO and diluted to 100ml with minimum essential medium. 1000, 500, 250, 125 62.5, 31.2, 15.6, 7.8 µg/ml solutions were prepared by diluting with distilled water.

MTT assay

Principle:

MTT assay is a standard colorimetric assay used for measuring the activity of the enzymes. It can also be used to determine cytotoxicity potential of medicinal agents and other toxic materials.

The assay is based on conversion of the MTT – [3-(4,5 – dimethyl thiazole -2-yl)-2,5-diphenyl tetrazolium bromide], a yellow tetrazole to a purple coloured formazan crystal by the active mitochondrial reductase (or cellular reductase) present in the viable cells. The purple colour thus formed is directly proportional to the viable cells present. This provides study of the cytotoxic activity of the test compounds. The absorbance of this coloured solution can be quantified by measuring at a wavelength of 500 to 600nm by spectrophotometer.

Procedure:

7.3.2.1. CYTOTOXICITY EVALUATION BY TETRAZOLIUM (MTT) ASSAY [61]

- The Chang liver monolayer cells were detached with Trypsin-ethylene Diamine tetra acetic acid (EDTA) to make single cell suspensions and the viable cells were counted using a haemocytometer and diluted with medium along with 5% FBS to give final density of 1×10^5cells/ml.
- Cells (1×10^5/ well) were plated in 5ml of medium/well in 96 well plates (Coster Corning, Rochester, NY).

- After 48 hours incubation the cell reaches the confluence. Then, cells were incubated with different concentrations of Silymarin, Hexane, Ethylacetate and Ethanol, for 24-48hrs at 37˚C.
- After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 1ml/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide cells (MTT) phosphate-buffered saline solution was added.
- After 4 hours incubation, 0.04M HCl/ isopropanol were added.
- Viability of cells were determined by measuring the absorbance at 570nm using UV spectrophotometer and wells not containing sample were treated as blank.
- Measurements were performed and the concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically.
- Triplicate was maintained for all concentrations.
- The effect of the samples on the proliferation of Chang Liver cells was expressed as the % cell viability, using the following:

$$\textbf{\% cell viability} = \frac{\textbf{A 570 of treated cells}}{\textbf{A 570 of control cells}} \times 100$$

7.3.2.2. *IN VITRO* HEPATOPROTECTIVE ACTIVITY USING VARIOUS EXTRACTS AGAINST PARACETAMOL INDUCED HEPATOTOXICITY (MTT ASSAY) [62]

- The Chang liver monolayer cells were detached with trypsin-ethylene diamine tetra-acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a Haemocytometer and diluted with medium along with 5% FBS to give final density of 1×10^5 cells/ml.
- Cells (1×10^5/well) were plated in 5ml of medium/well in 96 well plates (Costar Corning, Rochester, NY).
- After 48 hours incubation the cell reaches the confluence. Then, cells were challenged with Hepatotoxicant (Paracetamol) 125µg/ml and different concentrations of various extracts (Hexane, Ethyl acetate and Ethanol) and the Standard drug (Silymarin) were added. The cells were incubated for 24- 48 hours at 37˚C.
- After removal of the sample solution and washing with phosphate-buffered saline (pH-7.4), 1ml/well (5mg/ml) of 0.5% 3-(4,5- dimethyl -2- thiazolyl)-2, 5-diphenyl-terazolium bromide cells (MTT) phosphate – buffered saline solution was added.

- After 4 hours incubation, 0.04M HCl/ isopropanol was added.
- The absorbance at 570nm was measured with a UV- Spectrophotometer. The wells without sample acted as blank.
- Triplicate was maintained for all concentrations.

7.3.3. *IN-VIVO* STUDIES

Model – Paracetamol induced Hepatotoxicity in Wistar Albino rats

Selection of the active extract

- In the *in vitro* method, the ethyl acetate extract exhibited the high percentage of protection paracetamol induced Hepatotoxicity using Normal Chang liver cell line.
- Hence ethyl acetate extract was selected for *in vivo* study.

Materials and Methods

Plant extract

Ethyl acetate extract of berries of *Vitex agnus castus*

Drugs and chemicals

- Paracetamol (South East Pharmaceuticals, India)
- Silymarin (Sigma Aldrich)
- Carboxymethyl cellulose

Preparation of drug solutions

The paracetamol, ethylacetate extract and standard drug (Silymarin) were suspended in 1% Carboxymethyl cellulose and used for the study.

Animal selection and procurement

Healthy adult Wistar albino rats (weighing 100-150g) of either sex were procured from Animal Experimental Laboratory, Madras Medical College, Chennai-03. Approval from the Institutional Ethical Committee was obtained for carrying out the study (8/243/CPCSEA dated 22/11/13).

The procured animals were kept in a clean and dry, polycarbonate cages and maintained in a well-ventilated animal house. The temperature of experimental animal room was maintained at room temperature and the relative humidity was maintained at 50-60%. Lighting was maintained for 12hrs dark and 12hrs light. All the animals were kept in the cages for at least 5 days prior to dosing for acclimatization to the laboratory conditions. The

animals were fed with normal diet and water was given *ad libitum.* The animals were fasted overnight but allowed to access water initially before the start of the study.

7.3.3.1. ACUTE ORAL TOXICITY STUDIES [63]

The acute oral toxicity study was carried out for ethylacetate extract using OECD guidelines 423 (Organization of economic co-operation and development). A single dose of 2000mg/kg p.o. was given and this was used as a starting dose. After oral administration, the animals were observed every 1 hour for 24 hours to assess the general behaviour and mortality. They were further observed for 72 hours for toxic symptoms and mortality of the animal. The flow chart in figure depicts the procedure adopted for this method. **(Fig. 2)**

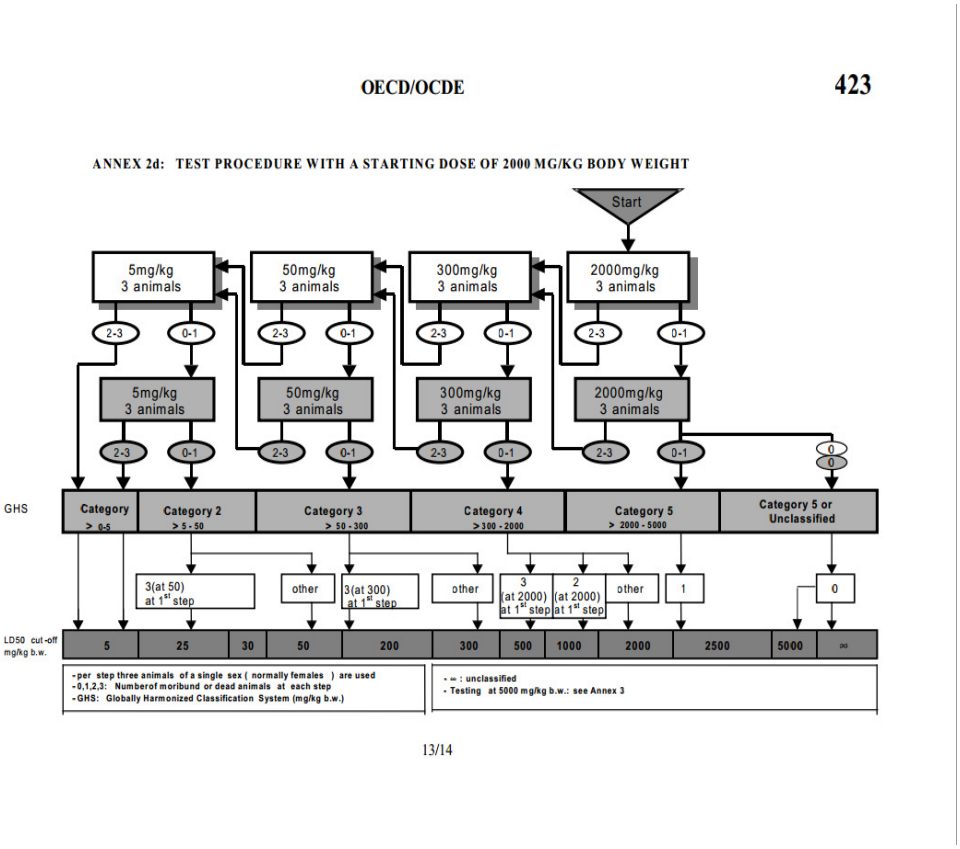


Fig. 2. Flow chart for acute toxicity studies

7.3.3.2. HEPATOPROTECTIVE ACTIVITY

Experimental method [29,31,64]

MODEL: Paracetamol induced hepatotoxicity in Wistar albino rats

Rats are divided into five groups consisting of 6 animals each.

Table 1. Paracetamol induced hepatotoxicity in Wistar albino rats

GROUPS		**TREATMENT SCHEDULE**
Group 1	Normal control	Normal food and water
Group 2	Disease control	Vehicle (water) followed by paracetamol(2g/kg) p.o single dose on 7th day
Group 3	Standard control	Silymarin (100mg/kg) p.o for 7 days + Paracetamol (2g/kg) p.o single dose on 7th day
Group 4	Test group I	Ethyl acetate extract 200mg/kg p.o for 7 days + Paracetamol (2g/kg) p.o single dose on 7th day
Group 5	Test group II	Ethyl acetate extract 400mg/kg p.o for 7 days + Parcetamol (2g/kg) p.o single dose on 7th day

On the 9th day of the experiment, the rats were fasted overnight and sacrificed by cervical dislocation method. The blood samples were collected by retro orbital puncture with glass capillary and allowed to clot and centrifuged for 15- 20 minutes at 2000 rpm and the serum collected was used for assay of marker enzymes SGOT, SGPT, ALP, total protein and total bilirubin.

Histopathology:

The abdomen was cut open and the liver was dissected out. A portion of liver tissue was taken in each group and was immediately put in 10% formasal (formalin diluted to 10% with normal saline) and then it was processed.

Sections were stained with Ehrlich's haematoxylin and eosin to view the necrotic lesions of liver microscopically.

Statistical analysis:

Data were expressed as mean ± S.E.M. Statistical comparison between the groups were done by one way analysis of variance (ANOVA) followed by t test. p values < 0.05 were considered significant.

