PHARMACOGNOSTICAL, PHYTOCHEMICAL , FORMULATION AND EVALUATION OF HYPOLIPIDEMIC AND ANTI-OBESITY ACTIVITY ON HEARTWOOD OF *Caesalpinia sappan* Linn.,

A dissertation submitted to

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY

CHENNAI - 600 032

In partial fulfillment of the requirements for the award of degree of

MASTER OF PHARMACY

IN

PHARMACOGNOSY

Submitted by

REG.NO:261420661

Under the guidance of

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Madras Medical College

Chennai – 600003.

APRIL 2016

COLLEGE OF PHARMACY
MADRAS MEDICAL COLLEGE
CHENNAI – 600 003
TAMIL NADU

CERTIFICATE

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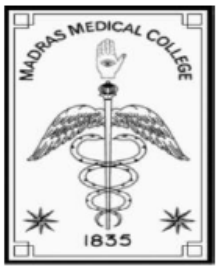

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INTRODUCTION

CORONARY ARTERY DISEASE[1-3]

Coronary artery disease (CAD), also known as ischemic heart disease (IHD) is a group of diseases that includes: coronary occlusion (**Atherosclerosis**), Angina pectoris, myocardial infarction and sudden coronary death. It is within the group of cardiovascular diseases of which it is the most common type. It is reported that almost 12 million people die of CHD disease each year all over the world.

Risk factors of CAD include:

- High blood pressure
- Diabetes
- **High blood cholesterol**
- **Obesity**
- Lack of exercise
- Smoking
- Lack of exercise
- Excessive alcohol
- Depression

CEREBRO VASCULAR ACCIDENT (CVA) [4, 5]

Brain receives blood from basilar artery and internal carotid artery. The obstruction of blood flow to the brain leading to permanent damage is called a cerebrovascular accident (CVA) or cerebral infarction (stroke). If the symptoms are only temporary, it is referred to as a Transient ischemic attack (TIA). Rupture of an artery within the brain (cerebral hemorrhage) is also called a **stroke** or cerebrovascular accident. **Atherosclerosis** (plaque in the arteries) is the leading cause of cerebral ischemia.

Risk factors for cerebrovascular disease include smoking, coronary artery disease, hypertension, diabetes, **hyperlipidemia**, peripheral vascular disease, atrial fibrillation, carotid disease and valvular heart disease.

Factors that may affect the rating of an applicant with a history of cerebrovascular accident include Current neurological residuals, hypertension, **high cholesterol levels**, and generalized atherosclerosis.

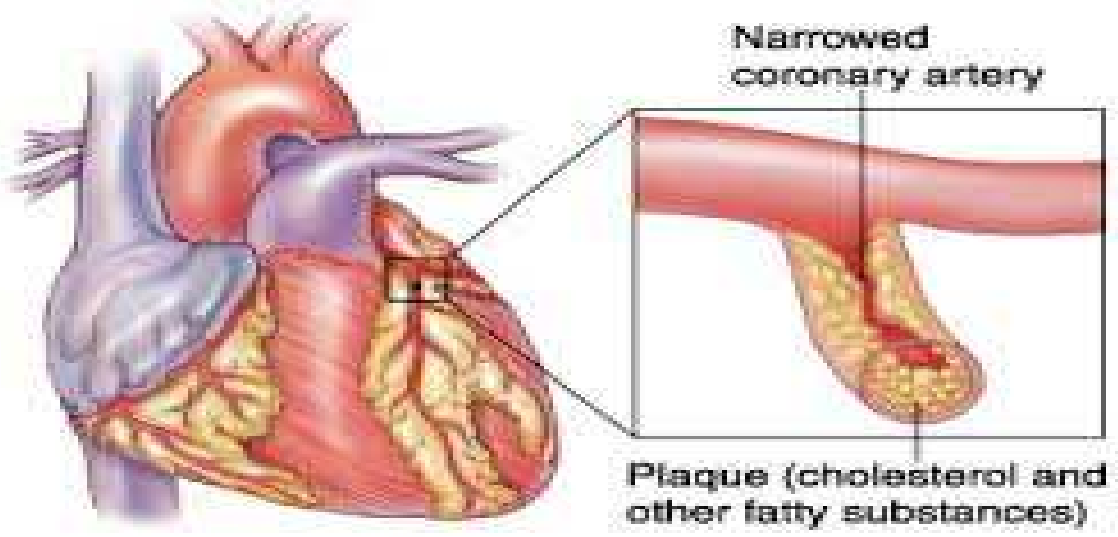


Fig. 1: CORONARY ARTERY DISEASE

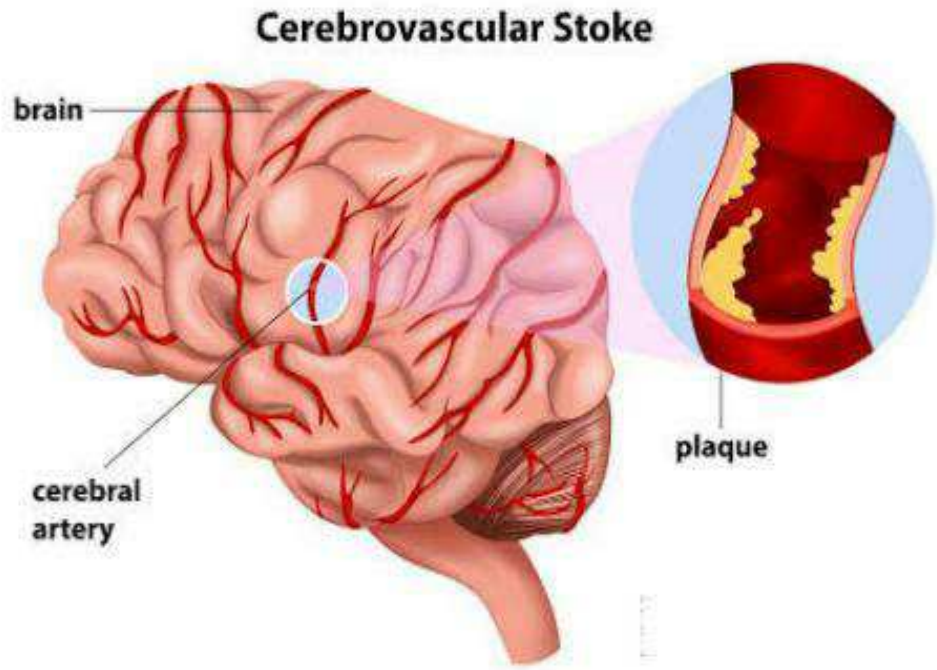


Fig 2: CEREBRO VASCULAR DISEASE

ATHEROSCLEROSIS[6,7]

Atherosclerosis (also known as atherosclerotic vascular disease or ASVD) is a specific form of arteriosclerosis in which an artery wall thickens as a result of invasion and accumulation of white blood cells (WBCs) (foam cells) and proliferation of intimal smooth muscle cell creating a "fibro fatty plaque".

Atherosclerosis is therefore a syndrome affecting arterial blood vessels due to a chronic inflammatory response of WBCs in the walls of arteries. This is promoted by low-density lipoproteins (**LDL**-plasma proteins that carry cholesterol and triglycerides) without adequate removal of fats and cholesterol from the macrophages by functional high-density lipoproteins (**HDL**). It is commonly referred to as a "hardening" or furring of the arteries. It is caused by the formation of multiple sclerosis.

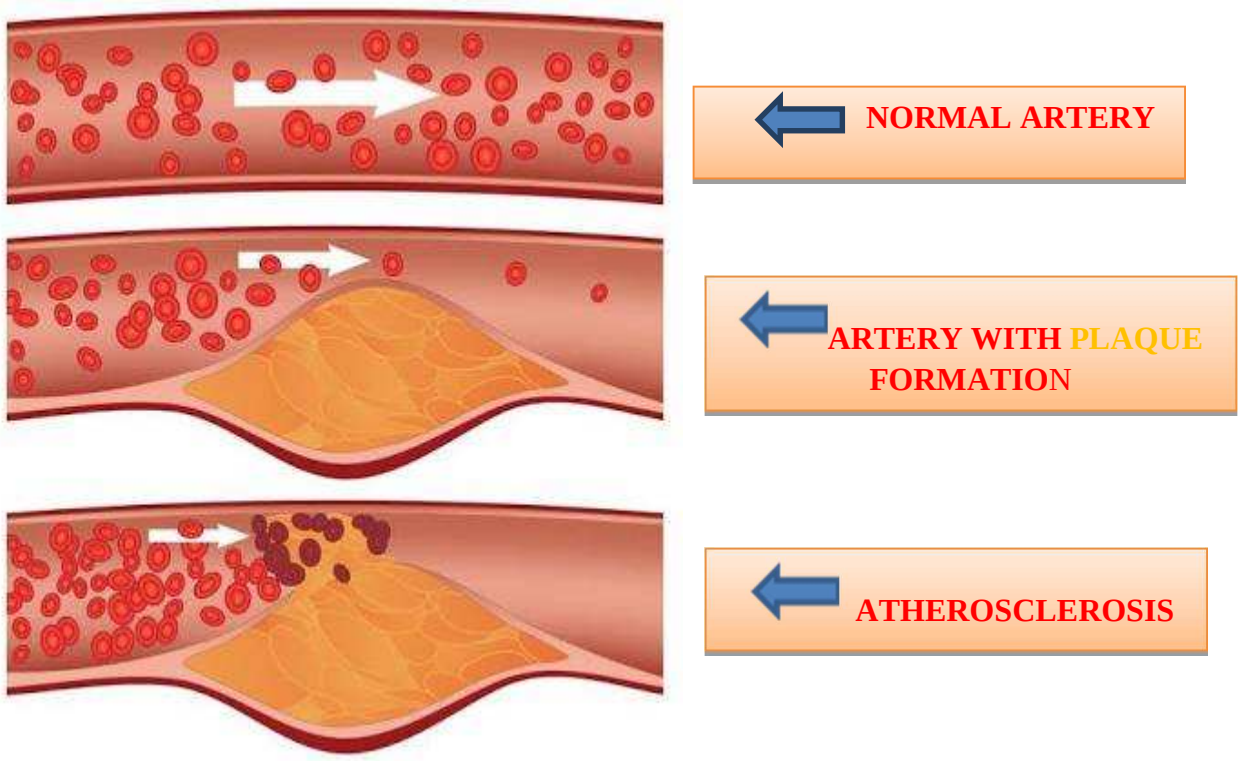


Fig 3 : Atherosclerosis

LIPIDS [8, 9]

It is the scientific quantitative term for fats in the blood. They are carried from one place to another by mixing with protein particles called Lipoproteins. Lipoproteins are divided into five major classes according to density such as

- ✓ Chylomicrons
- ✓ Very low density lipoproteins(VLDL)
- ✓ Intermediate density lipoprotein(IDL)
- ✓ Low density lipoprotein(LDL)(**Bad cholesterol**)
- ✓ High density lipoprotein(HDL)(**Good cholesterol**)

Table 1: Normal levels of lipids

Types	**Desirable mg/dL**
Cholesterol	< 200
LDL Cholesterol	< 130
HDL Cholesterol	≥60
Triglycerides	< 200

HYPERLIPIDEMIA AND OBESITY[10, 11]

Hyperlipidemia is one of the greatest risk factors which further lead to **coronary heart diseases, stroke, atherosclerosis** and **ischemic heart diseases**, which are the primary cause of death. Hyperlipidemia is a condition characterized by elevation of one or more lipids including cholesterol, cholesterol esters, phospholipids and triglycerides in the blood stream. It is also called as hyperlipoprotenemia, because these fatty substances travel in the blood attached to protein. Central to the pathogenesis of atherosclerosis is deposition of cholesterol in the arterial wall. Nearly all lipoproteins are involved in this process including cholesterol carried by very low density lipoproteins (VLDL), low density lipoproteins (LDL) and remnant lipoproteins. Hyperlipidaemia results in metabolic syndrome which is characterized by **obesity,** Insulin resistance and endothelial cell dysfunction which ultimately ends in Hypertension, Diabetes (or) Stroke.

Obesity is regarded as a social problem, associated with serious health risks and increased mortality. Obesity is difficult to define in quantitative term. It refers to the above average amount of fat contained in the body this in turn is dependent on the **lipid content of each fat cell** and on the total number of fat cells. WHO says obesity is related to **cardiovascular disease, hypertension, diabetes mellitus, cancer, osteoarthritis, pulmonary diseases,** as well as psychological issues, including social bias, prejudice discrimination and over eating.

According to WHO more than half of the total mortalities are associated with cardio vascular diseases. It is estimated that 12 million deaths per year occur from cardiovascular diseases, while one million of death in the European country occur due to obesity per year.

Table 2: Normal and abnormal levels of Lipids

Screening	Target
Cholesterol *(total)*	↓ 200 - Desirable 200 - 239 - Borderline ↑ 240 or above - High Risk
HDL	↑ 60 or above - Low Risk of Heart Disease 40 - 60 - Near Optimal ↓ 40 or below - High Risk of Heart Disease
LDL	↓ 100 or below - Low Risk of Heart Disease
Triglycerides	↓ 150 or below - Low Risk of Heart Disease

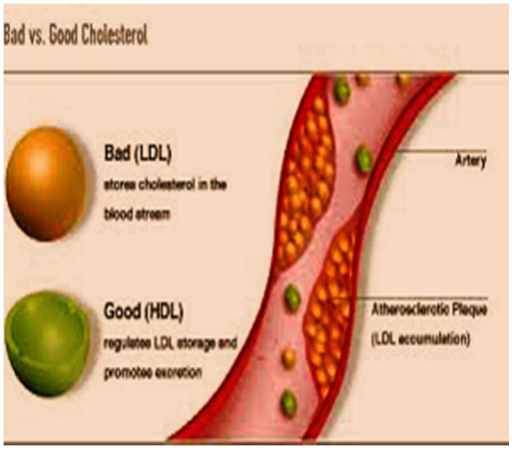


Fig 4: bad vs good cholesterol

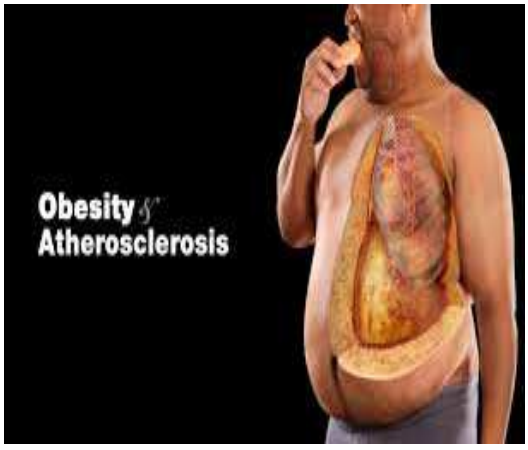


Fig 5: obesity

Currently available synthetic drugs for hyperlipidemia and obesity[8,12]

The consumption of synthetic drugs leads to hyperuricemia, diarrhoea, nausea, myositis, gastric irritation, flushing, dry skin and abnormal liver function. Though drugs therapies available for the treatment of hyperlipidemia includes use of drugs like Niacin, Fibrates (clofibrate, gemfibrozil), HMG-CoA reductase inhibitors (lovastatin, pravastatin, simvastatin and fluvastatin), Bile acid binding resins (cholestyramine and cholestipol) and Probucol but associated with lots of side effects.

Orlistat, Lorcaserin, Sibutramine, Rimonabant, Metformin, Exenatide ,Pramlintide and some other anti-obesity drug therapies available for the treatment of obesity with lots of side effects.

HERBAL MEDICINE[13, 14]

Herbal Medicine sometimes referred to as Herbalism or Botanical Medicine, is the use of herbs for their therapeutic or medicinal value. An herb is a plant or plant part valued for its medicinal, aromatic or savoury qualities. Herb plants produce and contain a variety of chemical substances that act upon the body.

Herbal medicine is the oldest form of healthcare known to mankind. Herbs had been used by all cultures throughout history. It was an integral part of the development of modern civilization. Much of the medicinal use of plants seems to have been developed through observations of wild animals, and by trial and error. As time went on, each tribe added the medicinal power of herbs in their area to its knowledgebase. They methodically collected information on herbs and developed well-defined herbal pharmacopoeias. Indeed, well into the 20th century much of the pharmacopoeia of scientific medicine was derived from the herbal lore of native peoples. Many drugs commonly used today are of herbal origin. Indeed, about 25 % of the prescription drugs dispensed in the United States contain at least one active ingredient derived from plant material. Some are made from plant extracts; others are synthesized to mimic a natural plant compound.

The World Health Organization (WHO) estimates that 4 billion people, 80 % of the world population, presently use herbal medicine for some aspect of primary health care. Herbal medicine is a major component in all indigenous peoples' traditional medicine and a common element in Ayurvedic, homeopathic, naturopathic, traditional oriental, and Native American

Indian medicine. WHO notes that of 119 plant-derived pharmaceutical medicines, about 74 % are used in modern medicine in ways that correlated directly with their traditional uses as plant medicines by native cultures. Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicinal value.

Some common herbs with hypolipidemic and obesity activity[15]

It has been reported that traditional systems have immune potential against various diseases. More than thirteen thousand plants have been studied for various pharmacological properties. An herbal treatment for hyperlipidemia and obesity has no side effects and is relatively cheap, locally available.

Table 3: Herbs with hypolipidemic and Anti-obesity activity

s.no	Plant name	Family	Part used	Active constituent
1	*Achyranthus aspera*	Amaranthaceae	Whole plant	Achyranthine Achyranthol
2	*Bauhinia purpurea*	Caesalpiniaceae	Leaf	Bio-flavonoids
3	*Cinnamomum tamala*	Lauraceae	Leaf	Eugenol Methyl eugenol Volatile oil
4	*Cissus quandrangularis*	Vitaceae	Stem	Anabolic phytosterol
5	*Commiphora mukul*	Burseraceae	Resin	Guggle sterol
6	**Garcinia cambogia*	Guttiferae/Clusiaceae	Peel of matured fruits	L-Hydroxy citric acid
7	*Gymnema sylvestre*	Asclepiadaceae	Leaf	Gymnemic acid
8	*Sapindus emarginatus*	Sapindaceae	Pericarp	Saponins
9	*Solanum melongena*	Solanaceae	Ripe fruit	Solanine Solanosterol Steroidal sapogenin
10	*Terminalia arjuna*	Combretaceae	Bark	Arjunolic acid

The present work is an attempt to determine the hypolipidemic and anti-obesity activity of *Caesalpinia sappan* heartwood. This plant has a folklore claim in the treatment of hyprecholesteremia but no studies have been reported so far.