8. RESULTS AND DISCUSSION

8.1. PHARMACOGNOSTICAL STUDIES

The results of Pharmacognostical studies are as follows:

8.1.1. Macroscopical features:

Berries

Colour - greenish black

Shape - round or ovoid

Odour - aromatic

Taste - bitter and aromatic

Size - 2 to 4mm in diameter

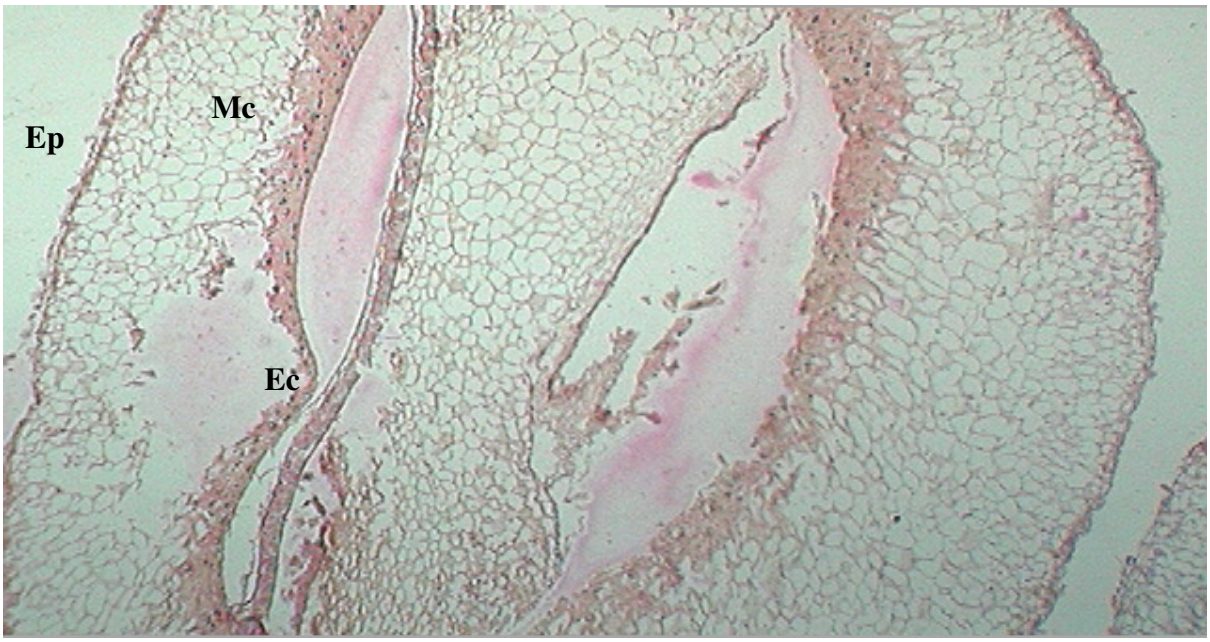
Surface - hairy

Fig. 3. *Vitex agnus-castus* berries

8.1.2. Microscopical features

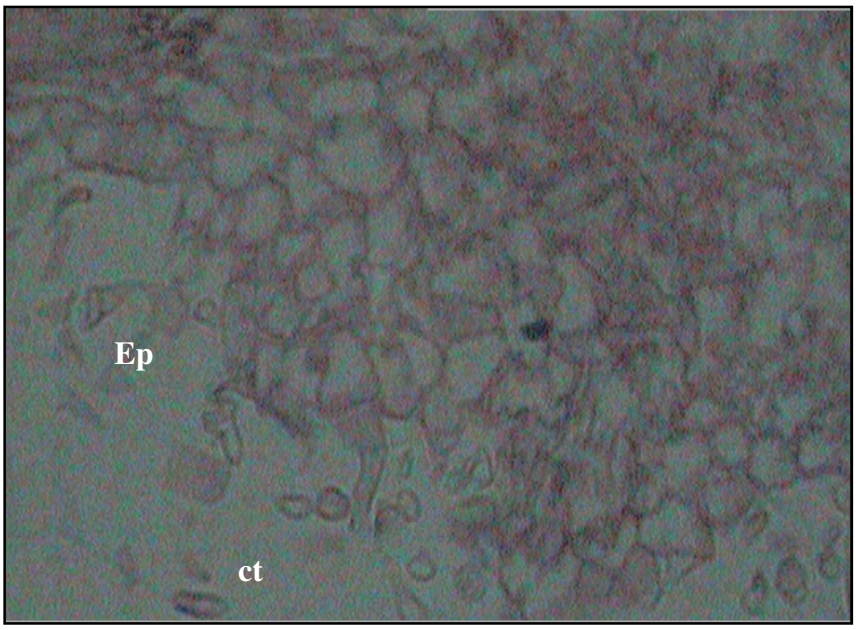
Transverse section:

Epicarp is composed of polygonal cells with thickened walls and some cells have shown simple pits. The epidermis shows the presence of covering and glandular trichomes. Glandular trichome is composed of single celled stalk and a four celled head. Mesocarp consists of isodiametric parenchyma cells with pitted walls. Outer mesocarp cells are brown in nature. Small vascular bundles are arranged in a circle. Small brachysclerides are seen in innermost cell layers. The cells are pigmented near the endosperm region. Starch grains are present in the endosperm region.



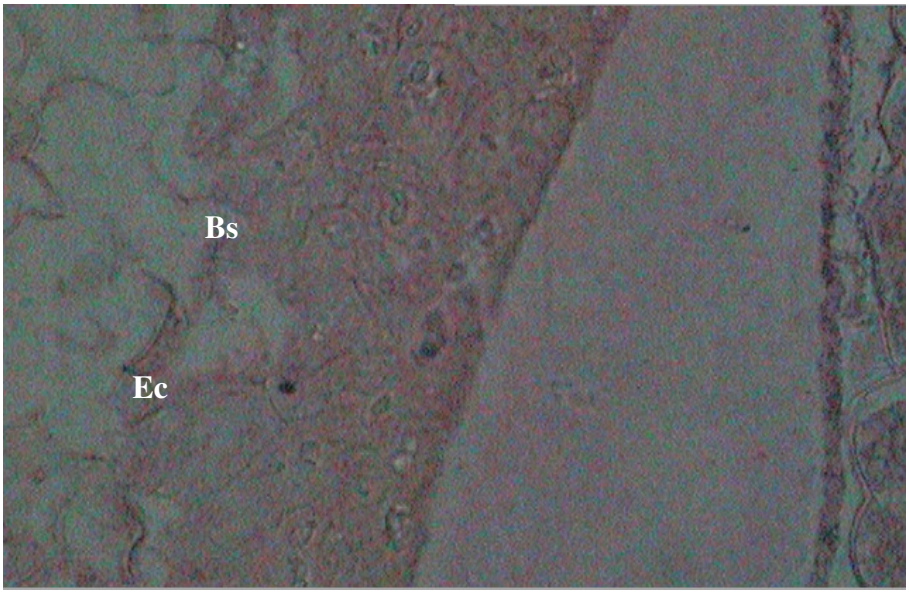
Ep – Epicarp, Mc – Mesocarp, Ec - Endocarp

Fig. 4. T.S. of Vitex (4X) entire fruit



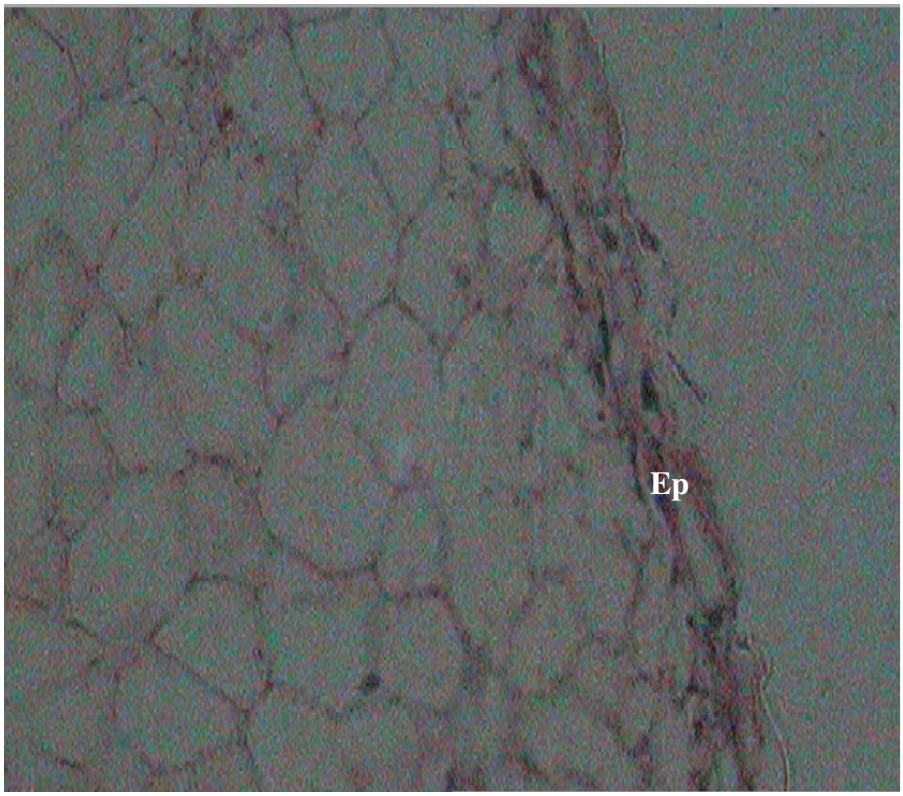
Ep – Epicarp, ct – covering trichome

Fig. 5. Vitex fruit (40X) epicarp



Ec – Endocarp, Bs – brachyscleride

Fig. 6. Vitex fruit endocarp (40X)