2. REVIEW OF LITERATURE

Pharmacognostical Review

1. **Shrishailappa Badami *et al* (2003)** carried out Pharmacognostical Evaluation of *Caesalpinia sappan* Heartwood[16].
2. **Mehrotra, S. and H.P. Sharma (1984)** carried out Pharmacognostic studies on sappan (*caesalpinia-sappan* linn) and its market samples[17].

Phytochemical Review

3. **Zhao, M.-B *et al* (2014)** isolated two new Phenolic compounds and a new minor homoisoflavonoid from the Heartwood of *Caesalpinia sappan*[18].
4. **Zhang,J.-Y *et al* (2012)** isolated two new cassane diterpenoids from the seeds of *Caesalpinia sappan* Linn[19] .
5. **Shu , S-H *et al* (2011)** studied about two novel biphenyl dimers from the heartwood of *Caesalpinia sappan* [20] **.**
6. **Fuke, C *et al* (1985)** isolated 2 aromatic-compounds related to brazilin from *caesalpinia-sappan*[21].
7. **Nigam , S.S *et al* (1977)** carried out the Chemical examination on the heart wood of *caesalpinia-sappan*[22]

Pharmacological Review

8. **Kim, S.-H *et al* (2015)** reported that Brazilin Isolated from *Caesalpinia sappan* Suppresses Nuclear Envelope Reassembly by Inhibiting Barrier-to-Auto integration Factor Phosphorylation[23.]

9. **Nirmal *et al* (2014)** reported the Anti-Propionibacterium acnes assay-guided purification of brazilin and preparation of brazilin rich extract from *Caesalpinia sappan* heartwood and then Wound healing activity of standardized brazilin rich extract[24].
10. **Zhang *et al*. (2010)** reported the Study of *caesalpinia sappan* aqueous extract's apoptosis-inducing effects on human ovarian cancer cell SKOV3[25]
11. **Yodsaoue *et al*. (2009)** reported the Anti-Allergic Activity of Principles from the Roots and Heartwood of *Caesalpinia sappan* on Antigen-Induced beta-hexosaminidase Release[26]
12. **Hemalatha K *et al* (2007)** reported the Anti-inflammatory and analgesic activity of *Caesalpinia sappan* heartwood[27].
13. **Chun , H *et al*(2002)** reported the inhibitory effects of butyl alcohol extract from *Caesalpinia sappan* L. on melanogenesis in Melan-a cells[28]
14. **Xie, Y.W *et al* (2000)** studied the Vasorelaxing effects of *Caesalpinia sappan* - Involvement of endogenous nitric oxide[29]
15. **Hikino, H *et al* (1977)** reported the antiinflammatory principles of *Caesalpinia-sappan* wood and of hematoxylon-*campechianum* wood[30].

3. ETHANOBOTANICAL SURVEY [31-35]

PLANT PROFILE

Plant name	:	*Caesalpinia sappan* Linn
Common name	:	Sappan wood/ Brazil wood
Synonym	:	*Binacaea sappan* / *Caesalpinia minutiflora*
Family	:	Caesalpiniaceae /Fabaceae

VERNACULAR NAMES

Tamil	:	Parthangi
English	:	Brazilwood
Malayalam	:	Sappanam
Kanadam	:	Patang
Telugu	:	Bakamu
Hindi	:	Patang
Gujarati	:	Bakam

TAXONOMICAL STATUS

Kingdom	:	Plantae
Subkingdom	:	Viridiplantae
Infra kingdom	:	Streptophyta
Division	:	Tracheophyta
Sub division	:	Spermatophyta
Class	:	Magnoliopsida
Super Order	:	Rosanae
Order	:	Fabales
Family	:	Caesalpiniaceae/ Fabaceae
Genus	:	*Caesalpinia* Linn.
Species	:	*Caesalpinia sappan* Linn- Sappan wood

Geographical distribution

Caesalpinia sappan plant is cultivated wild in tropical countries, locally abundant throughout the Philippines at low and medium altitudes.

Also occurs in India through Burma, Thailand Indo-China, southern China to Malaysia.

In India, it is found in west Bengal, Madhya Pradesh, Orissa.

Habit

Caesalpinia sappan plant enjoys the open altitude of 1000m above sea level and grows well in mountainous areas that are not too cold.

It is small shaped leguminous tree, 5-10m high; 15-25cm diameter with spiny trunk.

Morphology

Leaves are compound, up to 50 centimeters long. Pinnae are about 20, opposite, and 10 to 20 centimeters long. Leaflets are 20 to 30, obliquely oblong to oblong-rhomboid.

Flowers are yellow, on terminal panicles, 2 to 2.5 centimeters in diameter with densely wooly filaments.

Fruit is a hard, indehiscent, shiny pod, about 7 centimeters long, and 3.5 to 4 centimeters wide, with a hard recurved beak at the upper angle with seeds 3-4.

Wood has outer sapwood which is white pale buff colour and inner orange red heartwood.

Part used

Heartwood.

Chemical constituents

The plant contains wide varieties of compounds, viz: triterpenoids, flavanoids, Steroids, Oxygen heterocycles and aminoacids.

- Tannin is found in the leaves 19%, bark and fruit walls 44%, pods 40%.
- Leaves yield volatile oil, 0.16 to 0.25%; d-a-phellandrene, terpene, and methyl alcohol

- Stem yields a gum.
- Brazilin, the main constituent of plant is oxidised to produce Brazilein by air and light additional constituents include gallic and tannic acids.

- Decoction of **wood** and **bark** used for tuberculosis, atonic diarrhoea, dysentery, wounds, postpartum Tonic, skin infections, ulcers, and anaemia.
- **Heartwood** used against inflammation, diuretics**.**
- **Roots, stems** and **seeds** used as sedative and vulnerary.
- In Ayurveda, Heartwood useful in vitiated conditions of Pitta, burning sensations, wounds, Ulcers,leprosy, skin diseases, diarrhoea, dysentery, and diabetes.
- In Kerala, decoction of heartwood used for blood purifying, anti-thirst, and anti-diabetic.

Other uses

- Cancer
- Inhibition of melanocytes formation
- Immunomodulatory
- Hyperlipidemia
- Liver disorder
- Dye wood

BIOGRAPHY OF THE PLANT

Fig 6: *Caesalpinia sappan* whole plant view

Fig 7:*Caesalpinia sappan* plant

Fig 8 :*Caesalpinia sappan* spiny trunk

Fig 9: *Caesalpinia sappan* pods

Fig 10: *Caesalpinia sappan* flowers

Fig 11: Heartwood of *Caesalpinia sappan*

4. RATIONALE FOR SELECTION

- Cardiovascular disease (CVD), particularly coronary heart disease (CHD) and cerebro vascular accident (CVA) is the leading cause of medically certified death in both developed as well as developing countries.

- Hyperlipidemia and obesity is one of the greatest risk factors in the manifestation and development of CHD and CVA.

- Medicinal plants have been shown to play a major role in treating hyperlipidemia and obesity and considering this, the present study was designed to develop an herbal capsules formulation on heartwood of *Caesalpinia sappan* for hypolipidemic and anti-obesity activity.

- The plant *Caesalpinia sappan* Linn., belonging to the family Caesalpiniaceae was selected for the present work.

- The Ethanomedicinal uses claimed were tuberculosis, atonic diarrhoea, dysentery, wounds, postpartum Tonic, skin infections, ulcers, anaemia, inflammation, diuretics.

- No pharmacognostical work has been carried out on microscopical evaluation on the heartwood of *Caesalpinia sappan* .

- The hypolipidemic and anti -obesity activity was not scientifically validated.

- So the heartwood of the plant *Caesalpinia sappan* Linn., was selected for evaluation of hypolipidemic and anti-obesity activity.

5. AIM AND OBJECTIVES

- The objective of the study is to explore the Pharmacognostical, Phytochemical, Formulation and then Hypolipidemic and Anti-obesity Evaluation of herbal capsule on the Heartwood of *Caesalpinia sappan* Linn.,

- Collection and Authentication of the plant specimen.

- Establishing Pharmacognostical profile of the plant.

- Extraction of plant material by using, cold maceration with solvents Ethanol and water (1:1)

- Phytochemical screening and determination of bioactive constituents.

- Formulation and evaluation of Herbal capsules.

- Evaluation of Hypolipidemic and Anti- obesity activity by *In-vivo* models.

6. PLAN OF WORK

I. PHARMACOGNOSTICAL STUDIES

- Collection of plant material
- Authentication
- Macroscopy
- Microscopy
- Powder microscopy
- Physiochemical constants determination
- Qualitative analysis of heavy metals and inorganic elements
- Quantitative estimation of heavy metals and inorganic elements.

II. PHYTOCHEMICAL STUDIES

- Preparation of **Hydro-alcoholic** extract(1:1)
- Preliminary Phytochemical screening of powder and extract
- Fluorescence analysis of powder and extract
- Quantitative estimation of phytoconstituents.
- Thin layer chromatography of extract.
- High Performance Thin Layer Chromatography of extract.

III. FORMULATION OF HERBAL CAPSULE

- Raw material standardization
- Pre-formulation studies

  (trial batches -3, optimizing for flow characteristics**)**
- Formulation development and standardization
- Accelerated stability studies

  (at 40^0c and 75% RH for 3 months)

IV. PHARMACOLOGICAL STUDIES

- Acute toxicity studies of formulated herbal capsule.
- In vivo-Evaluation of Hypolipidemic and Anti-obesity activity.
  - High fat diet induced obesity in rats.
  - Measurement of body weight
  - Measurement of internal organs weight
  - Biochemical parameters
  - Atherogenic index
  - Histopathological examination of rat aorta and liver.

7. MATERIALS AND METHODS

7.1 PHARMACOGNOSTICAL STUDIES

Collection of Plant Material

The Heartwood of *Caesalpinia sappan Linn.,* was collected from Kulasekaram in Kanyakumari district, Tamilnadu in June- 2015.

Identification and Authentication of Plant Material

The plant material was authenticated by Botanist Dr.V. Chelladurai, Research officer- Botany, Central Council for Research in Ayurveda and Siddha, Tirunelveli. The heartwood were shade dried, Coarsely powdered and used for further studies.

7.1.1MACROSCOPY

The plant materials are categorized according to sensory characteristics. Organoleptic evaluation provides the simplest and quickest means to establish the identity, purity and quality of a particular sample. Hence, this observation is of primary important before any further testing can be carried out.

7.1.2 MICROSCOPY [36-47]

Staining method

Fixation of plant material

The sample or heartwood was cut fixed in FAA solution (Formalin 5ml + Acetic acid 5ml + 90ml of 70% Ethanol). The specimen was dehydrated after 24 hours of fixing. The heartwood were graded with series of tertiary butyl alcohol, as per the standard method.

Infiltration of the specimen - It was carried out by gradual addition of 58 – 60º C of melting pointed paraffin wax until Tertiary butyl alcohol (TBA) solution attained super saturation. The specimens were cast into paraffin blocks.

Sectioning

The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the sections was 10 - 12µ. Dewaxing of the sections were done by customary procedures. The sections were stained Toludine blue. Since toludine blue is a polychromatic stain, the sections were stained as per the method published by O'Brein*et al*. The staining results were remarkably good. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to subrein, violet to mucilage and blue to the protein bodies. Whenever necessary sections were also stained with safranin, fast green and iodine for starch.

PHOTO MICROGRAPHS

Microscopic descriptions of tissues were supplemented with photo micrographs whenever necessary. Photographs of different magnifications were taken with Nikon lab photo 2 microscopic units. For normal observations bright field 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. Descriptive terms of the anatomical features are as given in the standard anatomy books.

POWDER MICROSCOPY

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 reagent were used. Powder was stained with 1% phloroglucinol in 90% ethanol, concentrated hydrochloric acid and observed through microscope. All the lignified cells stained with pink colour.

7.1.3 PHYSIO – CHEMICAL EVALUATION [48-52]

Shade dried powdered plant material of heartwoods of *Caesalpinia sappan* Linn., was used for the determination of physio chemical constants in accordance with WHO guidelines.

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 left after incineration of the crude drug is designated as ash. The residue obtained usually represents the inorganic salts naturally occurring in the drug and adhering to it. An ash value determination furnishes the basis for judging the identity and cleanliness of any drug and gives information relative to its adulteration/contamination with inorganic matter.

TOTAL ASH

The determination of ash is useful for detecting low grade products, exhausted drugs & excess of sandy and earthy material. Total ash is useful to exclude drugs which have been coated with chalk, lime or calcium sulphate to improve their appearance. The total ash usually consists of carbonates, phosphates, silicates and silica which include 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

About 2-3 gm of the ground material was weighed accurately into previously ignited tared crucible, incinerated at a temperature not exceeding 450^0c until free from carbon. The charred mass was cooled in desicattor and weighed. The mass was exhausted with charred mass with hot water. The residue was collected on ashless filter paper and incinerated at a temperature not exceeding 450^0c. Then the content of total ash in mg/gm of air -dried material.

$$\text{Wt. of total ash} = \frac{\text{\% Total ash value}}{\text{Wt. of crude drug taken}} \times 100$$

Water soluble ash

The ash was boiled for 5min with 25ml of water. The insoluble matter was then collected in an ash less filter paper. It was washed with hot water and ignited for 15min at a temperature not exceeding 450°c. The weight of the insoluble matter was subtracted from the weight of the ash and the difference in weight represented the water soluble ash, the percentage of water soluble ash with reference to the air dried substances was calculated by the formula.

$$\% \text{ Water soluble ash value} = \frac{\text{Wt. of total ash} - \text{Wt . of water insoluble ash}}{\text{Wt. of crude drug taken}} \times 100$$

Acid insoluble ash

Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth. Acid-insoluble ash is useful for detecting the presence of excessive earthy material.

Procedure

To the total ash obtained, 25ml of dilute hydrochloric acid was added, covered with a watch glass and boiled gently for 5min on a burner. The watch glass was rinsed with 5ml of hot water and these washings were added to the crucible. The insoluble matter was collected on an ash less filter paper by filtration and the filter paper was rinsed repeatedly with hot water until the filtrate is neutral and free from acid. Filter paper containing the insoluble matter was transferred to the crucible, dried on a hot plate and ignited to a constant weight in the muffle furnace at 450-500°C. The silica crucible was removed from the muffle furnace and allowed to cool in a desiccator for 30min, and then weighed without delay. The content of acid insoluble ash was calculated.

$$\% \text{ Acid insoluble ash value} = \frac{\text{Wt . of water insoluble ash}}{\text{Wt. of crude drug taken}} \times 100$$

Sulphated ash

2-3gm 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 were 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 of sulphated ash with reference to the air- dried substance was then calculated.

DETERMINATION OF EXTRACTIVE VALUES

Extractive value gives idea about soluble chemical constituents in particular solvents. According to IP, alcohol, pet.ether and water soluble extractive values should be determined.

Determination of water soluble extractive

5gm of the powder was weighed and macerated with 100ml of chloroform water (95ml distilled water and 5ml chloroform) in a closed flask for 24 hours. It was shaken frequently for six hours and allowed to stand for eighteen hours. It was then filtered rapidly, taking precautions against loss of solvent and 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish. 2 ml of alcohol was added to the residue and it was dried at 105°C for 1 hour in the hot air oven and cooled in desiccators for 30min and weighed. The process was repeated till a constant weight was obtained; the percentage of water soluble extractive value with reference to the air dried drug was calculated.

$$\text{Water soluble extractive value} = \frac{\text{weight of the dried extract}}{\text{weight of the sample taken}} \times 100$$

Determination of alcohol soluble extractive

5gm of the powder was weighed and macerated with 100ml 90% ethanol in a closed flask for 24 hours. It was shaken frequently for six hours and allowed to stand for eighteen hours. It was then filtered rapidly, taking precautions against loss of solvent and 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish. It was dried at 105$^{\circ C}$ for 1hour in a hot air oven. The dish was cooled in desiccator and weighed. The process was repeated till the constant weight was obtained. The percentage of alcohol soluble extractive value with reference to the air dried drug was calculated.

$$\text{Alcohol soluble extractive value} = \frac{\text{weight of the dried extract}}{\text{weight of the sample taken}} \times 100$$

Determination of volatile ether soluble extractive

2gm of powdered drug was accurately weighed and extracted with anhydrous diethyl ether in a continuous extractive apparatus for 20hours. The ether solution was transferred to tarred porcelain dish and evaporated spontaneously. Then it was dried over phosphorous pentoxide for 18hours and the total ether extract was weighed. The extract was heated gradually and dried at 105$^{\circ C}$ to constant weight. The loss in weight represents the volatile portion of the extract.

Determination of moisture content (Loss on Drying)

Specified quantity of the substances was taken in a previously ignited and cooled silica crucible and the substance was evenly distributed by gentle side wise shaking. The crucible with the contents was weighed accurately. The loaded crucible and the lid were placed in the drying chamber (105$^{\circ C}$). The substance was heated for a specified period of time to a constant weight. The crucible was covered with the lid and allowed to cool in a desiccator at room temperature before weighing. Finally the crucible was weighed to calculate the loss on drying with reference to the air dried substance.

$$\%\ \text{Loss on drying} = \frac{\text{Loss in weight of the sample}}{\text{Weight of the sample}} \times 100$$

Determination of foaming index

1gm of the coarsely powdered drug was weighed and transferred to 500ml conical flask containing 100ml boiling water. The flask was maintained at temperature 80-90$^{\circ C}$ for about 30min. It was then cooled and filtered into a volumetric flask and sufficient water was added through the filtrate to make up the volume to 100ml. The decoction was poured into 10 stopper test tube (height 16cm, diameter 16mm) in successive portions of 1ml, 2ml, 3ml, 4ml up to 10ml and the volume of the liquid in each tube was adjusted with water to 10ml. The tubes were stoppered and shaked in a length wise motion for 15 seconds, two shakes per second. Allowed to stand for 15min and the height of the foam was measured. The results are assessed 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 dilution 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 used for preparing the dilution in the tube where foaming to a height of 1cm is observed.

Determination of swelling index

The swelling index is the volume in ml occupied by the swelling of 1gm of plant material under specified conditions. A specified quantity of the plant material were previously reduced to the required fineness was accurately weighed and transferred into a 25ml glass stoppered 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 in an upward direction. Unless otherwise indicated in the test procedure, add 25ml of water and shake the mixture thoroughly every 10min for 1hour, allowed to stand for 3 hours at room temperature. The volume in ml occupied by the plant material was measured including any sticky mucilage. Calculate the mean value of the individual determination, related to 1gm of plant material.

Plant minerals play a vital role in metabolism. 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, silver bring about toxic effects resulting in intoxification.

7.1.4 QUALITATIVE ESTIMATION OF INORGANIC ELEMENTS AND HEAVY METALS

To the ash of the drug material 50%v/v hydrochloric acid 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 is formed on addition of ammonia solution. It is slightly soluble in excess of the reagent. The precipitate dissolves readily in strong acid and base, but after boiling it becomes insoluble.