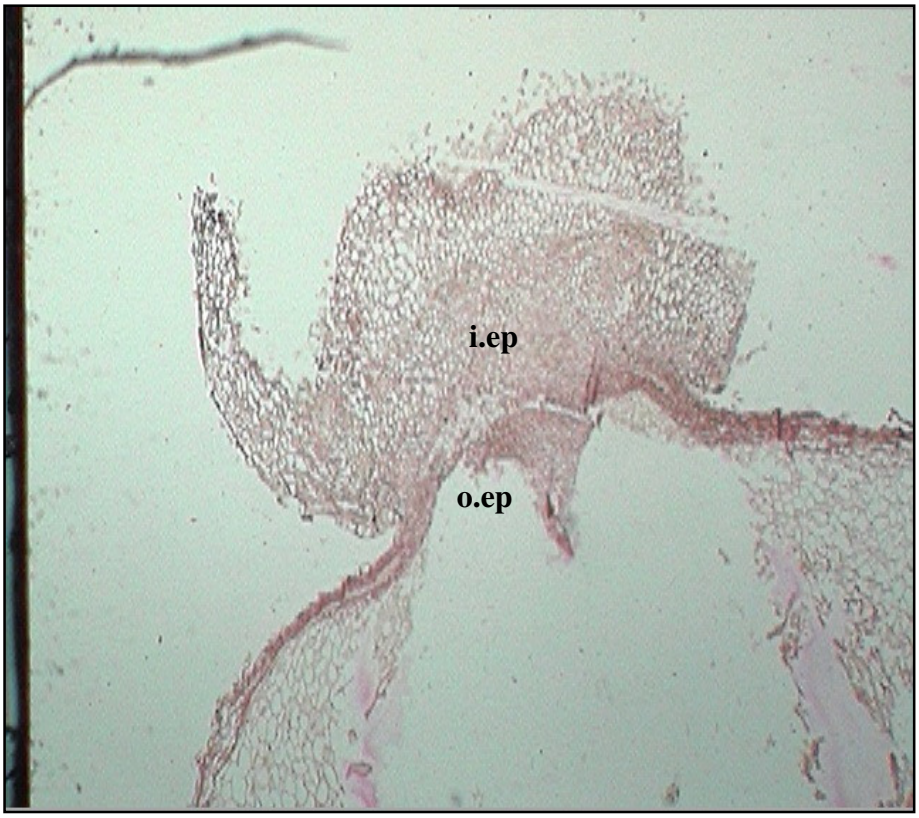


Ep - Epicarp

Fig. 7. Vitex fruit epicarp (40X)

Calyx:

Outer epidermis is composed of compactly arranged small polygonal cells, covering and glandular trichome. The covering trichomes consist of 1 to 4 cells and glandular trichomes consist of a very small unicellular stalk and a 4-celled glandular head. Stomata can also be seen on the outer epidermis. The inner epidermis is glabrous and composed of rectangular elongated cells with irregular cell walls. The inner epidermis shows sclerides in the longitudinal direction.



i.ep - inner epidermis, o.ep – outer epidermis

Fig. 8. T.S. of Calyx (4X)

8.1.3. Powder microscopy:

The powder microscopy showed the presence of mesocarp cells with starch grains, unicellular covering trichomes, xylem vessels, cortical parenchyma cells, brachysclerides, collenchyma cells and epicarp which are shown in the figures below.

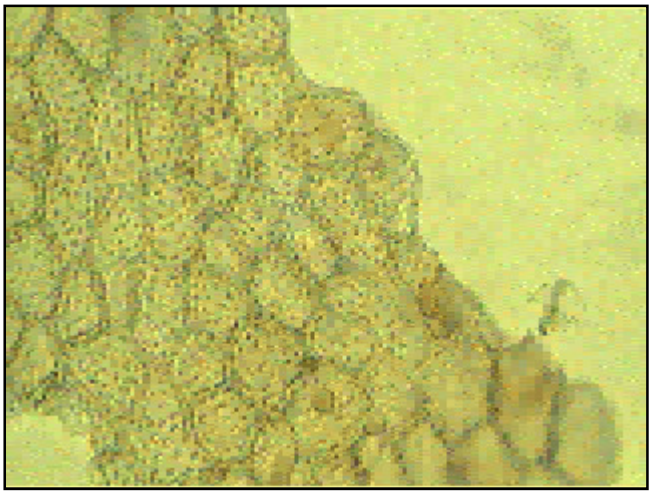


Fig. 9. Mesocarp cells with starch grains

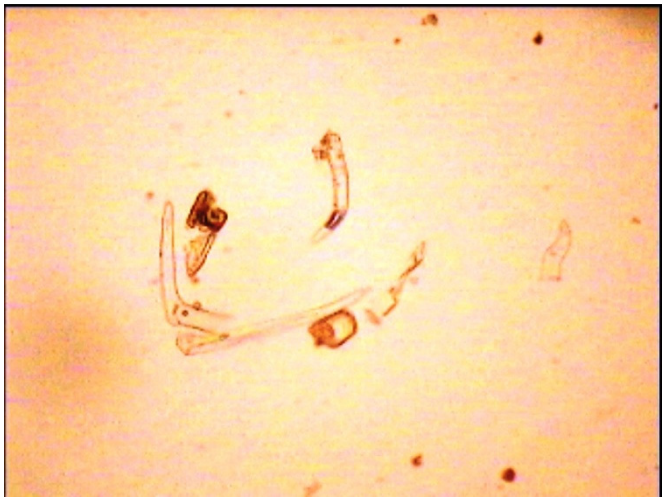


Fig. 10.Unicellular covering trichomes

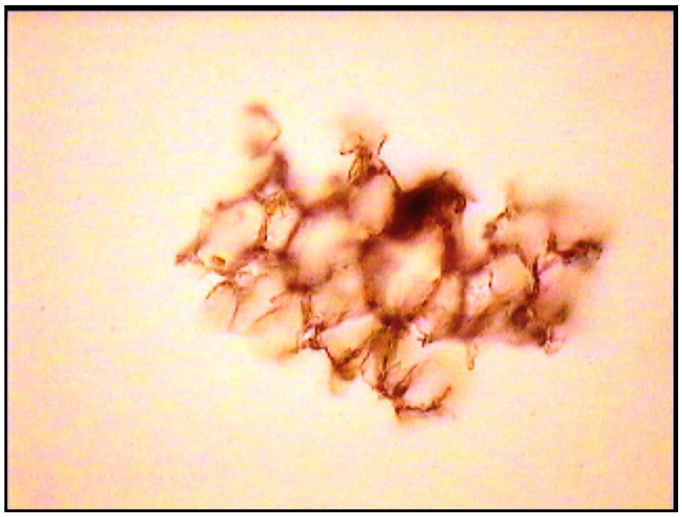
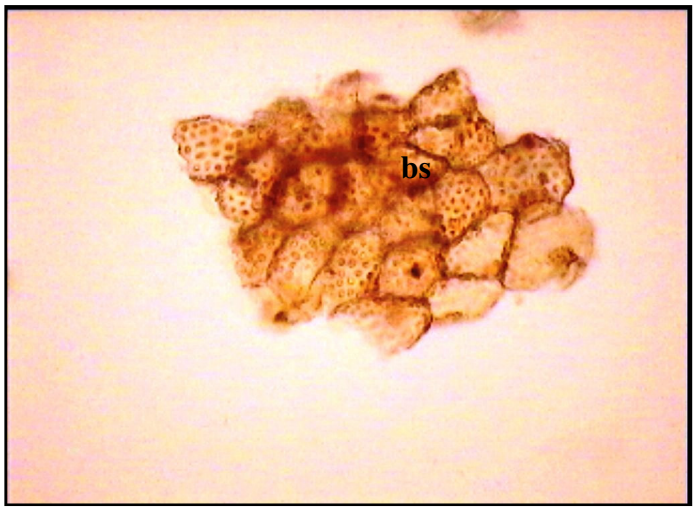


Fig. 11. Xylem vessels

- **Xylem – thickening of vascular elements**
- **Mesocarp showing cortical parenchyma with pigments**