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 sulpuric 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, yield a white precipitate after boiling and cooling the mixture (it is insoluble in ammonium chloride solution).

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

Chlorides: Chlorides, when treated with silver nitrate solution, yield a white crude precipitate which is insoluble in nitric acid, but soluble after being well washed with water, in diluted ammonia, from which it is re precipitated by the addition of nitric acid.

Copper: An excess of ammonia, 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 treated with ammonium carbonate solution and boiled, yield a white precipitate, but yield no precipitate in the presence of ammonium chloride solution.

Mercury: Solution of mercury 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 (slow from the side 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 with dilute ammonia solution and in dilute nitric acid yield yellow precipitate of normal silver ortho phosphate (distinction from meta and pyrophosphate) solution.

Potassium: Moderately strong 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 in nitric acid.

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

7.1.5 QUANTITATIVE ESTIMATION OF INORGANIC ELEMENTS

Inductive coupled plasma-Optical emission spectroscopy (ICP-OES)

It is an excellent multi-element technique with relatively good sensitivity and selectivity when configured correctly. This technique utilizes the plasma as an ion source or light emission source are capable of producing values.

QUANTITATIVE ANALYSIS OF HEAVY METALS

Instrumentation parameters:

Instrument name: Inductive coupled plasma-Optical emission spectroscopy

Instrument Model: PE Optima 5300DV 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.

Light source (Torch): Positioned horizontally in the sample compartment along the central axis of the spectrometer optics. Changing from axial to radial viewing is 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.

Standard alumina injector: 2.0mm inner diameter.

Spray chamber: Scott type

Nebulizer: Cross flow gem tip.

Preparation of sample by acid digestion method: 50mg of powder was treated with acid mixture of sulphuric acid: water in the ratio of 4:1in the Kjeldahl flask and heated continuously till the solution is colourless. The sample mixture was then transferred in a 25ml volumetric flask and made up to the volume with distilled water. Blank solution was prepared as above without sample.

The standards of Arsenic, Lead, Mercury and Cadmium were prepared as per the protocol and the calibration curve was developed for each of them.

Detection:

Samples were analyzed for the detection and quantification of the aluminium, calcium, chloride ,borate, silver, copper, potassium, magnesium, iron by Inductively Coupled Plasma Emission Spectrometry.

7.2 PHYTOCHEMICAL STUDIES

Phytochemical evaluation is used to determine the nature of phyto constituents present in the plant by using suitable chemical tests. It is essential to study the pharmacological activities of the plant. It can be done by confirmation with different chromatographic techniques like TLC ,HPLC and HPTLC. Therefore a complete investigation is required to characterize the Phyto constituents qualitatively and quantitatively.

7.2.1Preparation of Extracts [53]

About 200gm of coarsely pulverized heartwood was taken in a closed bottle and it was defatted with petroleum ether. The defatting was continued for 9-10 days with occasional shaking. The petroleum ether extract was filtered. The marc left after petroleum ether defatting was taken out and dried under shade to get a dry mass, then extracted with ethanol and water(50:50) by using cold maceration extraction. The extraction was continued for 9-10 days with occasional shaking. The hydro alcoholic extract was filtered, concentrated under reduced pressure to a semisolid mass and was made free from solvent. The final obtained extract was weighed; percentage yield was calculated and stored in a cool place. Extraction is the preliminary step involved in the phytochemical studies. It brings out the metabolites into the extracting solvent depends upon its polarity.

Extraction

The percentage yield, colour and consistency of the extract were recorded and preceded for further detailed phyto chemical and pharmacological screening.

7.2.2 PRELIMINARY PHYTOCHEMICAL SCREENING[54-60]

The chemical tests for various Phyto constituents in the dried powder and extracts on heartwood of *Caesalpinia sappan.*,were carried out as described below and the results were recorded.

Test for Alkaloids

Small quantity of the extract was treated with few drops of diluted hydrochloric acid and filtered. The filtrate was used for the followings,

a)Mayer's reagent (Potassium mercuric iodide solution)

Alkaloids give cream colour precipitate with mayer's reagent.

b)Dragendorff's reagent (Potassium bismuth iodide solution)

Alkaloids give reddish brown precipitate with Dragendorff's reagent.

c) Hager's reagent (Saturated solution of picric acid)

Alkaloids give yellow coloured precipitates with Hager's reagent.

d) Wagner's reagent (Solution of iodine in potassium iodide)

Alkaloids give reddish brown precipitate with wagner's reagent.

Detection of Proteins

Biuret test:The sample was treated with 5-8 drops of 10% w/w copper sulphate solution, violet color is formed.

Detection of Flavonoids

a) **Shinodatest :**Small quantity was dissolved in alcohol to this pieces to magnesium followed by concentrated hydrochloric acid were added dropwise and heated. Appearance of magenta colour shows the presence of flavonoids.

b) With aqueous sodium hydroxide solution: Small quantity of the extract was dissolved in aqueous sodium hydroxide and appearance of yellow colour indicates the presence of flavonoids.

Detection of Tannins

a) **Lead acetate test :**The test solution was mixed with basic lead acetate solution and examined for formation of a white precipitate.

b) **Ferric chloride test :**A few drops of 5% aqueous ferric chloride solution was added to 2ml of an aqueous extract of the drug and examined for the appearance of bluish black color.

Detection of fixed oils and fats:

a) **Spot 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.

b) **Saponification test:**Few drops of 0.5 % alcoholic potassium hydroxide were added to a small quantity of various extracts along with a drop of phenolphthalein. The mixture was heated on the water bath for 1-2 hr . Formation of soap with the alkali indicates the presence of fixed oils and fats.

Detection of Glycosides

a) **Borntrager'stest:**The powdered material was boiled with 1ml of sulphuric acid in a test tube for five minutes. Filtered while hot, cooled and shaken with equal volume of chloroform. The lower layer of solvent was separated and shaken with half of its volume of dilute ammonia. A rose pink to red colour is produced in the ammonical layer.

b) **Modified Borntrager'stest :**The test material was boiled with 2ml of the dilute sulphuric acid. This was treated with 2ml of 5% aqueous ferric chloride solution (freshly prepared) for 5 minutes, and shaken with equal volume of chloroform. The lower layer of solvent was separated and shaken with half of its volume of dilute ammonia. A rose pink to red colour is produced in the ammonical layer.

Detection of Steroids and Triterpenoids

a) **LibermannBurchardsTest :**The powdered drug was treated with few drops of acetic anhydride, boiled and cooled. Conc.sulphuric acid was added from the sides of the test tube, brown ring is formed at the junction of two layers and upper layer turns green which shows presence of steroids and formation of deep red color indicates presence of tri terpenoids.

b)Salkowski Test: The extract was treated with few drops of concentrated sulphuric acid, red color at lower layer indicates presence of steroids and formation of yellow colored lower layer indicates presence of tri terpenoids.

Detection of Carbohydrates

a) Molisch's test: To the test solution few drops of alcoholic alpha napthol and few drops of conc. sulphuric acid were added through the sides of test tube, purple to violet color ring appears at junction.

b)Fehling's test : The test solution was mixed with Fehling's I and II and heated and examined for the appearance of red coloration for the presence of sugar.

Detection of Saponins:A drop of sodium bicarbonate solution was added to the sample and the mixture was shaken vigorously and left for 3 minutes. Development of any honey comb like froth was examined.

7.2.3 FLUORESCENCE ANALYSIS [61, 62]

Many crude drug show the Fluorescence when the sample is exposed to UV radiation. Evaluation of crude drugs based on fluorescence in day light is not much used, as it is usually unreliable due to the weakness of the fluorescent effect. Fluorescent lamps are fitted with suitable filter, which eliminate visible radiation from the lamp and transmits UV radiation of definite wavelength. Several crude drugs show characteristic fluorescence useful for their evaluation.

7.2.4 QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

TOTAL SAPONNIN CONTENT [63]

20 gm of the powder of Heartwood was put into a conical flask and 100ml of 20% aqueous ethanol were added. The samples were heated ove r a hot water bath for 4 hrs with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200ml of 20% of ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separating funnel and 20ml diethyl ether was added and shaken vigorously. The

aqueous layer was recovered while the ether layer was discarded. The purification process was repeated.60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10ml of aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight. The saponnin content was calculated as percentage.

ESTIMATION OF FLAVONOIDS[64]:

Method : HPLC

Extraction solvent- Mixture of alcohol, water and HCl (50:20:8)

Mobile phase- A mixture of methanol, water and phosphoric acid (100:100:1)

Standard solutions- Accurately weighed quantities of USP quercetin RS, Rutin, Gallic acid, Thymoquinone, Gallangin to separate volumetric flasks, which is dissolved in methanol, and diluted quantitatively, and stepwise if necessary, with methanol to obtain standard solution 1 mg/ml, respectively.

Test solution – About 10g of hydroalcoholic extract were weighed to a 250 ml flask fitted with a reflux condenser. 78ml of extraction solvent added and refluxed on a hot water bath for 135 minutes. Then it was allowed to cool at room temperature. Decanted to a 100 ml volumetric flask. Then 20ml of methanol added to 250 ml flask, sonicated for 30 minutes, filtered and the filtrate was collected in 100 ml volumetric flask, the residue was washed on the filter with methanol. Then the washing was collected in the same 100 ml volumetric flask, diluted to volume and mixed.

Chromatographic system – The liquid chromatograph was equipped with a 270 nm detector and a 4.6mm x 25 cm column that contains packing L1. The flow rate was about 1.5ml per minute. Chromatograph standard solution 1 and the peak reponses was recorded.

Procedure – Separately injected equal volumes(about 20 µL) of each of the standard solution and the test solution into the chromatograph, chromatographs was recorded and areas were measured for the major peaks. The quantity of each flavonoids in sample was calculated and given.

ESTIMATION OF PHENOLIC CONTENT[65]

Method : HPLC

Column : C_{18}

Stationary phase: octadecylsilyl silica gel for chromatography R(5µm).

Mobile phase : glacial acetic acid R, tetrahydrofuran R, water R (2:5:40:60 v/v/v).

Flow rate : 0.9mL/min

Detection : spectrophotometer at 254nm

Injection : 20 µL

Run time : 30 min

Standard solution : Accurately weighed quantities of USP phenol standard in tetrahydrofuran R and dilute to 10ml with the mobile phase.

Then the quantitative amount of phenolic compounds present in both *Caesalpinia sappan* extract and herbal capsules were determined and reported.

7.2.5 CHROMATOGRAPHY [66,67]

Chromatography methods are important analytical tool in the separation, identification and estimation of components present in the plant.
Chromatographic fingerprinting has been in use for a long time for single chemical entity drug substances. Recently it has become one of the most powerful tools for quality control of herbal medicines. The use of chromatographic fingerprinting for herbal drugs tends to focus on identification and assessment of the stability of the chemical constituents observed by various chromatography techniques such as HPLC, TLC, HPTLC, GC, capillary electrophoresis.

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 reliable technique in which solute undergoes distribution between two phases, stationary 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 Stahl TLC spreader. 40gm of silica gel G was mixed with 85ml of water to prepare homogenous suspension and poured in the spreader. 0.25mm thickness of plates was prepared, air dried until the transparency of the layer disappeared, then dried at 110°C for 30 minutes and kept in desiccators.

Selection of mobile phase:

Solvent mixture was selected on the basis of the phyto constituents present in each extract. Factors such as nature of components, stationary phase, mobile phase, polarity, influence the rate of separation of constituents. From the vast analysis, best solvent was selected which showed good separation with
maximum number of components.

The Retardation Factor (Rf) is calculated using following formula,

$$Rf = \frac{\text{Distance travelled by solute from the origin}}{\text{Distance travelled by solvent from the origin}}$$

HPTLC FINGER PRINTING OF EXTRACT:

HPTLC is one of the versatile chromatographic method which helps in the 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.

Instrument Conditions:

Instrument : CAMAG HPTLC

HPTLC Applicator : CAMAG LINOMAT IV

HPTLC Scanner : CAMAG TLC SCANNER II

Sample : HYDROALCOHOLIC EXTRACT

Volume of injection : 20 µl

Mobile phase : Ethyl acetate : chloroform : methanol (5.3:1.5:0.5)

ƛ max : 200-400 nm

Lamp : Deuterium

Stationary phase : TLC Silica gel 60 F_{254} (Merck)

Equipment:

A Camag HPTLC system equipped with a sample applicator Linomat IV, Twin trough plate development chamber, TLC Scanner II.

Chromatographic conditions:

The estimation has been done using the following chromatographic conditions. Chromatography was performed on a 12 × 3 cm (H x W) pre-activated HPTLC silicagel 60 F_{254} 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 with solvent ethyl acetate.

HPTLC serves as a convenient tool for finding the distribution pattern of phyto constituents 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.

7.3 FORMULATION OF HERBAL CAPSULE

Raw material used for formulation development was tested for its identity, purity and quality by relevant preliminary tests. Formulation development studies were optimized with three different batches.

SAMPLING OF RAW MATERIALS[68]

From each container or package selected, three original samples were taken with care to avoid fragmentation. Samples were taken from the top, middle and bottom of the container. In the case of sacks and packages, the three samples were taken by hand, the first from a depth of not less than 10 cm from the top and the second and third from the middle and bottom after cutting into the side of the package. The three original samples were then combined into a pooled sample which was mixed carefully. The average sample was obtained by quartering the pooled sample.

7.3.1 ANALYSIS OF RAW MATERIAL

Raw materials were standardized as per WHO guidelines.

For crude herbal drugs, determination of foreign organic matter, loss on drying, total ash, acid insoluble ash, water soluble ash, sulphated ash, water soluble extractive, alcohol soluble extractive ,ether soluble extractive, heavy metals analysis, microbial load analysis were analysed according to Ayurvedic Pharmacopoeial procedure.

DETERMINATION OF FOREIGN ORGANIC MATTER [69]

Procedure: 100g of the drug sample was weighed and then it was spread out in a thin layer. The foreign matter was detected by inspection with the use of a lens. Foreign matter found were separated and weighed and the percentage was calculated.

$$\text{Foreign organic matter} = \frac{\text{Weight of the sample after inspection}}{\text{Weight of the sample before inspection}} \times 100$$

MICROBIAL LOAD ANALYSIS[70]

Materials and Methods

The following test is carried out for the estimation of number of viable aerobic microorganisms present and for detecting the presence of designated microbial species in the herbal medicines.

Procedure

1. Total aerobic viable counter

2. Yeast and moulds

3. *Escherichia coli*

4. Salmonellae

5. Pseudomonas

6. Staphylococcus

Procedure

Pre-treatment of the herbal material

The crude medicinal plant material or product was grinded and diluted with phosphate buffer pH 7.2 to eliminate any antimicrobial properties.

Total aerobic viable count

To one petri disk of 10cm in diameter, a mixture of 1 ml of the pre-treated plant material or product and 15cm of liquefied casein-soybean digest agar was added at 40^{o} C. the material was diluted to obtain an expected colony count of more than 300. At least two dishes were prepared using the same dilution. they were inverted and incubated at 30-35^{o} C for 72 hrs, unless a more reliable count was obtained in a shorter period of time. The number colonies formed was counted and result were calculated using the plate with the largest number of colonies, upto maximum of 300.

Total yeast and mould count

To one perti disk of 10cm in diameter , a mixture of 1 ml of the pre-treated plant material or product and 15cm of liquefied sabouraud glucose agar with antibiotic was added at 40^{o}C. the material was diluted to obtain an expected colony count of more than 100.At least two dishes were prepared using the same dilution and they were incubated them upright at 20-25^{o}C for five days , unless a more reliable count was obtained in a shorter period of time. The number colonies formed was counted and result were calculated using the plate with not more than 100 colonies.

Escherichia coli

1 ml of material was added to 100 ml of MacConkey broth and it was incubated at 43-45^{o}C for 24 hrs.

A subculture was prepared an a plat with MacConkey agar medium and incubated at 43-45^{o}C for 24 hrs. growth of red , generally non-mucoid colonies of Gram – negative road, sometime surrounded by a reddish zone of precipitation indicates the presence of *E.coli.*

Salmonella sp

The pre-treated plant material or product was incubated at 35-37^{o}C for 5-24 hours, as appropriate for enrichment.

Primary test

10 ml of the enriched culture was transferred to 100 ml tetrathionate bile brilliant green broth and was incubated at 42-43^{o}C for 24 hours. Subcultures were prepared on to agar media and brilliant green agar. Then they were incubated at 35-37^{o}C for 24 hours. The well-developed colourless colonies in deoxycholate citrate agar medium and small, transparent and colourless or opaque, pink or white.

Pseudomonas aeruginosa

1 ml of the prepared material was inoculated in 100 ml of soyaben-casein digest medium. Mixed and incubated at 35-37^{o}C for 24 -48 hours. Prepared on a subculture on a plate of cetrimide agar and incubated at 35-37^{o}C for 24 -48 hours.

If the no growth of microorganism is detected , the material passes the test. If growth of colonies of Gram-negative rods occurs, usually with a greenish fluorescence.

Staphylococcus aureus

Prepared material was incubated at 35-37ºC for 24 -48 hours. The material passes the test if no growth of microorganism is detected. Black colonies of Gram-positive cocci often surrounded by clear zones may indicate the presence of *staphylococcus aureus*.

7.3.2PREFORMULATION STUDIES[71]

To formulate any dosage forms, it is essential that fundamental physical and chemical properties of the drug powder are to be determined.

Definition:

Preformulation involves the application of biopharmaceutical principles to the physicochemical parameters of drug substance are characterized with the goal of designing Optimum drug delivery system. Before beginning the Preformulation programs the preformulation scientist must consider the following factors

- The amount of drug available.
- The physicochemical properties of the drug already known.
- Therapeutic category and anticipated dose of compound.
- The nature of information, a formulation should have or would like to have.

SELECTION OF EXCIPIENTS[72]

For the formulation of capsules in addition to the active ingredients, excipients like diluents (filler), binder, disintegrating agent, lubricant and preservatives are required. The choice of excipients was made keeping in mind the current Food and Drugs Administration (FDA) regulations.

Diluents:

Diluents/Fillers are added where the quantity of active ingredient is less (or) difficult to filling. Common tablet/capsule filler include Lactose, Dicalcium phosphate, Microcrystalline cellulose, etc.

Lubricants:

They reduce friction during the filling process. In addition, they aid in preventing adherence of capsule material. Magnesium Stearate, Stearic acid, Hydrogenised vegetable oils and talc are commonly used lubricants.

Glidants:

It is used to improve flow of the powder materials by reducing the friction between the particles. The most effective glidants are the Colloidal silicon dioxide, Talc and Starch.