bs – brachysclerides

Fig. 12. Cellular contents with parenchyma cells

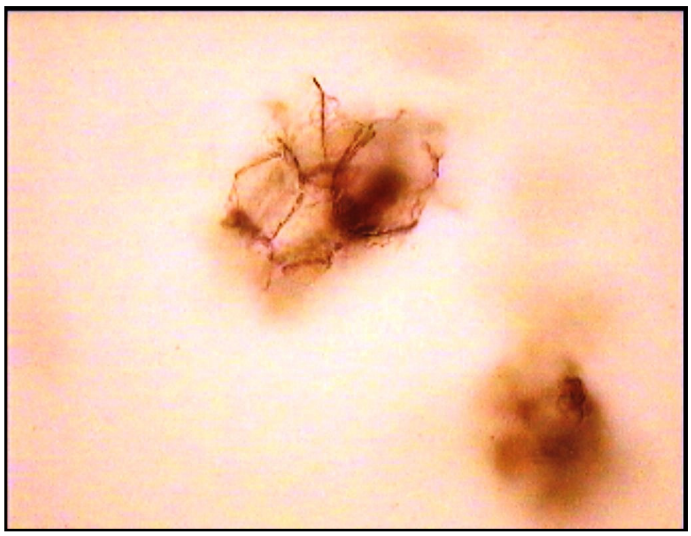


Fig. 13. Parenchyma cells with lignified cells

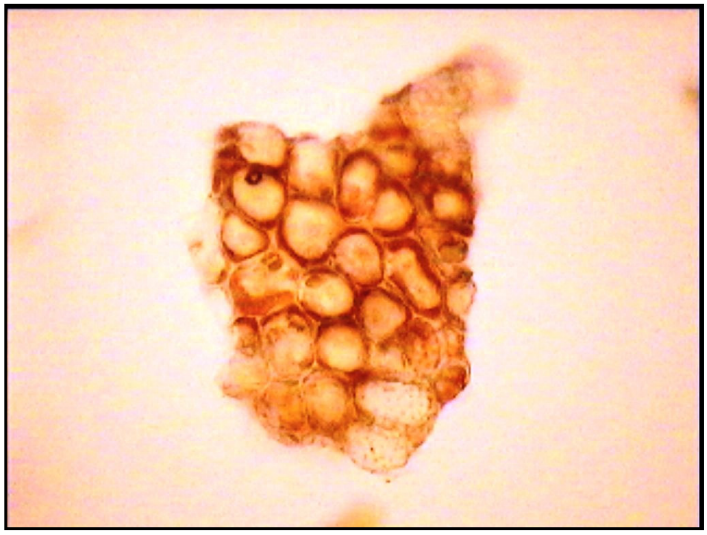


Fig. 14. Collenchyma with thickened wall

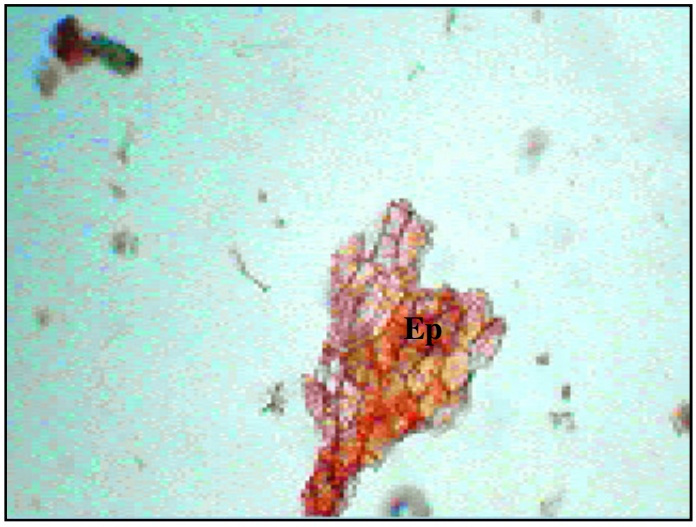


Fig. 15. Ep - Epicarp

8.1.4. Physicochemical constants

The physicochemical studies of the berries of *Vitex agnus-castus* showed the following results (**Table 2,3,4):**

Table 2. Physicochemical constants- ash values

S.No.	ASH VALUES	%W/W
1.	Total ash	10.55±0.69
2.	Water soluble ash	5.2±0.16
3.	Acid insoluble ash	3.4±0.17
4.	Sulphated ash	12.48±0.18

Values are expressed as Mean ± S.E.M. (n=3)

The total ash, water soluble ash, acid insoluble and sulphated ash of the berries of *Vitex agnus-castus* were found to be 10.55±0.69, 5.2±0.16, 3.4±0.17 and 12.48±0.18 respectively.

Table 3. Physicochemical constants- extractive values

S.No.	EXTRACTIVE VALUES	%W/W
1.	Alcohol soluble extractive value	10.86±0.75
2.	Water soluble extractive value	14.8±0.72
3.	Non-volatile ether soluble extractive value	4.6±1.04
4.	Volatile ether soluble extractive value	3.5±0.50

Values are expressed as Mean ± S.E.M.(n=3)

The alcohol soluble extractive and water soluble extractive were found to be 10.86±0.75 and 14.8±0.72. Non-volatile and volatile ether soluble extractives were found to be 4.6±1.04 and 3.5±0.50 respectively.

Table 4. Physicochemical constants

S.No.	PHYSICO-CHEMICAL CONSTANT	%W/W
III	**Loss on drying**	3.73±1.10
IV	**Foaming index**	<100
V	**Swelling index**	Nil

Values are expressed as Mean ± S.E.M. (n=3)

The loss on drying was found to be 3.73±1.10 and foaming index was less than 100. Swelling index was nil.

8.1.5. Qualitative analysis of inorganic elements and heavy metal analysis

The chemical studies on the powder showed the presence of several inorganic elements which tabulated in **Table 5.**

Table 5. Qualitative analysis of inorganic elements of *Vitex agnus-castus*

S.No.	INORGANIC ELEMENTS	REPORT
1.	Aluminium	+
2.	Arsenic	+
3.	Boron	-
4.	Calcium	+
5.	Carbonate	-
6.	Chlorides	-
7.	Copper	+
8.	Iron	+
9.	Lead	+
10.	Magnesium	+
11.	Sulphate	+
12.	Nitrate	-
13.	Phosphate	-
14.	Potassium	+

+ indicates presence, - indicates absence

The qualitative analysis showed the presence of copper, calcium, magnesium, potassium, aluminium, iron. The quantitative estimation of inorganic elements were carried out and tabulated in **Table 6.**

Table 6. Quantitative estimation of inorganic elements of *Vitex agnus-castus*

S.No.	INORGANIC ELEMENTS	TOTAL AMOUNT ppm
1.	Copper	0.185
2.	Calcium	0.261
3.	Magnesium	0.049
4.	Potassium	0.125
5.	Aluminium	0.145

8.1.6. Heavy metal analysis of *Vitex agnus-castus*

The quantification of the individual heavy metals was performed for the powdered berries of *Vitex agnus-castus* by ICP-OES technique given in **Table 7**.

Table 7. Heavy metal analysis of *Vitex agnus-castus*

S.No.	INORGANIC ELEMENTS	OSERVATION IN ppm	STANDARD LIMITS(ppm)
1.	Arsenic	0.008	5
2.	Cadmium	0.004	0.3
3.	Lead	0.020	10
4.	Iron	0.057	10

The study showed that the heavy metals arsenic, cadmium, lead and iron were well within the standard permitted limits of WHO.

8.2. PHYTOCHEMICAL STUDIES:

8.2.1. Percentage yield of successive solvent extraction of berries of *Vitex agnus-castus.*

Extraction was carried out with solvents of increased polarity for the berries of *Vitex agnus-castus* and percentage yield was determined and tabulated (**Table 8**).

Table 8. % yield of successive solvent extraction of berries of *Vitex agnus-castus.*

S.NO.	EXTRACTS	METHOD OF EXTRACTION	PHYSICAL NATURE	COLOUR	% YIELD (W/W)
1.	Hexane	Continuous hot percolation	Semi-solid	Pale green	8.4
2.	Ethyl acetate		Sticky	Greenish black	17.2
3.	Ethanol		Semi-solid	Brownish black	11.4

The percentage yield of ethyl acetate extract was more compared to the other extracts.

8.2.2. Preliminary phytochemical screening on the berries of *Vitex agnus-castus*

Qualitative phytochemical analysis for the powder and various extracts of the berries of *Vitex agnus-castus* was performed. The results are given in **Table 9.**

Table 9. Preliminary phytochemical screening on the berries of *Vitex agnus-castus*

S.No.	PHYTOCONSTITUENTS	POWDER	HEXANE EXTRACT	ETHYL ACETATE EXTRACT	ETHANOL EXTRACT
1.	Carbohydrates	+	-	+	+
2.	Flavonoid	+	-	+	+
3.	Glycosides	+	-	+	-
4.	Alkaloid	+	-	+	+
5.	Saponin	+	-	+	-
6.	Phytosterols	+	+	-	-
7.	Phenolic compounds	+	-	+	+
8.	Proteins	-	-	-	-
9.	Fixed oils and fats	-	-	-	-
10.	Tannins	+	-	+	-
11.	Triterpenoids	+	+	+	+
12.	Gums and mucilage	-	-	-	-

+ indicates presence, - indicates absence

The hexane extract of the berries showed the presence of phytosterols and triterpenoids. The ethyl acetate extract revealed maximum number of active constituents such as carbohydrates, flavonoids, glycosides, alkaloids, saponins, phenolic compounds, tannins and triterpinoids. The ethanol extract showed the presence of carbohydrates, flavonoids, alkaloids, phenolic compounds and triterpenoids.

8.2.3. Quantitative estimation of phytoconstituents

Total flavonoid content: The absorbance of the standard quercetin at different concentrations and the absorbance of ethyl acetate and ethanol were found out and tabulated in **Table 10.**

Table 10. Absorbance of quercetin, ethyl acetate & ethanol

QUERCETIN	
CONCENTRATION (mg/ml)	**ABSORBANCE**
20	0.15
40	0.22
60	0.28
80	0.36
100	0.47
Ethyl acetate	0.34
Ethanol	0.31

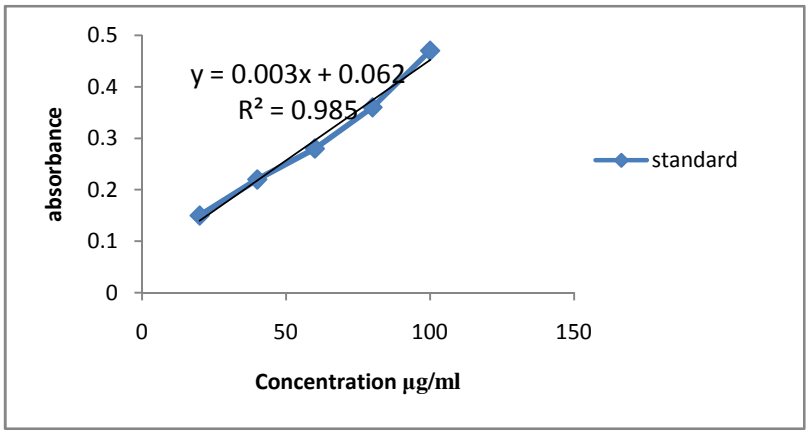


Fig. 16. Calibration curve of standard quercetin

The concentration of Flavonoid present in Ethyl acetate extract was found to be 92.66µg equivalent to Quercetin in 1mg(9.3%) and in Ethanol extract was found to be 8µg equivalent to Quercetin in 1mg(8%). The ethyl acetate extract was found to have higher amount of flavonoid than ethanol extract.