Preservatives:

The preservatives are added to herbal formulation to prevent contamination, deterioration and spoilage by bacteria, fungal and other microorganisms. The most effective preservatives are the sodium methyl paraben, sodium propyl paraben, sodium benzoate and bronopol.

selection of excipients in the formulation are given below

- Talc -glident/lubricant
- Microcrystalline cellulose -diluent/disintegerant
- Starch -binder/disintegerant
- Colloidal sillicon dioxide -glident
- Magnesium stearate - lubricant
- Bronopol & sodium methyl paraben -preservative

PREPARATION OF FORMULATION[73-76]

The dry hyroalcoholic extract of *Caesalpinia sappan* were dried in tray drier at 60^{o}c for 20 minutes. All excipients used in this formulation except preservatives were dried separately in tray drier at 100^{o}c for 30 minutes. All active ingredients were weighed according to the formula, mixed and lubricated with magnesium stearate followed by diluents and preservatives were mixed well. The mixture was blended thoroughly for 30 minutes. Then the powder was transferred to the polythene bags and labelled for further studies.

7.3.3DEVELOPMENT OF FORMULATION-TRIAL BATCHES

Three trial batches were formulated by varying the composition of the excipients proportions for excellent flow properties.

Table 4 : Development of formulation

s.no	Materials	Trial-1 (g)	Trial-2 (g)	Trial-3 (g)
1	*Caesalpinia sappan extract*	20	20	20
2	Talc	1.7	1.8	2.5
3	Mcc	0.8	0.9	1
4	Starch	2	2.5	2.6
5	Magnesium stearate	0.8	1	1.5
6	Colloidal sillicon dioxide	0.25	0.28	0.3
7	Bronopol	0.15	0.15	0.15
8	Sodium methyl paraben	0.15	0.15	0.15

Evaluation of blended powder

The blended powder of all trial batches were analysed for its flow characteristics like bulk density, tap density, compressibility index, Hausner's ratio and angle of repose.

Bulk density and tap density and Carr's index

A weighed quantity (15g) of powdered material was taken in a 50ml measuring cylinder. And recorded the initial volume (v_o). tapped the contents and recorded the powdered volumes after 50 taps(v_{50}).

Fluff density = w/v_o g/cc

Tapped density = w/v_{o50} g/cc

Carr's index = Tapped density- Fluff density/ Tapped density * 100

Value for Carr's index below 15 indicate excellent flowing material and value over 20-30 suggested poor flowing material.

Angle of repose

A funnel was fixed at a particular height (1.5, 2.5, 3.5 cm) on a burette stand. A white paper was placed below the funnel on the table. The powdered drug passed slowly through the funnel until it forms a pile. The radius of the pile was noted down.

Angle of repose of the powder material was calculated by using the formula:

$\tan\theta = h/r$

$\theta = \tan (h/r)$

where, h = height of the pile, r = radius.

Values for angle of repose $< 30^o$ usually indicate a free flowing material and angle $> 40^0$ suggest a poor flowing material.

Hauser's ratio

The basic procedure is to measure the unsettled apparent volume, V_0 and the final tap volume V_f , of the powder tapping the material until no further volume changes occur.

The Hausner's ratio was calculated as follows:

Hausner's ratio = V_0 / V_f

Hausner's ratio between 1.00 to 1.11 shows excellent flow and value more than 1.60 shows very poor flow.

Table 5: Final batch composition -250 mg/capsule

S.No	Ingredients	Quantity in mg
1	*Caesalpinia sappan*	200
2	Talc	25
3	Colloidal sillicon dioxide	3.7
4	Magnesium stearate	0.85
5	Micro crystalline cellulose	12.5
6	Starch	7.4
7	Sodium methyl paraben	0.25
8	Bronopol	0.25

From the 3 trial batches one optimized batch is selected for formulation based on above results. Trial batch 3 was found to be the perfect batch and it was selected for the consideration of further large scale manufacturing.

7.3.4 FORMULATION OF CAPSULES[77-82]

Capsules are small dosage form in which one or more medicinal and inert ingredients are enclosed in a small shell usually made of gelatin.

CAPSULE SIZE AND SELECTION OF FILLING METHOD

- The formulated granules were filled in "1" size capsules to an average net content t weight of 270 mg.

- The capsules were then de dusted, transferred into polybags, labelled and the

Samples were evaluated as per the testing requirements.

- After approval from QAD the capsules were packed as per the packing instructions.

- A hand operated gelatin capsule filling machine (Chamunda pharm machinery) was used in this study for encapsulation of capsules.
- from the final trial, samples were taken for accelerated stability studies as per the testing requirements.

Fig 12: HERBAL CAPSULE

7.3.5 STANDARDISATION OF HERBAL CAPSULES[83, 84]

The developed herbal capsules were standardized for its description, uniformity of weight, disintegeration time, moisture content, physicochemical parameters, phytochemical studies, fluorescence analysis. Standardization were crried out as per Indian pharmacopoeial procedures.

Quality control parameters

Description

The general appearance of a capsule, its visual identity and overall “elegance” is essential for consumer acceptance. The color, shape, odor and surface texture are all noted for the capsules prepared.

Uniformity of weight

20 individual units were selected at random and their content was weighed and their Average weight was calculated. Not more than two of the individual weights deviate from the average weight by more than the percentage shown in the table

Table: 6 Acceptance Criteria I.P Limit

Dosage form	Average limit	Deviation
capsules	< 300 mg	10%
	>300mg	7.5%

Disintegration test

Disintegration test was performed using the digital microprocessor based disintegration test apparatus (Veego, Mumbai).One capsule was introduced into each tube and added a disc to each tube. The assembly was suspended in the water in a 1000 ml beaker. The volume of water was such that the wire mesh at its highest point is at least 25 mm below the surface of water, and at its lower point was at least 25 mm above the bottom of the beaker. The apparatus was operated and maintained the temperature at 37±2°C. (Indian Pharmacopoeia, 2010).

Determination of moisture content

The loss on drying test is important when the herbal substance is known to be Hygroscopic. An excess of water in medicinal plant materials will encourage Microbial growth, the presence of fungi, insects deterioration. In modern Pharmaceutical technology, the water content provides information concerning the Shelf life and quality of the drugs.

$$\text{Moisture content (\%)} = \frac{\text{Final weight of the sample}}{\text{Initial weight of the sample}} \times 100$$

pH

1 g of capsule powder was taken and dissolved in 100 ml demineralized water. The pH value of the solution was determined by means of a digital PH meter. The pH meter was calibrated using buffers of 4, 9 and 7 PH. The electrodes were immersed in the test solution and PH was measured.

7.3.6 ACCELERATED STABILITY STUDIES OF THE CAPSULES[85-87]

Definition

Stability is defined as the extent to which a product retains, within specified limits and throughout its period of storage and use (i.e., its shelf-life) the same properties and characteristics that it possessed at the time of its manufacture.

Accelerated testing

Studies designed to increase the rate of chemical degradation or physical change of a drug substance or drug product by using exaggerated storage conditions as per of the formula stability studies. Date from the studies , in addition to long tern stability studies, can be used to assess longer term chemical effect at non acceleration and to evaluate the effect of short –term excursions outside the label store conditions such a might occurs during shipping. result from accelerated testing studies are not always predictive of physical changes.

Conditions of Stability studies

- Accelerated condition of 40° C ± 2° C / 75% RH ± 5% RH
- Long term condition of 25° C ± 2° C / 60% RH ± 5 % RH
- Long term / intermediate condition of 30° C ± 2° C / 75 % RH ± 5% RH

The ICH Harmonized Tripartite Guideline provides a general indication on the requirements for stability testing of new drug substances and products. The main thrust of the stability guideline centers on criteria for setting up stability protocols.

Climatic zones:

The four zones in the world that are distinguished by their characteristic prevalent annual climatic

Table : 7 Climatic zones and derived storage conditions.

ZONE	CONDITION	TEMPERATURE	HUMIDITY
Zone 1	Temperature	21ºc	45% RH
Zone 2	Sub-tropical with possible humidity	25ºc	60% RH
Zone 3	Hot/ dry	30ºc	35% RH
Zone 4	Hot / humid	40ºc	70% RH

As our country comes under zone IV, the following conditions were maintained throughout the study

Accelerated stability condition : Accelerated stability study were carried out of storage condition at **40° C ± 2° C of humidity 70% RH for 3 month(time period covered).**

7.4 PHARMACOLOGICAL STUDIES

7.4.1. ACUTE TOXICITY STUDY (OECD 423 GUIDELINES) [88,89]

Animals were kept in the lab for one week to acclimatize to laboratory conditions before starting the experiment, they were allowed to free access of water and standard rat feed.

Organization for Economic co-operation and development (OECD) regulates guideline for oral acute toxicity study. It is an International Organization which work with the aim of reducing both the number of animals and the level of pain associated with acute toxicity testing. Organization for Economic co-operation and development (OECD) regulates guideline for oral acute toxicity study. It is an International Organization which work with the aim of reducing both the number of animals and the level of pain associated with acute toxicity testing.

Following are the main type of guideline followed by OECD

Guideline 420, fixed dose procedure (5 animals used)

Guide 423, Acute toxic class (3 Animals used)

Guide 425, Up and Down method (1 Animal used)

Toxicity – Acute toxic class method (OECD 423 GUIDELINES)

The acute oral toxicity study was carried out as per the guidelines set by Organization for Economic Co-operation and Development (OECD), received from Committee for the Purpose of Control and Supervision of Experiments of Animals (CPCSEA), Ministry of Social justice and Empowerment, Government of India.

The method is based on biometric evaluation with fixed doses (5,50,300 and 2000 mg/kg b.w., p.o) and results allow the substance to be ranked and classified according to Globally Hormonised System (GHS) classification of extract which causes hypolipidemic and anti-obesity.

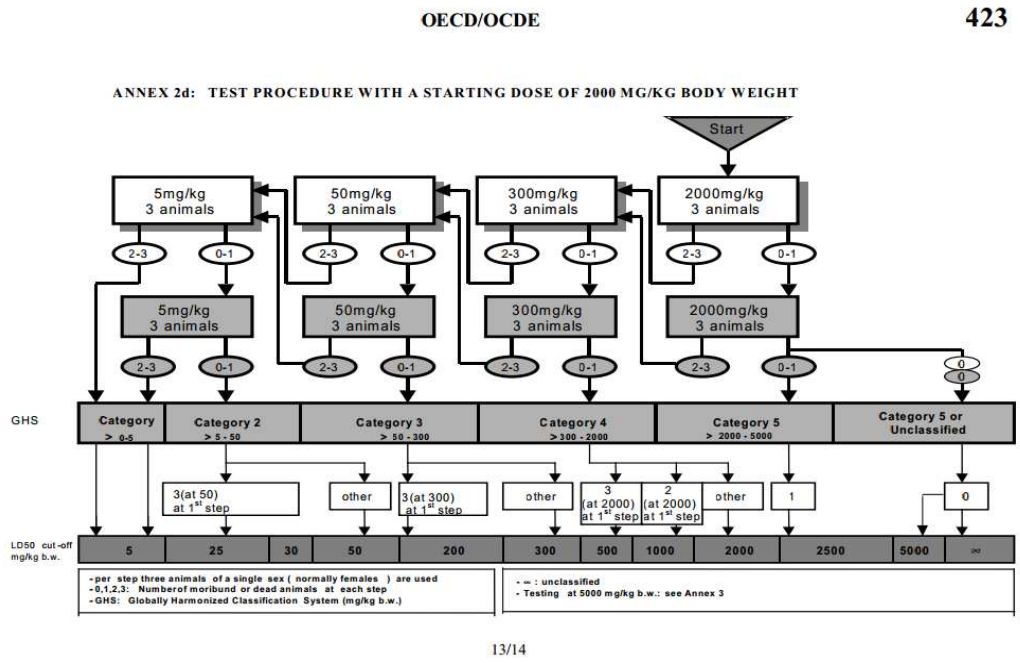


Fig 13 : OECD guidelines

Healthy female albino rats were selected and the animal were procured from the animal house of the Institution were used. Animals were divided into 2 groups of 3animals each. The starting were deprived of diet for four hours and water was given *ad libitum*. The animals dose level 2000 mg/kg bw.,p.o of the extracts of tablet was administrated.

The animals were kept under direct observation for first four hours and thereafter for 24 hours and were observed for mortality. The animals were then kept under observation for 14 days.

Body weight of rats before and at the end of the termination was observed and any changes in awareness, mood, motor activity , CNS excitation , Motor coordination, muscle tone , reflexes were noted. The onset of toxicity and signs of toxicity was also if any noted.

There was no death as per the guidelines, so the study was repeated with the same dose to confirm the result.

The protocol for conducting the *In Vivo* study in either sex of adult *albino wistar* rat was approved by the Institutional Ethical Committee (ICE) of the Madras Medical College, Chennai - 600003, India **Approval no: Vide 14/243/CPCSEA. Dated :10.08.2015**

EXPERIMENTAL DESIGN[90-92]

7.4.2 *IN -VIVO* EVALUATION OF HYPOLIPIDEMIC AND ANTI-OBESITY ACTIVITY (HIGH FAT INDUCED OBESITY IN RATS MODEL)

Experimental animals

Thirty *albino wistar* rats of thirty weighing 140-160 gm were randomly divided into 5 groups of six animals each and kept in their cages for 1 week prior dosing to allow for acclimatization to the laboratory conditions, with free access to food and water, *ad libitum*. The study was carried out after obtaining the Animal ethics committee (**14/243/CPCSEA. Dated :10.08.2015**).

Chemicals

Cholesterol, coconut oil and Atorvastatin, chemicals used for the study were of analytical

grade.

Dose selection

The content of HECS herbal capsules was found to be safe at the dose of 2000mg/kg in the acute toxicity study. Hence for *In-vivo* evaluation, two doses of the hydro alcoholic extract of *Caesalpinia sappan* (HECS) herbal capsule were selected as 200mg and 400mg/kg, p.o. Atorvastatin was calculated based on a human dose of 1mg /kg, p.o.

Experimental design

Rats were divided into five groups containing six animals each. The schedule of grouping and treatment is given below in table 8

Table 8 :HIGH FAT DIET(HFD) INDUCED HYPERLIPIDEMIA AND OBESITY MODEL

GROUP	NAME OF THE GROUP	TREATMENT SCHEDULE
Group 1	Normal control	Normal food and vehicle p.o for 60 days
Group 2	Disease control	HFD and vehicle p.o for 60 days
Group 3	Standard control	HFD and vehicle for p.o 60 days and atorvastatin 1mg/kg from 31-60th days.
Group 4	Test group 1	HFD and vehicle for 60 days and HECS herbal capsule 200mg/kg p.o from 31-60th days.
Group 5	Test group 2	HFD and vehicle for 60 days and HECS herbal capsule 400mg/kg p.o from 31-60th days.

Hyperlipidaemia was induced by feeding a high fat diet that consists of 58% fat, 25% protein and 17% carbohydrate , lard(13%), vitamins, minerals and cholesterol 400mg/kg in coconut oil to all healthy rats except group 1(normal control rats)for 60days.

7.4.3 EVALUATION PARAMETERS

A) Body weight

The body weight (g) was recorded on day1, day 30 and day 60 using a digital weighing balance in each group animal**s.** The changes in body weight were calculated.

B) Organs weight

The animals were sacrificed on 60th day by cervical dislocation and then the different organs (kidney, liver and heart) were removed dried and then weighed.

C) Biochemical lipid connstituents parameters

2ml of the blood was collected from retro orbital sinus puncture, and all the animals were sacrificed by cervical dislocation, and then the organs were removed and weighed. The collected blood was allowed to clot for 30 minutes, centrifuged and then used for evaluating the lipid constituents and biochemical parameters.

Biochemical lipid constituents/parameters — The main biochemical parameters recommended by the National Cholesterol Education Program (NCEP) guidelines (2002) for lipid screening as follows

- Total Cholesterol (TC)
- Low Density Lipoprotein Cholesterol (LDL)
- Very Low Density Lipoprotein
- Cholesterol (VLDL)
- High Density Lipoprotein Cholesterol (HDL)
- Triglycerides (TG)

were evaluated from the serum. From the values, atherogenic index (TC:HDL) and LDL:HDL ratio were calculated using the formula.

serum glutamate oxaloacetate transferase(SGOT) and serum glutamate pyruvate transferase(SGPT) by standard method were also evaluated from serum using standard methods.

Cardiac risk indicators — The cardiac risk ratios recommended by NCEP guidelines (2002) were estimated by calculating the TC: HDL ratio (Atherogenic Index) and LDL: HDL ratio. The Friedewald formula was used to calculate serum low-density lipoprotein cholesterol(LDL-C) values and atherogenic index as follows:

LDL-C= TC– (HDL-C+TG/5)

Atherogenic index(AI)=(total cholesterol–HDL-C)/ HDL-C

7.4.4 HISTOPATHOLOGICAL EXAMINATION:

A small portion of aorta and liver was taken from 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 find out the Atherosclerotic lesions in aorta and to find out cellular degeneration and necrosis in liver .

Statistical Analysis

Results were expressed as Mean ± SEM. The data was analyzed using one way analysis of variance (ANOVA) followed by Dennett's test. P values <0.01 were considered as Significant.

8. RESULTS AND DISCUSSION

7.1 PHARMACOGNOSTICAL STUDIES

7.1.1) Table 9: Macroscopical characters of *Caesalpinia sappan* Heartwood

s.no	Parameters	Observation
1	Colour	Orange- red
2	Taste	Bitter
3	Odour	Slight
4	Surface	Grained
5	Texture	Fine and even
6	Shape	Straight

7.1.2 MICROSCOPIC FEATURES

Transverse section (TS) of the wood:

Wood is porous (having the pores or vessels) The growth – rings are fairly distinct. The vessels are circular or ovate . They are mostly solitary or less frequently radial multiples of two to four vessels. The vessels have very thick walls which are lignified. Most of the vessels are open and some have gum deposits. The vessels are 100-200 µm wide.

Axial parenchyma :-

Parenchyma cells wich are vertically oriented are called axial parenchyma cells. The axial parenchyma is abundant . They occur as thin tangential layer along boundary of the growth ring.Such parenchyma cells are called paratracheal parenchyma . The parenchyma cells are thick walled, lignified and have wide lumen.

The xylem rays are seen in several closely running radial lines of parenchyma cells. The ray parenchyma cells are radially elongated, narrow lumened and thick walled. They run in straight lines. Xylem fibres form the ground tissue of the wood. The fibres are angular in sectional view. They are compact; they have very thick, lignified walls. The cell lumen is reduced to a narrow hole.