Total Phenol content: The absorbance of the standard gallic acid at different concentrations and the absorbance of ethyl acetate and ethanol were found out and tabulated in **Table 11.**

Table 11. Absorbance of gallic acid, ethyl acetate & ethanol

GALLIC ACID	
CONCENTRATION (mg/ml)	**ABSORBANCE**
2	0.05
4	0.08
6	0.12
8	0.17
10	0.22
Ethyl acetate	0.19
Ethanol	0.13

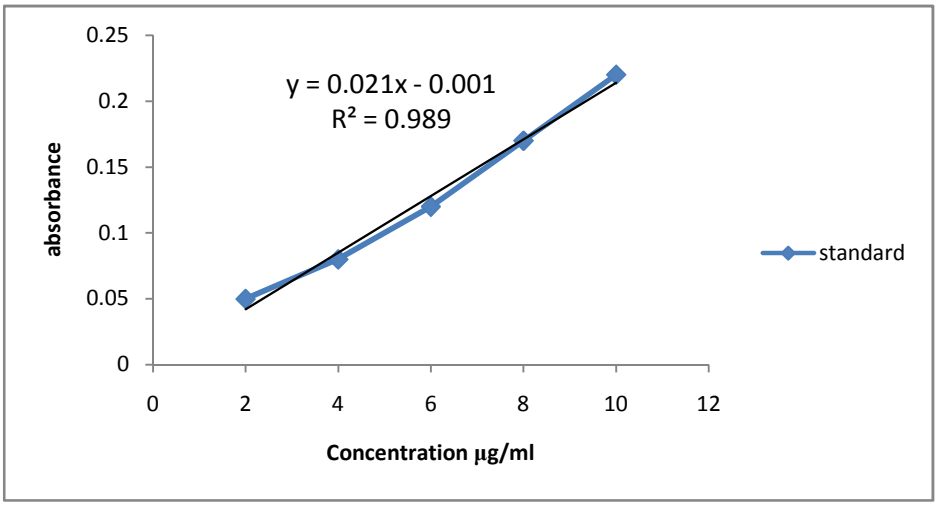


Fig. 17. Calibration curve of standard gallic acid

The concentration of phenol present in Ethyl acetate extract was found to be 9µg equivalent to Gallic acid in 1mg(0.9%) and in Ethanol extract was found to be 6.12µg equivalent to Gallic acid in 1mg(0.61%). The ethyl acetate extract was found to have higher amount of phenols than ethanol extract.

8.2.4. Fluorescence analysis

The fluorescence analysis of powdered drug and various extract of *Vitex agnus-castus* are tabulated in **Table 12.**

Table 12. Fluorescence analysis of powder of *Vitex agnus-castus.*

S.No.	POWDERED DRUG	DAY LIGHT	UV LIGHT	
			SHORT	LONG
1.	Powder	Greenish brown	Dark brown	Brown
2.	Powder +water	Light brown	Brownish green	Dark brown
3.	Powder +1N HCl	Light brown	Brownish black	Brown
4.	Powder +1N H_2SO_4	Brownish black	Reddish black	Light brown
5.	Powder +1N HNO_3	Pale brown	Reddish green	Yellowish green
6.	Powder +CH_3COOH	Brown	Reddish brown	Pale green
7.	Powder +1N NaOH	Green	Reddish brown	Pale green
8.	Powder +alc.NaOH	Greenish brown	Greenish black	Green
9.	Powder +1N KOH	Green	Greenish brown	Green
10.	Powder +alc.KOH	Brownish green	Reddish brown	Pale green
11.	Powder +NH_3	Greenish yellow	Green	Brownish red
12.	Powder +I_2	Green	Reddish green	Brownish green
13.	Powder +$FeCl_3$	Pale brown	Greenish black	Greenish brown
14.	Powder +ethanol	Light brown	Greenish black	Greenish brown

Table 13. Fluorescence analysis of the various extracts of *Vitex agnus-castus.*

S.No.	EXTRACTS	DAYLIGHT	UV LIGHT	
			SHORT	LONG
1.	Hexane	Pale green	Reddish brown	Green
2.	Ethyl acetate	Greenish black	Black	Light brown
3.	Ethanol	Brownish black	Brownish black	Light brown

No characteristic fluorescence was observed in powdered sample as well the extracts.

8.2.5. Thin layer chromatography

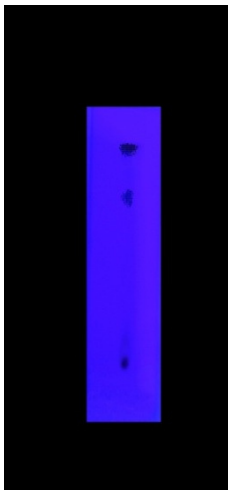
Since the both the ethyl acetate and ethanol extract showed the presence of flavonoids and alkaloids, TLC was carried out with mobile phases for these two phytoconstituents and detected under UV chamber.

Table 14. TLC of Flavonoids

EXRTACTS	SOLVENT SYSTEM	NO.OF SPOTS	R_F VALUE
Ethyl acetate	Ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:27)	2	0.36 (spot 1) 0.61 (spot 2)
Ethanol		2	0.32 (spot 1) 0.54 (spot 2)

The ethylacetate extract showed two spots with Rf value 0.36 and 0.61. The ethanol extract also showed two spots with Rf value 0.32 and 0.54. **Fig.18.1, 18.2**

Ethyl acetate extract



Ethanol extract

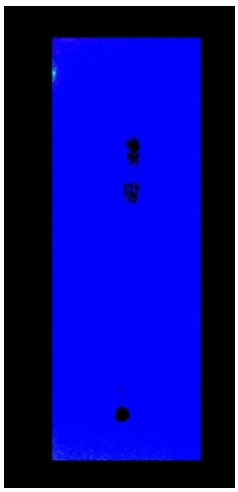
Fig. 18.1-18.2 TLC of flavonoids

Table 15. TLC of Alkaloids

EXTRACTS	SOLVENT SYSTEM	NO.OF SPOTS	R_F VALUE
Ethyl acetate	Toluene: ethyl acetate: diethyl amine (70:20:10)	2	0.45 (spot 1) 0.63 (spot 2)
Ethanol		2	0.42 (spot 1) 0.60 (spot 2)

The ethylacetate extract showed two spots with Rf value 0.45 and 0.63. The ethanol extract also showed two spots with Rf value 0.42 and 0.60. **Fig.19.1, 19.2**

Ethyl acetate extract



Ethanol extract

Fig. 19.1-19.2 TLC of alkaloids

8.2.6. High performance thin layer chromatography

TLC was run for the ethyl acetate before performing the HPTLC **(Fig. 19).**

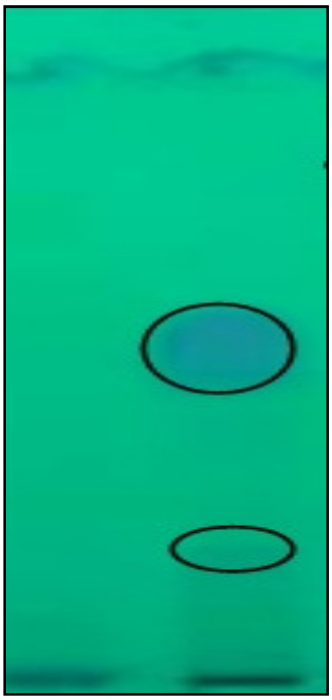


Fig. 20. TLC of Ethyl acetate extract

Ethyl acetate: hexane :: 6:4

TLC showed the presence of two spots.

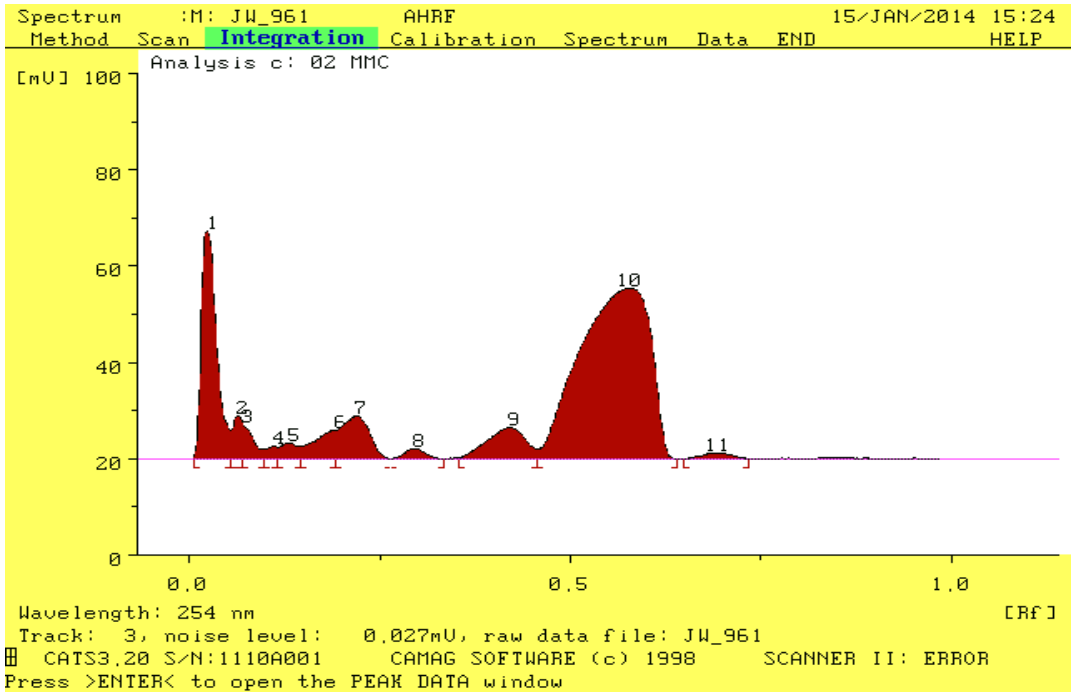


Fig. 21. HPTLC chromatogram of ethyl acetate extract of *Vitex agnus-castus*

Table 16. Rf values and area of ethyl acetate extract of *Vitex agnus-castus*

S. No.	Rf	HEIGHT	AREA	LAMBDA
1	0.02	41.5	718.9	278
2	0.06	9.0	78.9	273
3	0.07	7.2	75.3	253
4	0.11	2.6	27.8	379
5	0.13	3.3	58.3	400
6	0.19	6.0	127.0	294
7	0.21	8.9	259.4	293
8	0.29	2.2	44.7	288
9	0.42	6.5	256.8	294
10	0.58	35.6	2352.2	253
11	0.69	1.3	39.4	356

The HPTLC chromatogram shows the presence of 11 peaks with their corresponding area and this indicates the presence of a number of phytoconstituents in the Ethyl acetate extract.