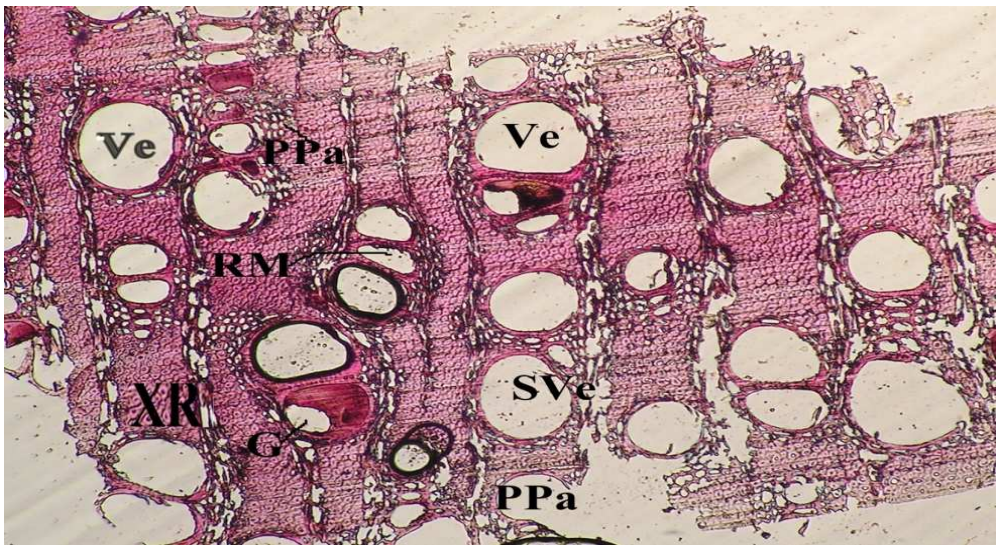


Fig14: TS of wood showing vessel distributions.

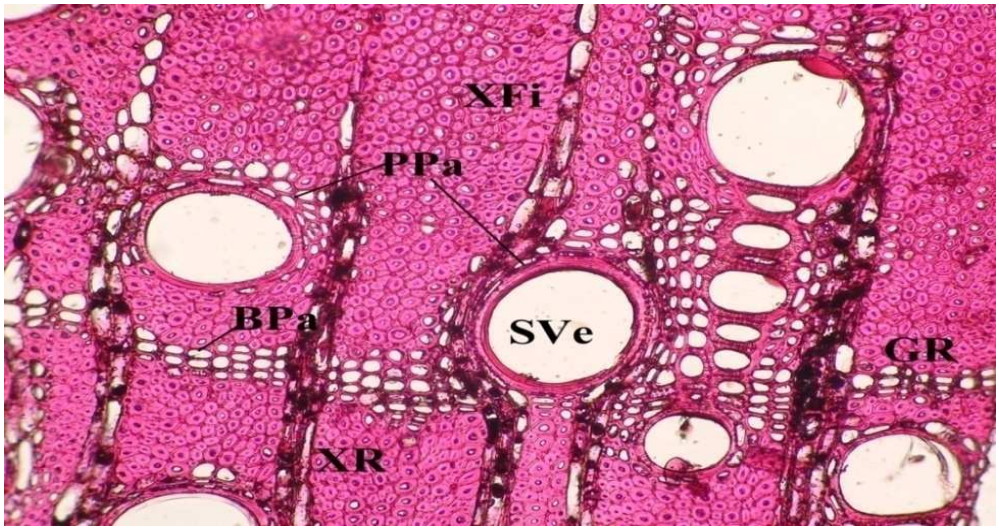


Fig 15: TS of wood showing solitary vessels, growth ring.

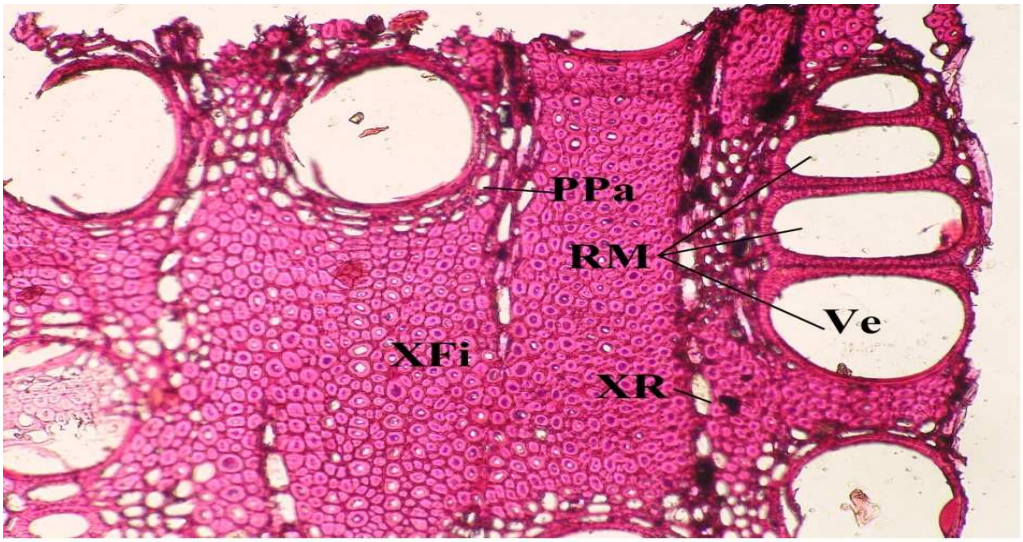


Fig 16: TS of wood showing radial multiples of vessels.

G- gum deposition on the crosswall of the vessels; **PPa**- paratracheal parenchyma; **BPa**-boundary parenchyma; **RM**- radial multiples of vessels; **Ve**- vessels ; **XF**- xylem fibres;
XP- xylem parenchyma; **XR**- xylem ray; **GR**-growth ring; **SVe**-solitary vessel.

Tangential longitudinal section(TLS view)

In TLS view, the xylem rays spindle shaped; the vessels, axial parenchyma and xylem fibres are seen in vertical alignment.The rays are random in vertical orientation. So the rays are uniseriate , biseriate or multiseriate . Thus the rays exhibit variations in seriation.The rays are either homocellular or heterocellular. The homocellular rays have uniform type of cells. The heterocellular rays have two type cells: the cells in the middle part of the rays isodiametric; the cell at the ray are vertically elongated. The isodiametric cells in the middle part of the rays are called procumbet cells; those at the ends of the rays are 300-600 µm in height and 50-100 µm thick. The ray cells are narrow and thick walled.

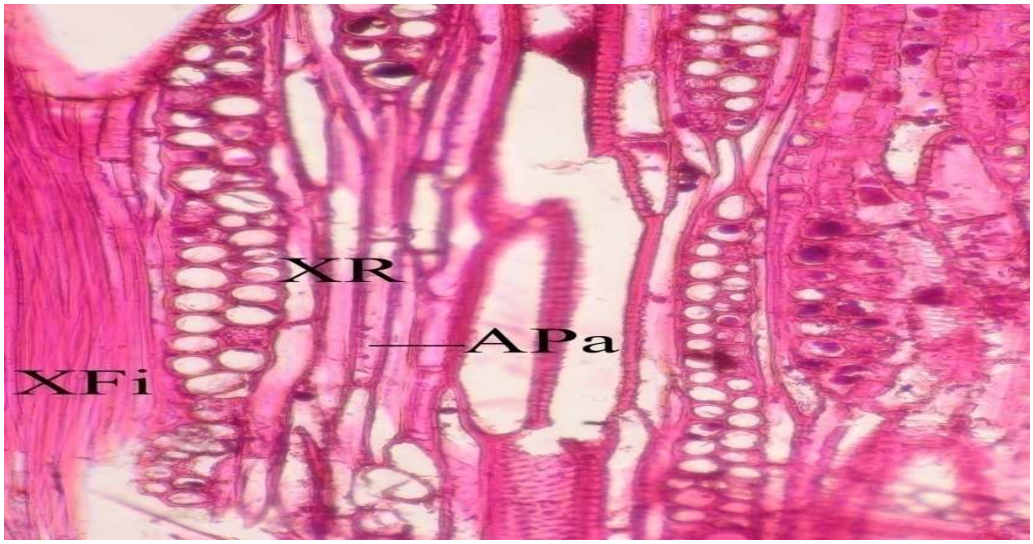


Fig 17: TLS of wood showing axial parenchyma

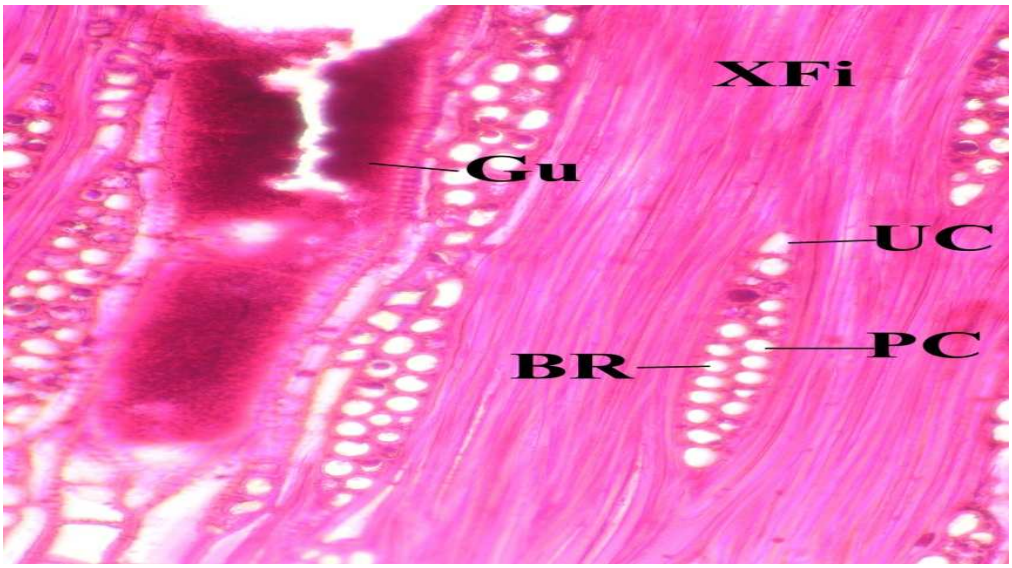


Fig 18: TLS of wood showing gum, biseriate xylem rays.

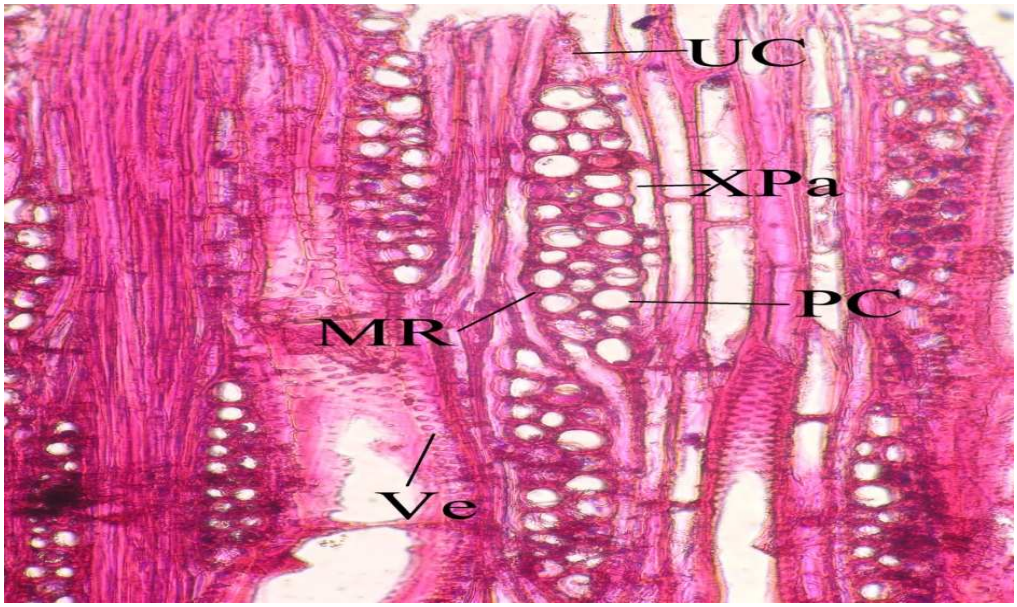


Fig 19: TLS of wood showing horizontal ribbon shaped ray.

GPI- gum plate; **Ve**- vessel; **XFi**- xylem fibres; **XPa**- xylem parenchyma; **XR**- xylem rays

APa- axial parenchyma; **BR**- biseriate ray; **Gu**-gum; **MR**- multiseriate ray; **Pc**- procumbent cells; **UC**- upright cells; **Ve**-vessel; **XFi**- xylem fibres; **XPa**- xylem parenchyma ; **XR**- xylem ray.

Radial longitudinal sections of the wood(RLS view)

In RLS view, the xylem rays appear as wide and flat horizontal ribbon like bands. The xylem fibres, parenchyma and the vessels are at vertical rows at right angles to the rays. The procumbent cells of the ray are horizontally elongated, narrow and thick walled. The ray cells have dense simple pits. The upright cells are short, and squarish in shape.The axial parenchyma cells are long in vertical plane; they occur clusters of 2-4 rows. The cells have minute circular pits.

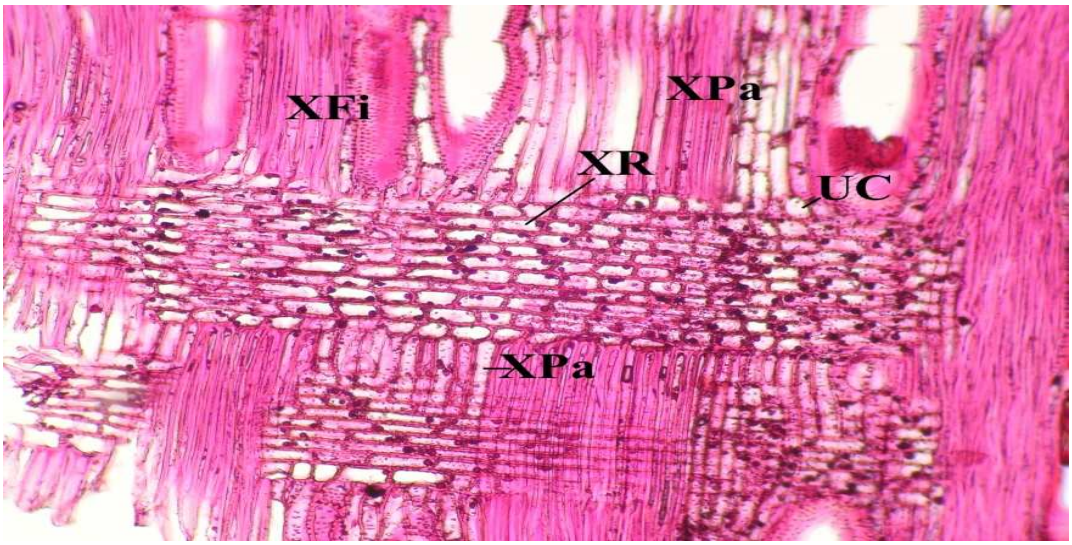


Fig 20: RLS of wood showing horizontal ribbon shaped rays

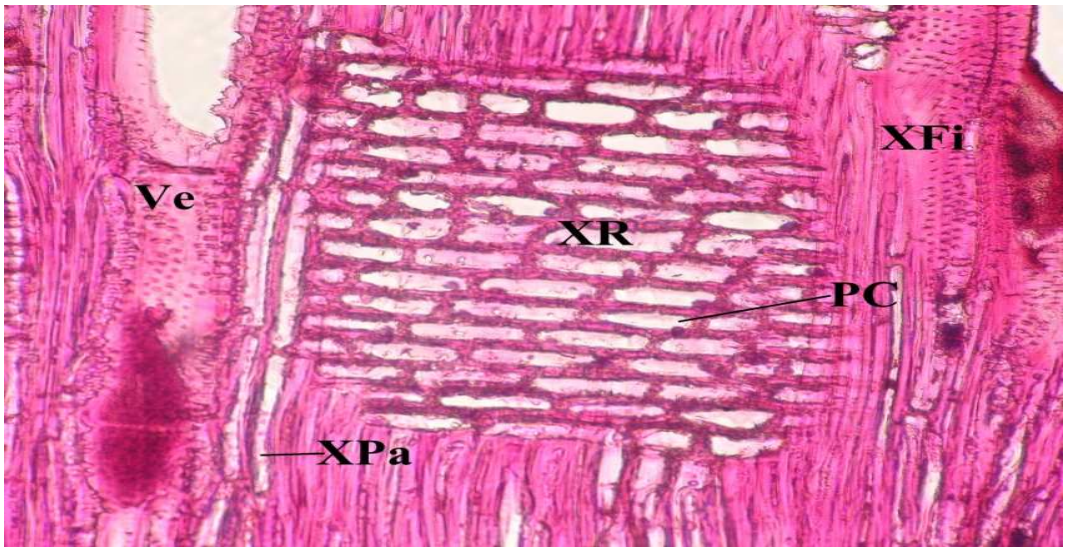


Fig 21: RLS of wood showing ray cells enlarged.

Pc- procumbent cells; **Ve**- vessel; **XFi**- xylem fibres; **XPa**- xylem parenchyma; **XR**- xylem ray ; **Uc** – upright cells

Powder microscopic observations

The powder preparations of the wood shows the following elements when observed under the microscope

(i) Fibres:-Long, thin needle shaped fibres are abundant in the powder. They have thick lignified walls and much reduced lumen. The fibres are 600- 800 µm long and 20µm thick.

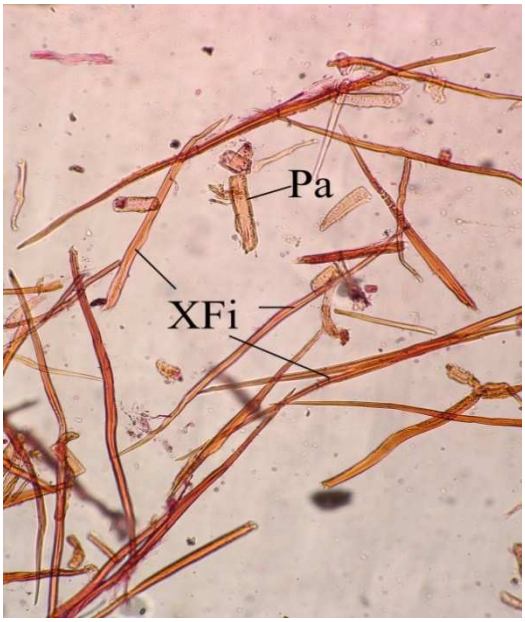


Fig 22: xylem fibers

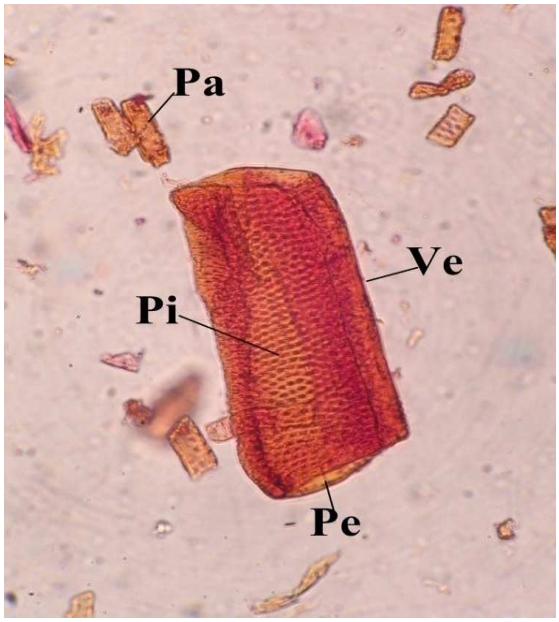


Fig23: pitted vessel

Pa- parenchyma; **XFi-**xylem fibres. **Pe-** perforation; **Pi-** pits; **Ve-** vessel element.

(ii) vessel elements:-

Vessel elements are fairly common in the powder . vessel elements of the powder shows long thick vessel elements with prominent bordered pits, short wide vessel element and wide, short barrel shaped vessel element were identified.. These vessels elements are 250 µm long and 140µm wide .There are also wide, very short and drum shaped vessel elements which are 130µm long and 250 µm wide. The vessel elements have dense multiseriate elliptical bordered pits(Fig.22).

(iii) **Parenchyma cells** :-

Rectangular , short parenchyma cells are occasionally seen. The cells are thick walled with dense simple pits. The cells are 90 µm long and 15 µm wide (Fig.23).

7.1.3 PHYSICO CHEMICAL CONSTANTS

Table 10: Physicochemical constants on heartwood of *Caesalpinia sappan* Linn.,

S.NO	PHYSIO-CHEMICAL CONSTANTS	RESULTS (%w/w)
I .	**ASH VALUE**	
1.	Total ash	1.22±0.02
2.	Water soluble ash	0.38±0.03
3.	Acid insoluble ash	0.13±0.01
4.	Sulphated ash	1.14±0.05
II .	**EXTRACTIVE VALUE**	
1.	Water soluble extractive	8.89±0.26
2.	Alcohol soluble extractive	4.80±0.3
3.	Ether soluble extractive	3.11±0.34
III .	**Loss on drying**	3.91 ±0.31
IV .	**Foaming index**	<100
V .	**Swelling index**	NIL
Values are expressed as Mean ± SD, n=3		

1.4 INORGANIC ELEMENTS AND HEAVY METAL ANALYSIS

Table 11: Qualitative estimation of inorganic elements of *Caesalpinia sappan* Linn.,

s.no	INORGANIC ELEMENTS	OBSERVATIONS
1.	Aluminium	+
2.	Chloride	+
3.	Copper	+
4.	Calcium	+
5.	Iron	+
6.	Borate	+
7.	Potassium	+
8.	Silver	+
9.	Phosphate	-
10.	Nitrate	-
11.	Sulphate	-

7.1.5 Table 12: Quantitative estimation of inorganic elements of *Caesalpinia sappan* Linn.,

S.NO	INORGANIC ELEMENT	TOTAL AMOUNT (%W/W)
1	Aluminium	0.028
2	Chloride	0.052
3	Copper	0.009
4	Calcium	0.010
5	Iron	0.028
6	Borate	0.005
7	Potassium	0.020
8	Silver	0.007

Quantitative estimation of Heavy metals by ICP OES method

The quantification of the individual heavy metals was analyzed for the powdered mixture of *Caesalpinia sappan* Linn., by ICP-OES technique the following metals like arsenic, lead, cadmium were detected and quantified, results are given in the following table

Table 13: Quantitative estimation of Heavy metals

S.No	Element	Results (ppm)	Specification as per WHO Guidelines
1.	Mercury	Not detected	Not more than 0.5ppm
2.	Arsenic	Not detected	Not more than 5.0ppm
3.	Lead	0.002	Not more than 10ppm
4.	Cadmium	Not detected	Not more than 0.3ppm

The above observation showed that the material is within the limits as per WHO standard and it is safe to consume internally.

7.2 PHYTOCHEMICAL STUDIES

7.2.1 Table 14: Percentage yield of Hydroalcoholic extract of *Caesalpinia sappan* Linn.,

EXTRACT	METHOD OF EXTRACTION	PHYSICAL NATURE	COLOUR	YIELD (%W/W)
Hydro-alcoholic	Cold maceration	Crystalline powder	Reddish Brown	12

7.2.2 QUALITATIVE PHYTOCHEMICAL ANALYSIS

Table 15: Qualitative Phytochemical analysis

Note: + indicates presence, - indicates absence .

S.No	Chemical constituents	Powdered drug	Hydroalcoholic extract
1.	Steroids	+	-
2.	Glycosides	+	+
3.	Saponins	+	+
4.	Flavonoids	+	+
5.	Tannins	+	+
6.	Phenolic compounds	+	+
7.	Proteins	+	+
8.	Alkaloid	-	-
9.	Carbohydrates	+	+
10.	Terpenoids	+	+
11.	Fixed oils and Fats	+	-

7.2.3 QUANTITATIVE ESTMATION OF PHYTOCONSTITUENTS

The *Caesalpinia sappan* Linn., was found to contain various phytochemical constituents and hence it is desirable to quantify few of them in order to establish a standard to maintain its quality. Among them the estimation of total Saponins , Flavonoids and phenolic content in the hydroalcoholic extract were decided to be taken as parameters. Samples were drawn from three random samples of *Caesalpinia sappan* Linn., and the total Saponins, Flavonoids and phenolic content present in them were estimated by HPLC method.

Estimation of flavonoids by HPLC method

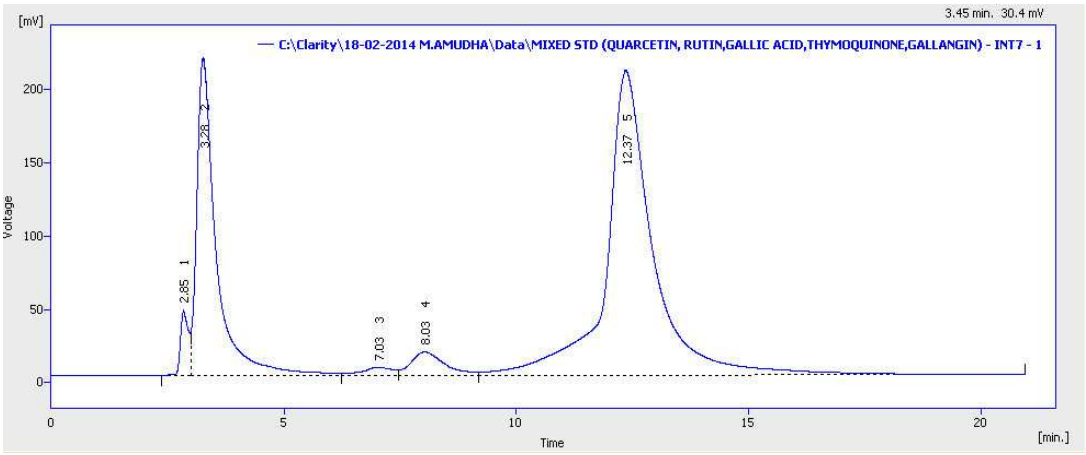


Fig 24: Graphical representation of Standard curves for flavonoids

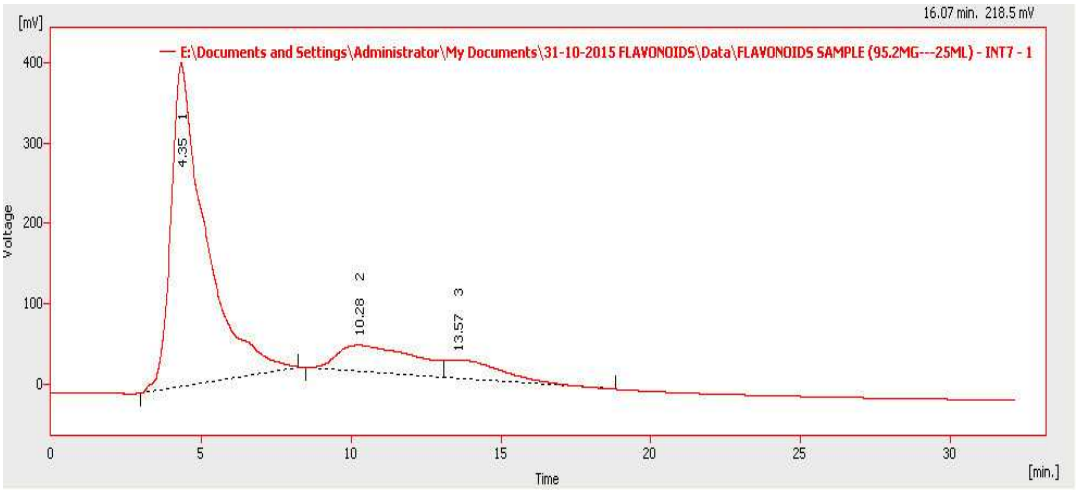


Fig 25: Graphical representation of Hydroalcoholic extract curve for flavonoids

Table 16: Quantitative estimation of flavonoids present in hydroalcoholic extract.

Flavonoids	Hydoalcoholic extract per gm.
Rutin	0.934mg
Quercetin	0.184mg
Gallangin	0.158mg

Estimation of phenolic content by HPLC method

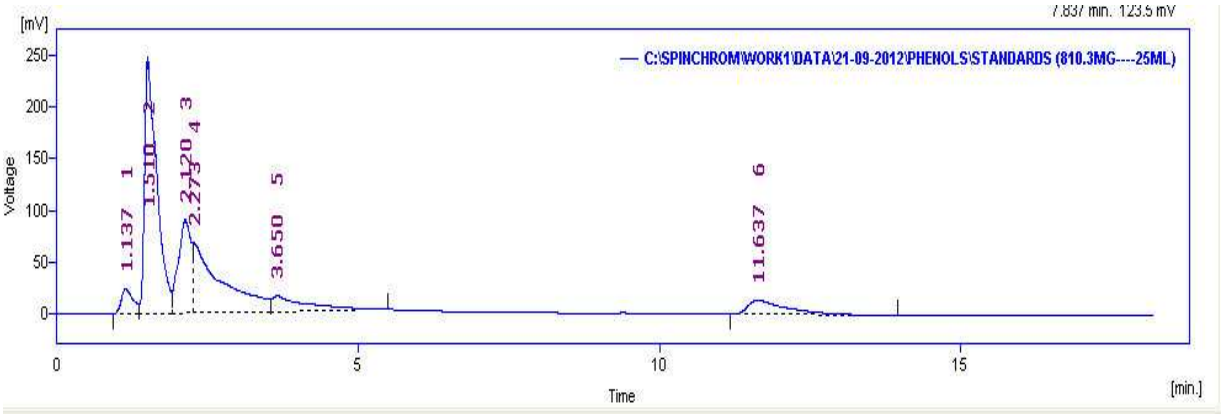


Fig26: Graphical representation of Standard curves for phenolic content

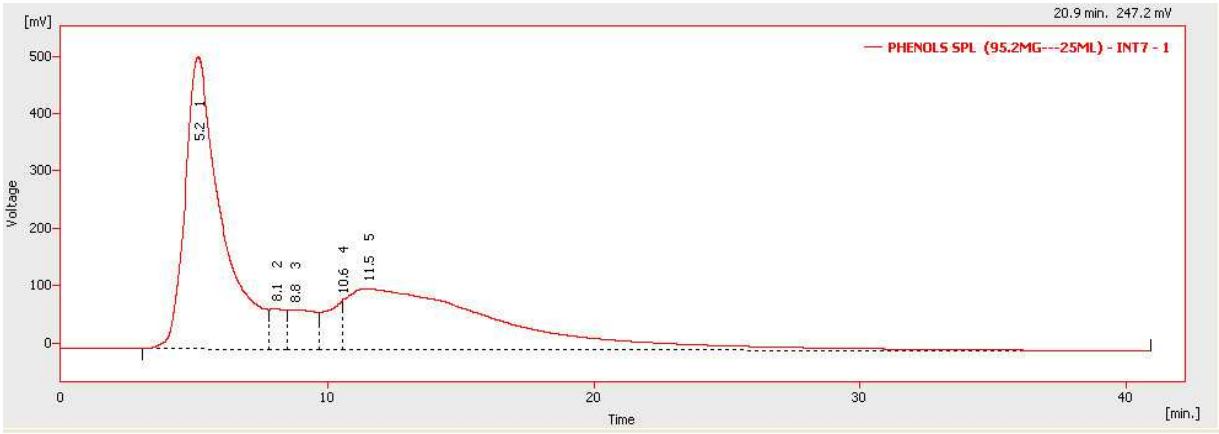


Fig 27: Graphical representation of Hydroalcoholic extract curve for phenolic content

Table 17: showing quantitative estimation of phenolic content present in hydroalcoholic extract.

Phenols	Sappan Extract Per gm.
Gallic acid	0.519mg
Tannic acid	0.083mg
Ascorbic acid	0.987mg

ESTIMATION OF TOTAL SAPONIN CONTENT

Table 18: QUANTITATIVE ESTIMATION OF SAPONIN

Parameters	Values (µg/ml)
Total saponins	8.24 ±0.15

7.2.4 FLORESCENCE ANALYSIS

Table 19:Fluorescence characteristic of powdered samples and extract

S.NO	TREATMENT	DAY LIGHT	SHORT UV (254nm)	LONG UV (366nm)
1.	Powder	Orange red	Yellow	Brown
2.	**Powder + water**	Orange red	Yellow	Brown
3.	**Powder + NaOH**	Red	Reddish brown	Violet
4.	**Powder + Hcl**	Orange	Yellowish brown	Brown
5.	**Powder + Acetic acid**	Orange	Yellow	Dark brown
6.	**Powder + Alc.NaOH**	Red	Brown	Violet
7.	**Powder + Picric acid**	Light orange	Brown	**Green fluorescence**
8.	**Powder + Sulphuric acid**	Black	Brown	Black
9.	**Powder + Nitric acid**	Orange brown	Brown	Cherry brown
10.	**Powder + Iodine**	Brown	Black	Dark brown
11.	**Powder + $Fecl_3$**	Brownish black	Black	Brown
12	**Hydroalcoholic extract**	Reddish brown	Black	Brown

The green fluorescence observed above indicates the presence of fluorescence compounds in *Caesalpinia sappan* .

7.2.5 THIN LAYER CHROMATOGRAPHY OF EXTRACTS

Table 20: Thin layer chromatography of hydroalcoholic extract at different solvent system.

S.No	Phyto-constituents	Solvent system	Detecting agent	No. of spots	R_f value
1.	Saponin	chloroform: Glacial Acetic acid:methanol:Water (6.4:3.2:1.2:0.8)	Iodine chamber	5	0.28 0.40 0.54 0.71 0.80
2.	Flavonoids	Ethyl acetate: Formic acid: Glacial Acetic acid: Water(100:11:11:26)	Iodine chamber	3	0.72 0.83 0.91

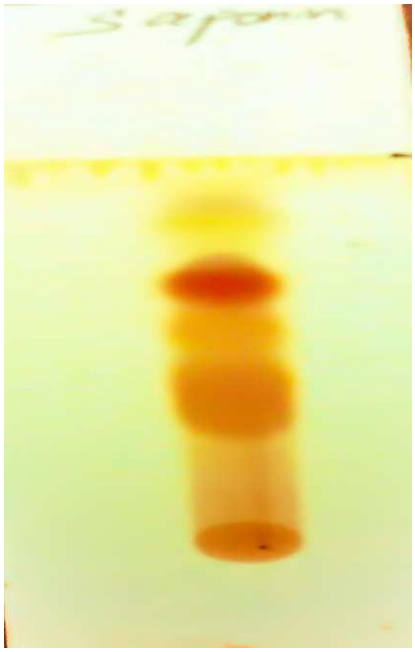


Fig 28: TLC of extract at Saponin solvent system

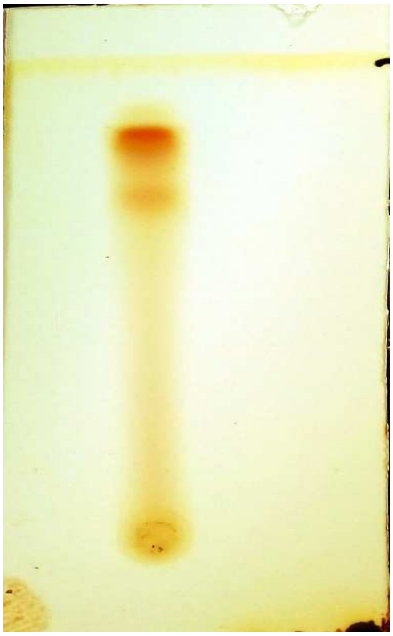


Fig 29:TLC of extract flavonoid solvent system.

HPTLC FINGER PRINT PROFILE

HPTLC Finger print Data of hydroalcoholic Extract of *caesalpinia sappan* Linn.,

High performance thin layer chromatography (HPTLC) finger printing was performed with the hydroalcoholic extract of ***caesalpinia sappan* Linn.,**. The chromatographic conditions were carried as detailed in material and method of this study. There were 13 peaks observed with different R_f Values and different heights. Percentage of areas was also obtained from the chromatogram.