8.3. PHARMACOLOGICAL STUDIES

8.3.1. ANTI OXIDANT ACTIVITY

8.3.1.1. Hydrogen peroxide scavenging method: The percentage inhibition of the standard and the various extracts were found out and tabulated in **Table 17.**

Table 17. H_2O_2 scavenging method

S. No.	CONCENTRATION µg/ml	% INHIBITION			
		STANDARD (ASCORBIC ACID)	ETHYL ACETATE EXRTACT	ETHANOL EXTRACT	HEXANE EXTRACT
1.	10	13.54	13.35	12.89	9.12
2.	20	26.79	25.95	24.72	22.35
3.	30	48.93	47.76	46.91	43.67
4.	40	59.21	58.89	57.54	48.53
5.	50	71.36	69.45	62.53	57.35

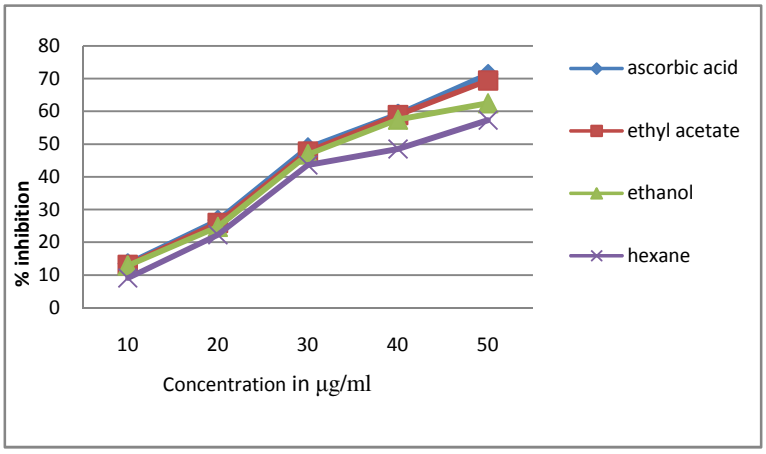


Fig. 22. H_2O_2 scavenging assay

IC_{50} values were found to be:

Standard (Ascorbic acid) - 33.53µg/ml, Ethyl acetate extract - 34.02µg/ml, Ethanol extract - 36.03µg/ml and Hexane extract - 40.30µg/ml.

From the results it is clear that the ethyl acetate extract shows the most potent antioxidant activity compared to the other two extracts. The IC_{50} value of the ethyl acetate extract is equivalent to that of the standard drug Ascorbic acid.

8.3.1.2. Reducing power ability assay: The percentage inhibition of the standard and the various extracts were found out and tabulated in **Table 18.**

Table 18. Reducing power ability assay

S. No.	CONCENTRATION (µg/ml)	% INHIBITION			
		STANDARD (ASCORBIC ACID)	ETHYL ACETATE EXTRACT	ETHANOL EXTRACT	HEXANE EXTRACT
1.	10	14.17	13.95	13.12	12.45
2.	20	27.56	25.86	25.67	22.89
3.	30	49.53	48.75	47.34	42.73
4.	40	60.27	59.12	53.62	49.12
5.	50	72.31	70.45	68.45	57.91

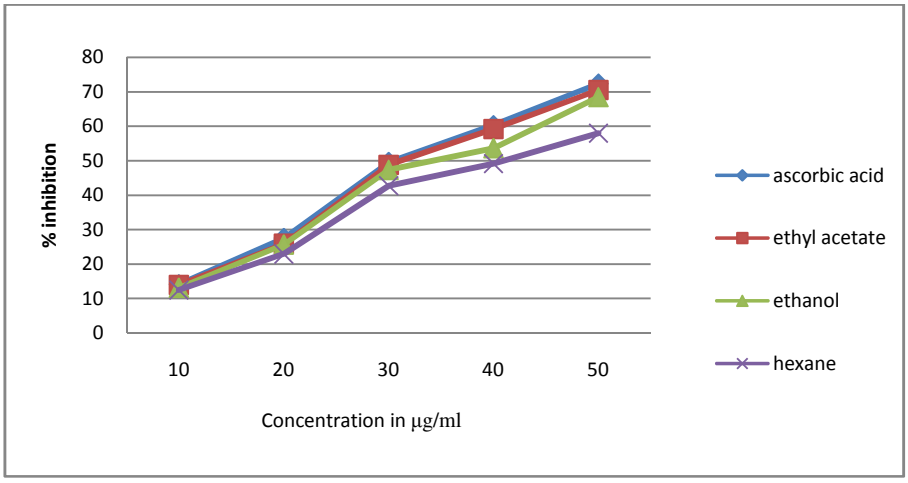


Fig. 23. Reducing power ability assay

IC_{50} values were found to be:

Standard (Ascorbic acid) - 33.53µg/ml, Ethyl acetate extract - 34.02µg/ml, Ethanol extract - 36.03µg/ml and Hexane extract - 41.09µg/ml.

From the results it is clear that the ethyl acetate extract shows the most potent antioxidant activity compared to the other two extracts. The IC_{50} value of the ethyl acetate extract is equivalent to that of the standard drug Ascorbic acid.

8.3.2. *In vitro* studies:

8.3.2.1. Cytotoxicity evaluation by tetrazolium (MTT) assay

The cytotoxicity studies were carried out for the standard silymarin and different extracts of the berries of *Vitex agnus- castus* on the Chang liver cell line over different concentration range (1000, 500, 250, 125, 62.5, 32.2, 15.6, 7.8µg/ml) and its CTC_{50} value was found out.**(Table 19)**

Table 19. Cytotoxicity studies

S.No.	CONCENTRATION (mg/ml)	% CELL VIABILITY			
		SILYMARIN	HEXANE EXRTACT	ETHYL ACETATE EXTRACT	HEXANE EXTRACT
1.	1000	6.77	15.25	11.86	8.47
2.	500	18..64	25.42	22.03	18.64
3.	250	32.20	35.59	33.89	32.20
4.	125	40.67	42.37	44.06	40.67
5.	62.5	51.84	47.45	50.84	48.15
6.	31.2	69.49	54.23	62.71	57.62
7.	15.6	79.66	62.71	76.27	71.18
8.	7.8	89.83	69.49	88.13	83.05
9.	Cell control	100	100	100	100

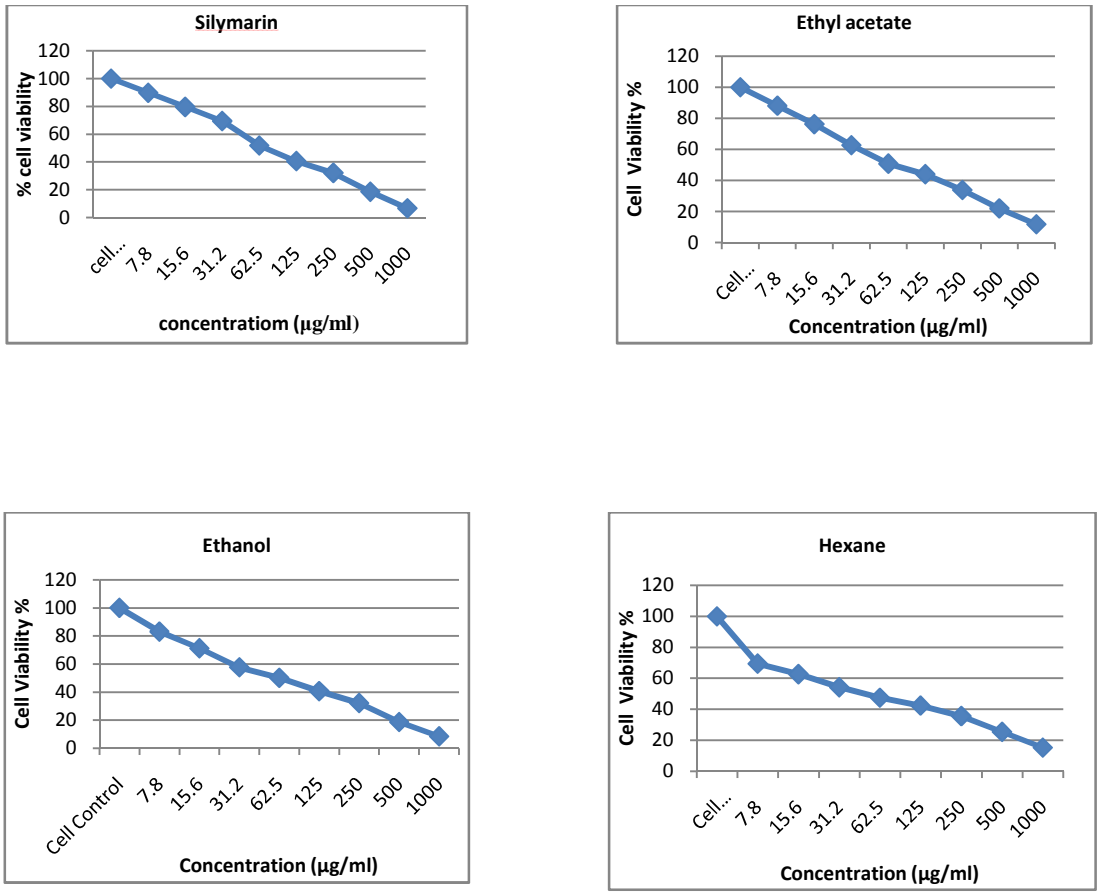


Fig. 24. Cytotoxicity of different extracts on Chang cell line

The cell viability decreased with increase in concentration of the test compounds. The CTC_{50} values of the extracts were found to be 62.5µg/ml which is similar to that of the standard silymarin thus proving that the extracts have no toxicity against the normal cell line.

8.3.2.2. *In vitro* hepatoprotective activity using various extracts against Paracetamol induced hepatotoxicity :

The Chang liver cells were first challenged with the paracetamol at the concentration of 125µg/ml in which 39.92% cells are viable. Then the cell line was treated with standard silymarin and different extracts of *Vitex agnus-castus* in the concentration of 100, 50, 25, 10µg/ml to assess the percentage cell viability and the values are tabulated in **Table 20**.