SOLVENT SYSTEM

Table 21: Solvent system for HPTLC

Extract	Solvent System
Hydroalcoholic extract	Ethyl acetate : chloroform : methanol (5.3:1.5:0.5)

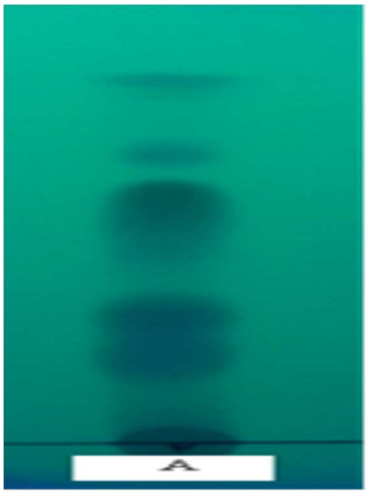


Fig 30: HPTLC Finger Print Data

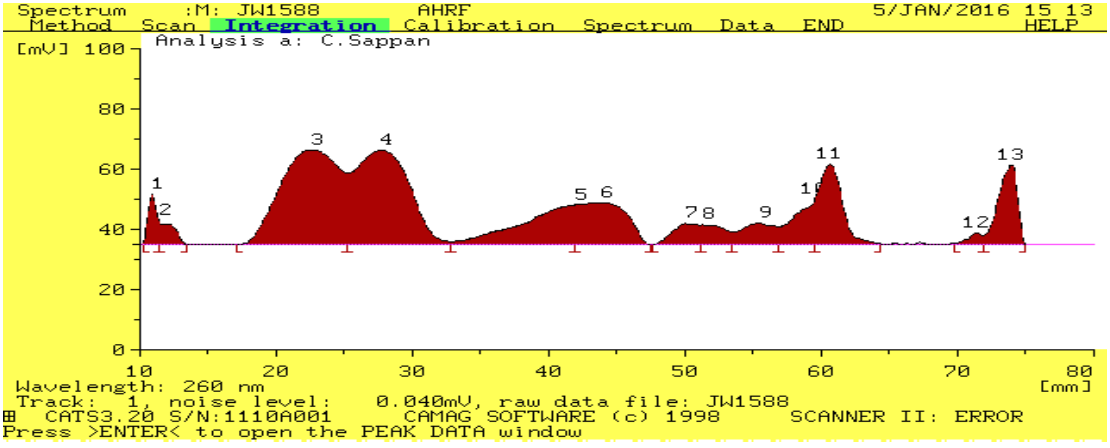


Fig 31: Graphical representation of chromatographic finger printing analysis of the extract.

Table 22: HPTLC profile for Hydroalcoholic extract

S.No	Rf	Height	Area	Lamda Max
1	0.10	16.7	112.1	359
2	0.12	7.8	99.2	205
3	0.23	31.5	1590.9	256
4	0.28	31.2	1449.0	255
5	0.42	13.1	579.1	400
6	0.44	13.9	584.3	400
7	0.50	6.9	153.9	277
8	0.51	6.5	130.9	277
9	0.56	7.0	201.5	277
10	0.60	15.2	262.6	277
11	0.61	26.5	516.4	315
12	0.72	3.9	44.5	277
13	0.74	26.4	425.3	200

7.3PREPARATION OF FORMUULATION

The raw materials were sampled, authenticated and analysed for their compliance to quality standards as established by WHO guidelines, pharmacopoeial and other standard reference books.

7.3.1RAW MATERIAL STANDARDIZATION

Table 23: Foreign organic matter determination

Plant name	Observation (w/w)	Limit
Caesalpinia sappan	0.44	NMT 2

Results are reported as mean ± standard deviation; (n=3): NMT- not more than.

The raw material standardization of organoleptic evaluation, microscopical evaluation and physico chemical evaluation were already given in the pharmacognostical and phytochemical studies of the plant.

MICROBIAL LOAD ANALYSIS

Tests carried out for the estimation of number of viable aerobic microorganisms present

Table 24: microbial load analysis of the hydroalcoholic extract of *Caesalpiinia sappan*

S.NO	PARAMETERS	*CAESALPINIA SAPPAN* EXTRACT
1	Total aerobic count (NMT 1000cfu/g)	50cfu/g
2	Yeast and mould count (NMT 100 cfu/g)	1 cfu/g
3	*E.Coli* (To be absent)	Absent
4	*Salmonella* (To be absent)	Absent
5	*Pseudomonas*(To be absent)	Absent
6	*Staphylococcus* (To be absent)	Absent

7.3.2PREFORMULATION STUDIES

3 trial batches of the herbal formulation were prepared and tested for preformulation parameters like bulk density, tap density, Carr's index, Hausner's ratio and Angle of repose. The results observed is shown in table.

Table 25 Preformulation studies- trial batches

s.no	Materials	Trial1 (g)	Trial-2 (g)	Trial-3 (g)
\1	*Caesalpinia sappan extract*	20	20	20
2	Talc	1.7	1.8	2.5
3	Mcc	0.8	0.9	1
4	Starch	2	2.5	2.6
5	Magnesium stearate	0.8	1	1.5
6	Colloidal sillicon dioxide	0.25	0.28	0.3
7	Bronopol	0.15	0.15	0.15
8	Sodium methyl paraben	0.15	0.15	0.15

Table 26 a: Evaluation of inprocess parameters

Parameters	**Trial-1**	**Trial-2**	**Trial-3**
Bulk density (g/cm)	0.42±0.01	0.38±0.05	0.35±0.04
Tap density (g/cm)	0.45±0.03	0.47±0.01	0.50±0.04
Compressibility index(%w/w)	26.83±0.66	23.26±2.54	13.06±1.12
Hausner ratio	1.35±0.15	1.22±0.02	1.13±0.01
Angle of repose(^{o})	40.42±2.57	39.36±2.67	34.66±0.18

All values are expressed as standard mean deviation ±, where n=3

Table 26b: Evaluation of inprocess parameters.

Parameters	**Trial-1**	**Trial-2**	**Trial-3**
Flow property	Normal	Fair	Perfect
Filling	Uniform	Uniform	Uniform
Weight	Not uniform	Not Uniform	Uniform
Moisture content	Satisfied	Satisfied	Perfect
Disintegration time	Within the limit	Within the limit	Perfect

As per the standards, the flow property of the blend to be filled in the capsules should be in good range and was confirmed by the above parameters. Trial batch- 3 showed excellent flow characters and that batch was taken for capsule filling.

The trial 3 flow properties were Excellent and all parameter were within the Specified limits. So, third trial was chosen for further studies.

7.3.5 STANDARDISATION OF FINISHED FORMULATION

The final batch was tested for organoleptic characters , physical and physic chemical parameters. The results observed are shown in table

Table 27:Organoleptic characters

Name of test	**Observations**
Description	Pale brown powder contained in purple cap/ transparent body “1” size capsule
Colour	Reddish brown powder
Odour	Characteristic odour
Taste	Bitter

Table 28: Physical parameters

Name of the test	Observations
Moisture content	3.6%±0.22
Uniformity of weight	268mg±4.5mg
Disintegration time	3.32(min)±0.34
pH(1% aqueous solution)	7.33±0.21

Results (n-=3) are reported as mean ± standard deviation.

- ✓ 1% aqueous solution of herbal formulation showed acidic pH.
- ✓ The average weight of the capsules was calculated as per I.P and the obtained value was with in the limit (±10%).
- ✓ Sample were taken randomly (3times) to specify quantity, the moisture content was calculated as per trail and error by KFR titration method. The result were given in the above table .
- ✓ Disintegration time of the herbal capsule was performed as per I.P and the obtained value showed that the capsule will be disintegrated within the prescribed time for the absorption.
- ✓ The uniformity of weight of the capsules was calculated as per the I.P and obtained value was within limit(±7.5).
- ✓ The formulated herbal capsule weight were the lower limit is noted as 248 mg and the upper limit is noted as 287mg.

PRELIMINARY PHYTOCHEMICAL SCREENING OF CAPSULES

The herbal formulation was found to contain various phytochemical constituents and hence it is desirable to quantify few of them in order to establish a standard to maintain its quality. Among them the estimation of total phenolics, Flavanoids in the aqueous extract were decided to be taken as parameters. Samples were drawn from three random samples of herbal capsules and the phenolics, Flavonoids content present in them were estimated by HPLC method and it is given below.

Table 29: preliminary screening of HECS herbal capsule

S.No	Chemical constituents	Powdered drug	HECS herbal capsule
1.	Steroids	+	-
2.	Glycosides	+	+
3.	Saponins	+	+
4.	Flavonoids	+	+
5.	Tannins	+	+
6.	Phenolic compounds	+	+
7.	Proteins	+	+
8.	Alkaloids	-	-
9	Carbohydrates	+	+
10.	Terpenoids	+	+
11.	Fixed oils and Fats	+	-

The results were established a scientific data which can be used for the identification of the crude drugs.

QUANTITATIVE ESTIMATION OF PHYTOCONSITUENTS OF HERBAL CAPSULES

Estimation of flavonoids for HECS herbal capsule:

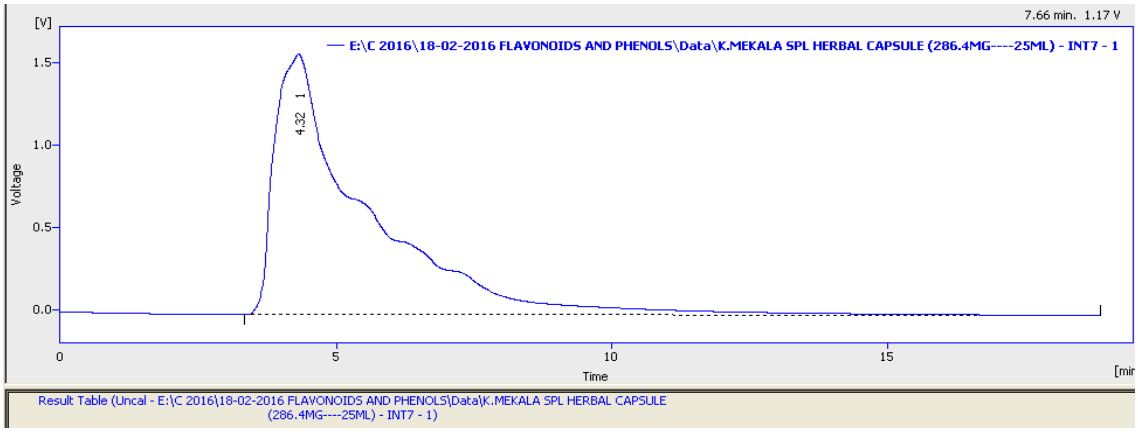


Fig 32: Graphical representation of HECS herbal capsule for flavonoids

Table 30: Quantitative estimation of of flavonoids present in each herbal capsule

FLAVONOIDS	HECS HERBAL CAPSULE (250 MG)
Rutin	0.258mg
Quercetin	0.091mg
Gallangin	0.056mg

Estimation of phenolic compounds for HECS herbal capsule :

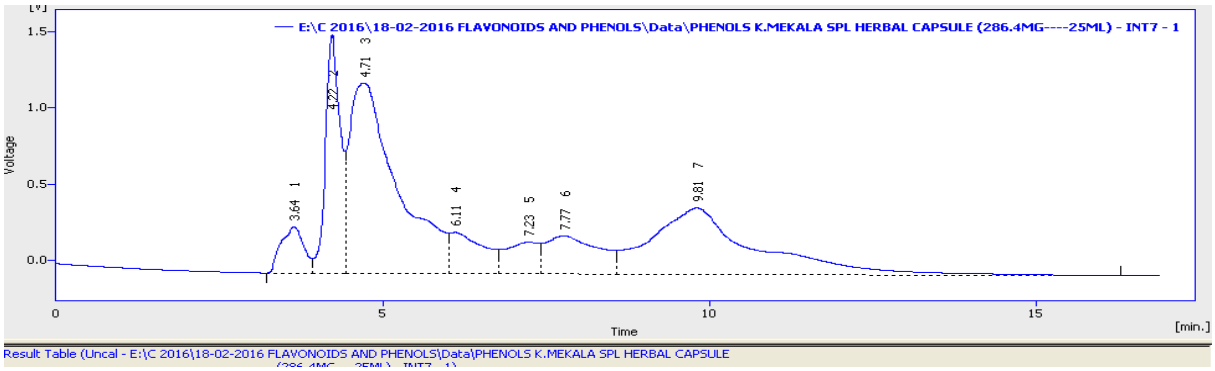


Fig 33: Graphical representation of Estimation of phenolic compounds for HECS herbal capsule

Table 31: quantitative estimation of phenolic compounds in each capsule

Phenolic compounds	HECS herbal capsule (250mg)
Gallic acid	0.224mg
Ascorbic acid	0.481mg
Tannic acid	0.138mg

From the results obtained it is determined that the average content of phenolics, Flavonoids were present in the herbal formulation.

7.4 PHARMACOLOGICAL STUDIES

7.4.1 ACUTE TOXICITY STUDIES

Behavioural and physical observation of *Caesalpinia sappan* Linn., treated rats (2000mg/kg body weight)

Table 32: Observation of Acute toxicity study

OBSERVATION	30min	4 hrs	14 hrs	24 hrs
Body weight	NC	NC	NC	NC
Cage side observation	N	N	N	N
Motor activity	N	N	N	N
Convulsions	A	A	A	A
Piloerection	A	A	A	A
Righting reflex	P	P	P	P
Lacrimation	N	N	N	N
Salivation	N	N	N	N
Respiration	N	N	N	N
Skin color	N	N	N	N
Diarrhoea	A	A	A	A
Loss of corneal reflex	N	N	N	N
Loss of pinnal reflex	N	N	N	N
Grooming	A	A	A	A
Sedation	N	N	N	N
Excitation	N	N	N	N
Aggression	N	N	N	N

Note : NC-no change, A- absent, N- normal, P- present

Acute toxicity studies were carried out as per the OECD Guidelines 423 and the HECS herbal capsules were found to be no morbidity and mortality upto 2000 mg/Kg body weight. Hence 1/10th and 1/20th of the dose (200 and 400mg/kg) were taken for the study

7.4.2IN-VIVO HYPOLIPIDEMIC AND ANTI-OBESITY ACTIVITY

Changes in body weight from initial and final are tabulated in table 33

Table 33: changes in body weight

Groups	Changes in body weight			% increase in body weight
	1st day	30th day	60th day	
Group 1 (Normal control)	150±7.07	153±6.32	154±5.49	2.5%
Group 2 (Disease control)	154±8.01	183.3±12.11[a]	210±14.14[a]	36.36%
Group 3 (Standard control)	160±14.14	185±10.48[ab]	166±8.01[ab]	4.1%
Group 4 (low dose 200mg/kg)	168±14.71	202±14.71[ab]	188±17.51[ab]	11.9%
Group 5 (high dose 400mg/kg)	166±11.14	200±12.64[ab]	174.16±8.16[ab]	4.9%

Values are expressed as mean ± SEM(n=6)

'a' values are significantly different from normal control at P< 0.05

'b' values are significantly different from disease control at P <0.05

Data are analysed by one way ANOVA followed by DUNNETT'S t-test.

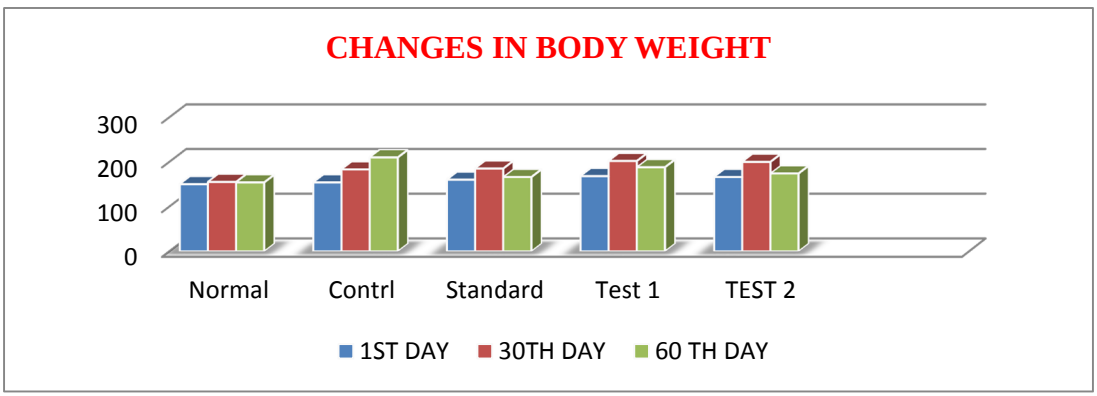


Fig: 34 Graphical representation of change in body weight

It is seen that there was a considerable increase in the body weight of animals which was treated with HFD induced obesity. This increase in body weight was much reduced in animals concomitantly treated with Atorvaststin and HECS herbal capsule in 2 doses of 200mg/kg and 400mg/kg.

Effect of HECS herbal capsule on (different organs weight) HFD induced obesity in rats

Various different organs weight were evaluated for all five groups and tabulated in Table -

Table 34: Evaluation of organs weight

Groups	Different organ weights(g)			
	heart	Liver	Kidney	
			Right	Left
Normal control	0.57±0.21	5.28±1.12	0.6±0.09	0.58±0.07
Disease control	0.69±0.02[a]	6.13±2.45[a]	0.66±0.7[a]	0.56±0.5[a]
Standard control	0.56±0.12[ab]	4.32±3.20[ab]	0.57±0.45[ab]	0.52±0.72[ab]
Test group 1	0.65±0.2[ab]	5.59±1.70[ab]	0.62±1.02[ab]	0.53±0.86[ab]
Test group 2	0.59±0.31[ab]	5.02±1.45[ab]	0.61±1.04[ab]	0.50±0.04[ab]

Values represents mean ± SEM (n=6);

'a' values are significantly different from normal control at P< 0.05

'b' values are significantly different from disease control at P <0.05

Data are analysed by one way ANOVA followed by DUNNETT'S t-test.

It is seen that HECS herbal capsule remarkably decreases the organ weight of rats .

7.4.3Various lipid profile parameters were evaluated for all five groups and tabulated in table - **HECS herbal capsules were analysed for lipids parameters .**

Table 35: Effect of HECS herbal capsule on lipid profile

GROUPS	TOTAL CHOLESTEROL (mg/dL)	TRIGLYCERIDES (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)
Group 1	62.55 ± 5.54	73.32±5.57	23.22±2.31	24.67±1.63	14.66±2.7
Group 2	195.2±10.56^{ab}	115±5.47^{ab}	17.71±6.10^{ab}	154.42±7.52^{ab}	23±2.01^{ab}
Group 3	65.43±2.62^{ab}	72±11.12^{ab}	24.30±3.10^{ab}	25.66±3.32^{ab}	14.4±2.10^{ab}
Group 4	78.56±6.46^{ab}	85.24±5.55^{ab}	19.62±4.56^{ab}	42.42±3.66^{ab}	7.84±2.68^{ab}
Group 5	69.72±5.62^{ab}	76.5±5.69^{ab}	24.14±3.13^{ab}	30.33±3.51^{ab}	15.3±2.11^{ab}

Values are expressed as mean ± SEM

Data are analysed by one way ANOVA followed by DUNNETT'S t-test.