Table 20. *In vitro* hepatoprotective activity

S.No.	TREATMENT	CONCENTRATION Mg/ml	% CELL VIABILITY
1.	Control		100
2.	Paracetamol	125	39.92±1.02
3.	Paracetamol + Silymarin	10	55.9±1.15
		25	60.4±1.04
		50	65.8±1.43
		100	71.3±1.09
4.	Paracetamol + ethyl acetate extract	10	49.8±1.24
		25	58.3±1.31
		50	62.5±1.57
		100	72.9±1.43
5.	Paracetamol + Ethanol extract	10	36.6±1.83
		25	47.9±1.26
		50	52.5±1.74
		100	58.7±1.89
6.	Paracetamol + Hexane extract	10	32.6±1.55
		25	41.3±1.21
		50	49.2±1.92
		100	57.1±1.34

Values are expressed as Mean ± S.E.M. (n=3)

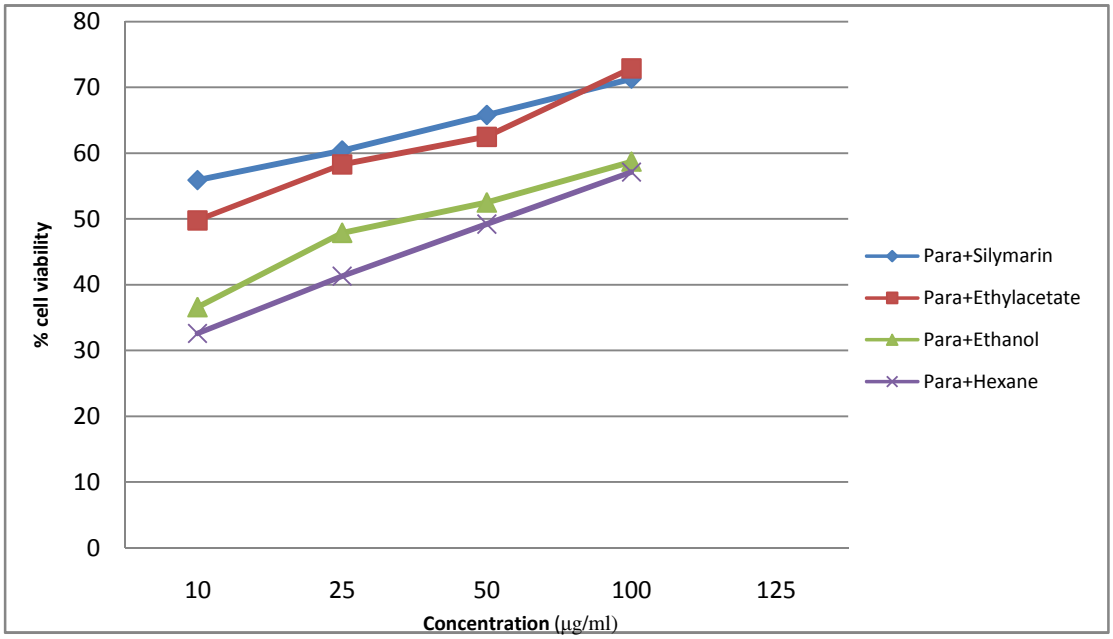


Fig. 25. *In vitro* hepatoprotective activity of different extracts using paracetamol induced toxicity

In the silymarin treated group, the cell viability was 73.1% at the highest concentration of 100µg/ml. The **ethyl acetate** extract showed significant increase in cell viability of 72.9% which is relatively close to that of the standard compared to the other two extracts. Hence ethyl acetate extract was selected for *in vivo* studies.

8.3.3. *In vivo* studies

8.3.3.1. Acute oral toxicity studies

No toxic symptoms and mortality was observed when ethyl acetate extract was administered at the dose level of 2000mg/kg. Hence 1/10th and 1/5th of this dose (200 and 400mg/kg) was taken for this study.

8.3.3.2. Hepatoprotective activity

Body weight

The animal body weight was calculated on 1st and 9th day of the study period and tabulated in **Table 21.**

Table 21. Change in body weight

TREATMENT GROUPS	ANIMAL BODY WEIGHT IN gms	
	1st day	9th day
Group I-control	133.33±0.14	135.47±0.17
Group II-disease control	138.33±0.48	136.04±0.35
Group III- standard control	135.00±0.52	134.00±0.43
Group IV-test group I	140.33±0.23	137.21±0.51
Group V-test group II	130.00±0.12	128.00±0.25

Values are expressed as Mean ±S.E.M. (n=6)

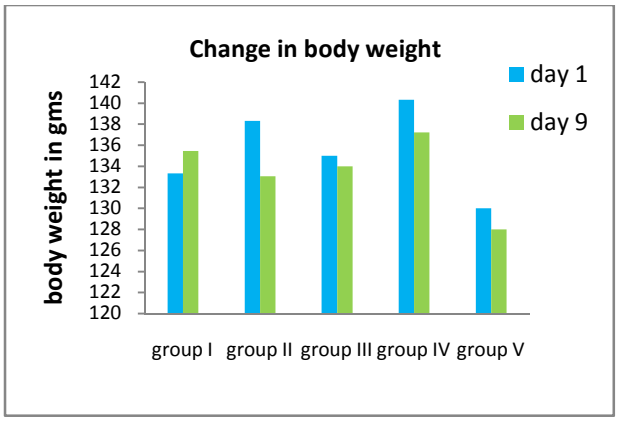


Fig. 26. Change in body weight

The disease control group showed slight decrease in body weight compared to the treated groups.

Evaluation of biochemical parameters

The biochemical parameters such as SGOT, SGPT, ALP, total bilirubin and total protein were found out. (**Table 22 and Fig.27)**

Table 22. Evaluation of biochemical parameters

Biochemical parameters	**SGOT (U/L)**	**SGPT (U/L)**	**ALP (U/L)**	**Total bilirubin (gm/dl)**	**Total protein (gm/dl)**
Group I	55.35±0.12	51.67±0.09	27.42±0.13	0.60±0.001	7.16±0.001
Group II	110.44±0.09 [a]	120.63±0.12 [a]	96.44±0.11 [a]	1.35±0.010 [a]	5.85±0.010 [a]
Group III	60.52±0.13 [ab]	56.54±0.15 [ab]	35.48±0.09 [ab]	0.63±0.001[ab]	7.44±0.001[ab]
Group IV	78.27±0.06 [ab]	68.45±0.13 [ab]	58.54±0.12 [ab]	0.74±0.001 [ab]	6.73±0.001 [ab]
Group V	63.36±0.08 [ab]	61.69±0.10 [ab]	39.42±0.08 [ab]	0.65±0.001[ab]	7.82±0.001[ab]

Values are expressed as Mean±S.E.M.

Data were analysed by one way ANNOVA followed by Dunnett's t-test.

'a' values were significantly different from normal control at P<0.01.

'b' values were significantly different from disease control at P<0.01.

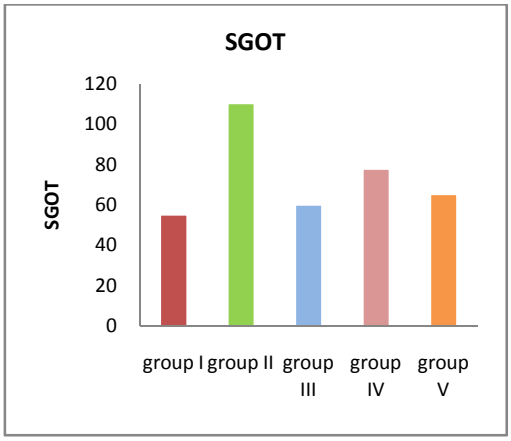


Fig. 27.1

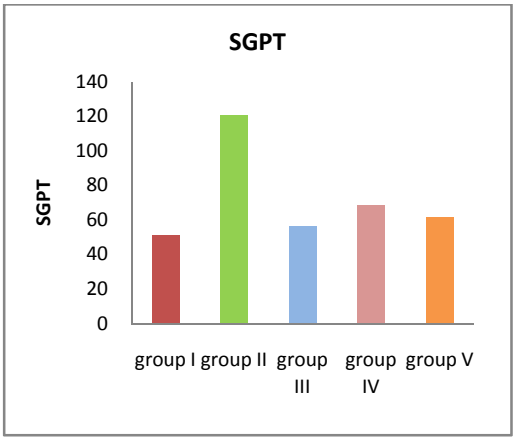


Fig. 27.2

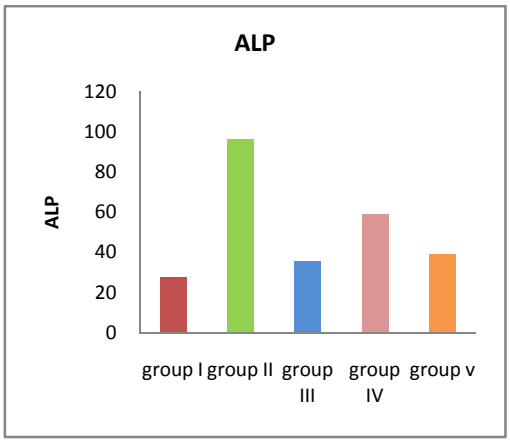


Fig. 27.3

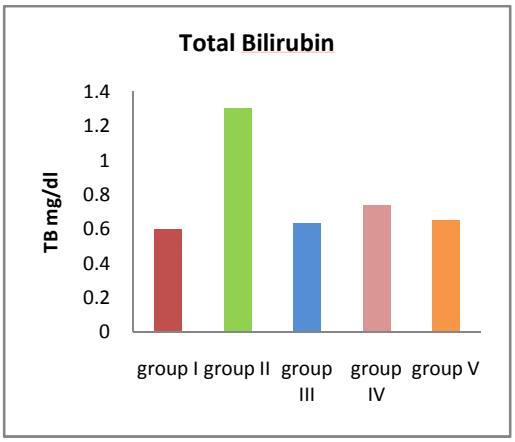


Fig.27.4

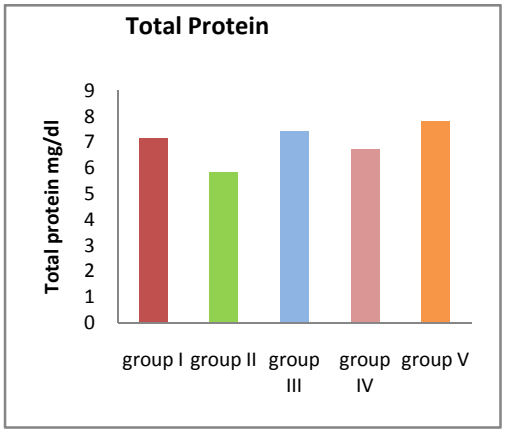


Fig.27.5

Fig. 27. Graphical representation of biochemical parameters

Estimation of the liver enzymes (SGOT, SGPT, ALP and TB) showed that in the paracetamol challenged group, there was a significant ($p<0.01$) rise in the levels of these enzymes. This is an indicator for hepatotoxicity. In the silymarin treated group, the enzyme levels were significantly reduced ($p<0.01$). In the drug treated groups also there was a significant decrease in the enzyme levels.

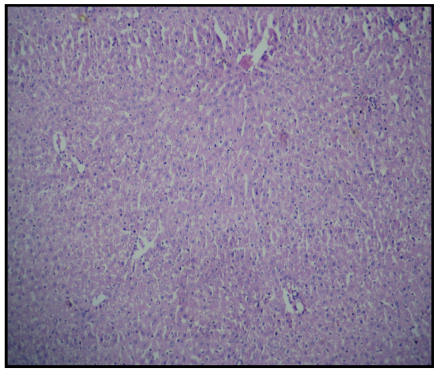
There was significant decrease in total protein levels (TP) in disease control group compared to the normal control group. The decrease in TP level in hepatotoxicity is mainly due to the presence of para proteins or decreased antibody production. Extract 200mg/kg and 400mg/kg treated groups showed significant ($p<0.01$) increase in TP level. This indicates that the extract, at both the doses tested, showed significant hepatoprotective activity.

Eventhough standard and extract treated groups were significantly different from control, the values were reduced when compared to disease control group.

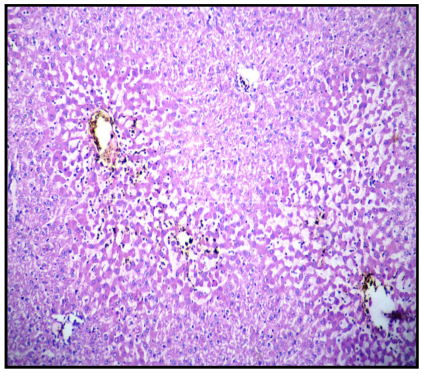
Histopathological studies

Histopathological sections of various groups are shown in **Fig. 28.**

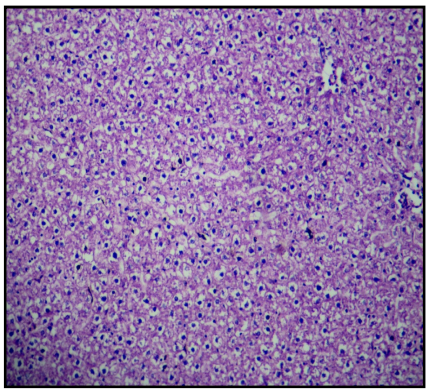
GROUP 1- Normal control



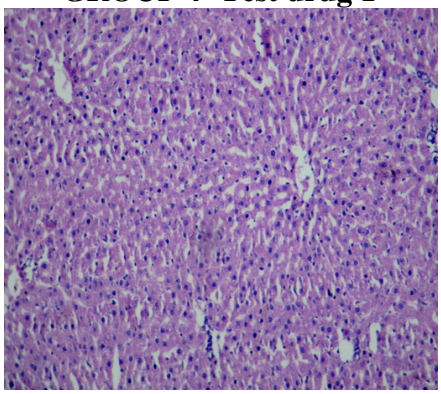
GROUP 2- Disease control



GROUP 3- Standard control



GROUP 4- Test drug 1



GROUP 5- Test drug 2

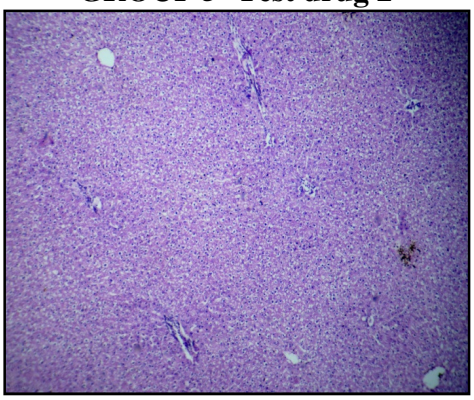


Fig. 28. Histopathological studies

Histopathological studies reveal that liver shows normal hepatocellular arrangement in normal control group. The paracetamol treated group shows loss of hepatic architecture with intense peripheral central vein necrosis and crowding of central vein. A normal hepatic architecture with hepatic rearrangement is seen in the silymarin pre-treated group. Group treated with 200mg/kg of berries extract reduces the hepatic injury, showing mild degree of necrosis and group treated with 400mg/kg of the berries extract reduces hepatic injury and necrosis clearly indicating the protection offered by the drug.

The presence flavonoids, glycosides and alkaloids contribute to hepatoprotective activity in many plants. The presence of flavonoids such as quercetin in *Phyllanthus emblica*[65], luteolin, apigenin in *Equisetum arvense*[66], citromitin, tangeretin in *Citrus depressa*[67], glycosides such as acubin from *Plantago asiatica,* picroside I and II from *Picorrhiza kurroa*[68] and alkaloids such as steroidal alkaloids in *Hygrophila auriculata*[69] showed potent liver protecting activity. In this study, the berries of *Vitex agnus*-castus have been shown to be rich in flavonoids, glycosides and alkaloids. Hence, the hepatoprotective activity of *Vitex agnus-castus* may be due to the presence of these phytoconstituents.

9. SUMMARY AND CONCLUSION

Vitex agnus-castus (Verbanaceae), generally called as the "women's herb" has a long term tradition of being used as a complementary medicine in Europe and also has a folklore claim for treating enlarged liver. It is also reported to possess antioxidant activity which are known to enhance hepatoprotective activity. In the present study hepatoprotective activity on the berries of *Vitex agnus-castus* is reported.

The pharmacognostical studies revealed various distinguishing characters present in the plant. The morphological studies showed that berries are greenish black, round to ovoid in shape with hairy surface and has a aromatic odour and bitter taste.

The microscopical sections of the berries showed the presence of **polygonal cells with thickened cell walls, simple pits, covering and glandular trichomes** in the **epidermis** of the **epicarp. The mesocarp** consists of **isodiametric parenchyma cells** and **endocarp** showed the presence of **brachysclerides.**

The powder microscopy revealed the presence of **starch grains, unicellular covering trichomes, xylem vessels, cortical parenchyma cells, brachysclerides and collenchymas cells.**

Standardisation of the drug was done by performing various physicochemical constants such as ash value, extractive values, loss on drying, foaming index and swelling index. The qualitative and quantitative estimation of the powdered drug was done which showed the presence of **aluminium, calcium, copper, iron, magnesium, sulphate and potassium** which were within the Pharmacopoeial limits.

Extraction was carried out with solvents of increasing polarity for the berries of *Vitex agnus-castus* and the percentage yield of ethyl acetate extract was found to be more compared to the other extracts.

Preliminary phytochemical screening of the extracts was performed. The **hexane extract** showed the presence of **phytosterols and triterpenoids**. The **ethyl acetate extract** revealed maximum number of active constituents such as **carbohydrates, flavonoids, glycosides, alkaloids, saponins, phenolic compounds, tannins and triterpenoids.** The **ethanol extract** showed the presence of **carbohydrates, flavonoids, alkaloids, phenolic compounds and triterpenoids.**

Quantification of flavonoids and phenols were carried out. The concentration of **flavonoid** present in **ethyl acetate** and **ethanol extract** was found to be **92.66µg equivalent to Quercetin in 1mg(9.3%) and 8µg equivalent to Quercetin in 1mg(8%)**. The concentration of **phenol** present in **ethyl acetate** and **ethanol extract** was found to be **9µg equivalent to Gallic acid in 1mg(0.9%) and 6.12µg equivalent to Gallic acid in 1mg(0.61%).**

The fluorescence analysis of powder and the various extracts were carried out and no fluorescence was observed.

The chromatography was performed, in which the ethyl acetate and ethanol extract each showed two spots for **flavonoid** mobile phase with **Rf value 0.36, 0.61, 0.32 and 0.54**. For **alkaloid** mobile phase also two spots each observed for both the extracts with **Rf value 0.45, 0.63, 0.42 and 0.60.** The **HPTLC chromatogram** of the **ethyl acetate** showed the presence of **11 peaks** with their corresponding areas.

The antioxidant activity was carried out by **H_2O_2 scavenging method** and **reducing power ability assay.** The IC_{50} value of the **ethyl acetate extract** in both the methods was found to be almost equivalent to that of the standard drug Ascorbic acid and showed the most potent antioxidant activity compared to the other two extracts.

Toxicity studies on normal Chang liver cell line showed that all the extracts were non-toxic. The *in vitro* hepatoprotective studies using paracetamol induced hepatotoxicity on Chang liver cell line showed that the **ethyl acetate extract** offered maximum protection against hepatotoxicity caused by paracetamol. The cell viability of the ethyl acetate and paracetamol treated group was 72.9% as against the cell viability of paracetamol treated group which was only 39.92%.

The *in vivo* studies were performed using two doses (200 and 400mg/kg) of the ethyl acetate extract. Both the doses tested showed significant hepatoprotective activity indicating that the plant possesses hepatoprotective activity.

This study substantiates our hypothesis that *Vitex agnus*-castus may be a useful hepatoprotective plant. Further studies on isolation of the phytoconstituents responsible for the activity are suggested.

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