'a' values are significantly different from normal control at P< 0.05

'b' values are significantly different from disease control at P <0.05

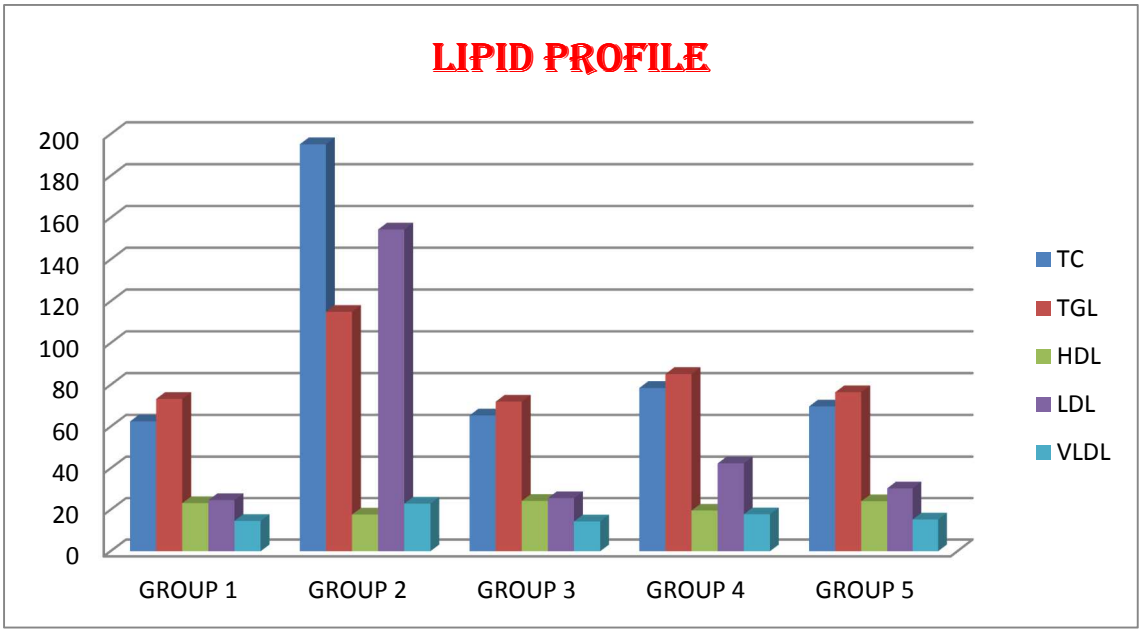


Fig 35 : Graphical representation of lipid profile

It is seen that HFD treated groups, the lipid values were significantly higher than the control animals. Treatment with atorvastatin significantly reduced the lipid levels. Treatment with the HECS herbal capsule , at both doses also significantly reduced the lipid levels and increased the HDL level. At the high dose of 400mg/kg the protection offered was better.

Table 36: Effect of HECS herbal capsule on atherogenic index(AI) and LDL/HDL

GROUPS	AI	LDL/HDL
Group 1	1.69±0.23	1.06±0.70
Group 2	10.02±1.62[a]	8.69±1.23[a]
Group 3	1.69±1.01[ab]	1.05±1.07[ab]
Group 4	3.00±1.36[ab]	2.16±0.80[ab]
Group 5	1.88±1.03[ab]	1.25±1.12[ab]

Values are expressed as mean ± SEM

'a' values are significantly different from normal control at $p < 0.05$

'b' values are significantly different from diseased control at $p < 0.05$

Data are analysed by one way ANOVA followed by DUNNETT'S t-test.

The atherogenic index is an indicator of cardiovascular disease. The HFD treated group showed an increase level of atherogenic index compared to normal group. Herbal capsule treated groups showed decrease level of atherogenic index as compared to disease group.

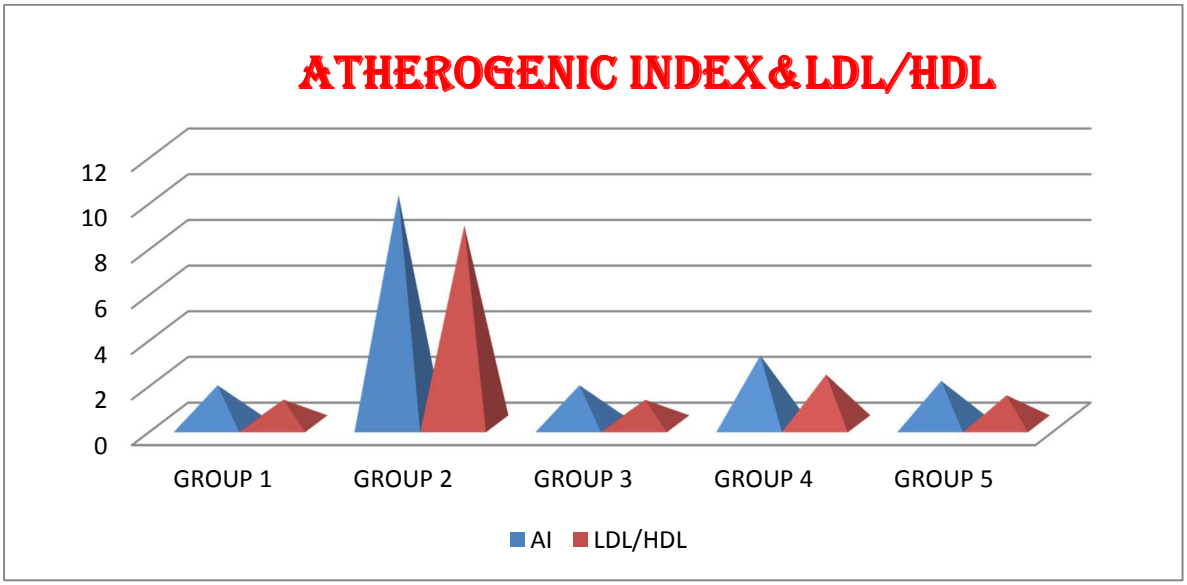


Fig 36: Graphical representation of atherogenic index

Table 37: Effect of HECS herbal capsule of Liver function test

GROUPS	SGPT (IU/L)	SGOT (IU/L)
Group 1	23.77±3.08	51.22±2.21
Group 2	62.84±2.17[a]	112.70±1.71[a]
Group 3	29.46±1.88[ab]	82.06±2.01[ab]
Group 4	38.03±2.19[ab]	84.44±1.45[ab]
Group 5	29.05±3.31[ab]	79.73±3.6[ab]

Values are expressed as mean ± SEM

'a' values are significantly different from normal control at $p < 0.05$

'b' values are significantly different from diseased control at $p < 0.05$

Data are analysed by one way ANOVA followed by DUNNETT'S t-test.

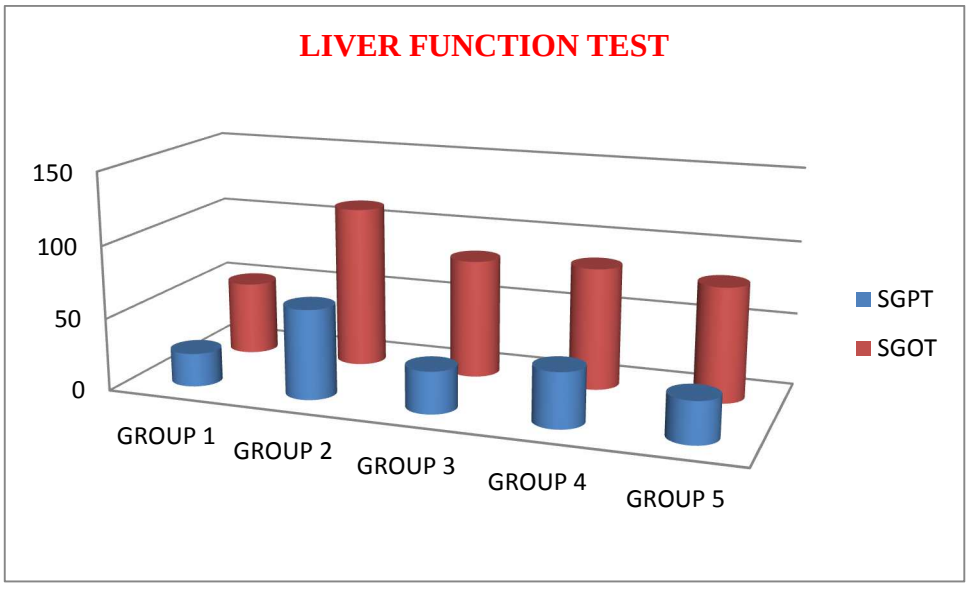


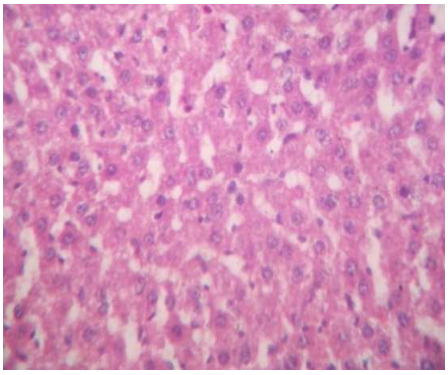
Fig 37: Graphical representation of liver function test

Liver function parameters such as SGOT and SGPT also showed a significant increase in animals fed with cholesterol. These levels decreased significantly in the standard and extract treated groups.

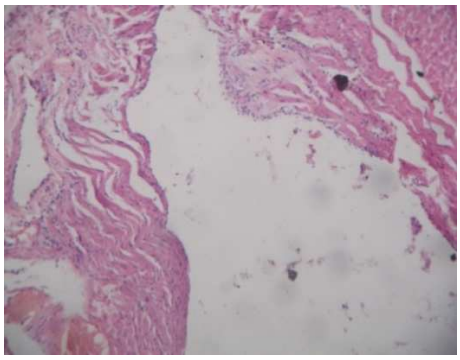
7.4.4 Histopathological reports are given in figure 38 & 39

Fig 38: HISTOPATHOLOGY OF LIVER

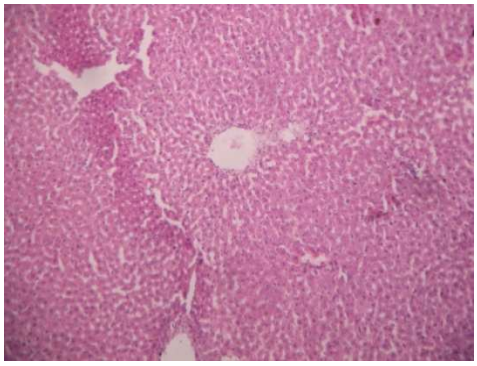
NORMAL CONTROL



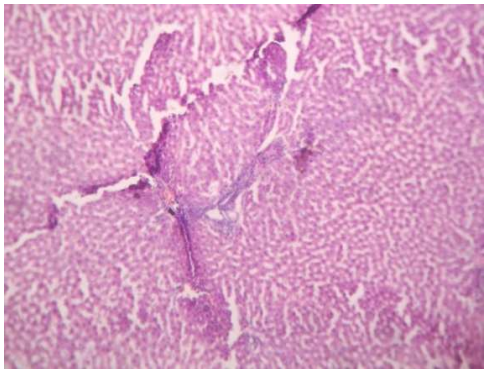
DISEASE CONTROL



STANDARD CONTROL



TEST GROUP 1



TEST GROUP 2

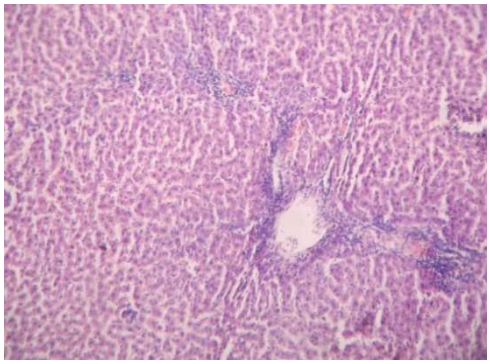
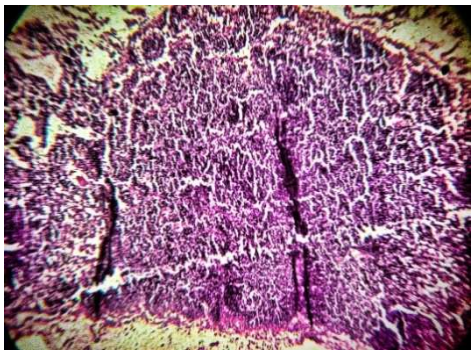


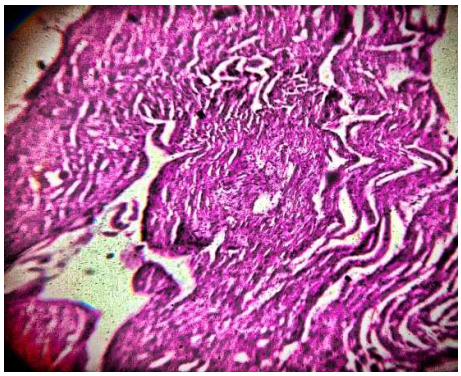
FIG 39:HISTOPATHOLOGY OF RAT AORTA

NORMAL CONTROL

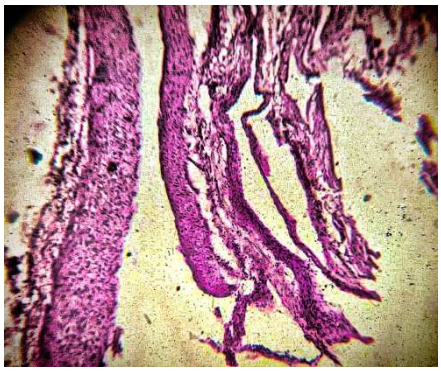


DISEASE CONTROL

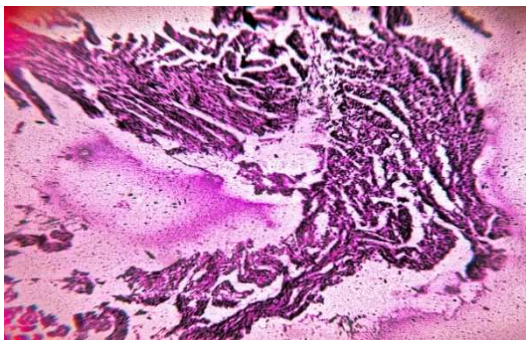
STANDARD CONTROL



TEST GROUP 1



TEST GROUP 2



Histopathology of liver:

The high fat diet induced obesity and abnormal lipid metabolism all collectively are associated with inflammation , congestion, and non-alcoholic fatty liver disease leading to hepatic failure causing a boost in SGPT, SGOT level in the serum.

- Histopathological studies showed that the liver section was normal in normal control group.
- The HFD treated group showed marked ballooning, cellular degeneration and inflammation.
- These changes were absent in Atorvastatin treated standard group.
- HECS herbal capsule (200mg/kg) treated test group 1 showed decrease in cellular degeneration and inflammation when compared to control group.
- The test group 2 treated with HECS herbal capsule 400mg/kg showed maximum suppression of cellular degeneration and inflammation.

Histopathology of aorta

HFD intakes were shown to contribute to syndromes such as hyperlipidemia, glucose intolerance,hypertension and atherosclerosis. Atherosclerosis are main cause of cardiovascular and cerebro vascular diseases.

- Histopathological studies showed that the aorta section was normal in normal control group.
- The HFD treated group showed marked atheromatous thickening (plaque) in the intima and atheromatous inflammatory changes.
- These changes were absent in Atorvastatin treated group.
- The HECS herbal capsule (200mg/kg) treated group showed decrease in atheromatous plaque size and inflammatory changes as compared to HFD treated group.
- Wheareas 400 mg/kg of extract treated group showed maximum level of suppression of atheromatous plaque size and atheromatous inflammatory changes.

DISCUSSION

PHARMACOGNOSTICAL STUDIES

Pharmacognostical studies play a key factor in establishing the authenticity of the plant material. The botanical identity of the heartwood was established by examining its anatomical features.

The macroscopic and microscopic features of the heartwood of *Caesalpinia sappan* were studied. Some of the characteristic features of the heartwood are ;

- The anatomical examination of *Caesalpinia sappan* Linn., heartwood exhibited important microscopical features like multiseriate medullary rays in TLS view and ribbon shaped medullary rays in RLS view, pitted vessels, numerous xylem fibers, large vesicles and parenchymatous cells.
- Physiochemical parameters are mainly used in judging the purity and quality of the powdered drug. Ash values of a drug give an idea of the earthy matter or inorganic elements and other impurities present along with the drug.
- Ash value is an important parameters to find the low grade product and exhausted drugs. A high ash value is an indicator of substitution, contamination and adulteration. The total ash value was found to be 1.22±0.02 %w/w. The acid insoluble, water soluble and sulphated ash was found to be 0.13±0.01%,0.38±0.03 % and 1.14±0.05% respectively.
- Extractive value is helpful for the prediction of nature of constituents. The water soluble, alcohol soluble, ether soluble (volatile oil) was found to be 8.89±0.26%, 4.88±0.3%, 3.11±0.34%.
- Loss on drying determines the amount of volatile matter present and it was found to be 3.91±0.31 %. There is no swelling index which indicates that the powder was devoid of sticky mucilage and the foaming index was found to be less than 100.

- Presence of inorganic elements and heavy metals were analysed qualitatively and quantitatively. The inorganic metal analysis showed the absence of toxic metals. The quantification of heavy metals shows the presence of elements within the limit.

The detailed pharmacognostical studies on the heartwood of *Caesalpinia sappan* Linn., provides information on the standardization parameters and physiochemical parameters which is essential for the identification of raw material and also used to differentiate the plant from its adulterants and substitutes.

From the above results a complete profile of pharmacognostical parameters was evolved.

PHYTOCHEMICAL STUDIES

Since herbal medicines are prepared from materials of plant origin they are prone to contamination, deterioration and variation in composition. Hence, before proceeding to clinical studies, scientists need a tool to authenticate plants and also to detect their potency.

A lot of analytical techniques have been developed for quality control of drugs from plant origin. Therefore it is very important to undertake phytochemical investigations along with biological screening to understand therapeutic efficacy of medicinal plants and also to develop quality parameters.

- In this analysis different polarity of Phytoconstituents were sorted out from the coarsely powdered heartwood of *Caesalpinia sappan* Linn., by using solvents of ethanol and water (1:1) of hydroalcoholic extract by using cold maceration extraction.

- Qualitative preliminary phytochemical analysis was performed initially with different respective chemical detecting agent to detect the Phytoconstituents nature and their presence in each extract and powder.

- Quantitative estimation of saponins, phenolic contents, flavanoids, and fluorescence analysis of extracts with different chemical reagents were carried out. flavonoids and phenolic content by HPLC method and saponin content by gravimetric method. Rutin, quercetin and gallangin were found to be present in quantitative amount in the flavonoids estimation and in the phenolic content estimation gallic acid, tannic acid and ascorbic acid was found to be present in the hydroalcoholic extract of the plant.

- Qualitative chromatographic analysis of hydroalcoholic extract were done by using Thin Layer Chromatography to separate and identify the single or mixture of constituents present in extract. The following solvents system was used to separate the phytoconstituents by using Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:26).

- High performance thin layer chromatography (HPTLC) finger printing was performed with the hydroalcoholiic extract . There were 13 peaks observed with different R_f values and different heights. Percentage of areas were also obtained from the chromatogram.

  The phytochemical studies showed the presence of flavonoids, phenolic content and saponins .This may be responsible for the potent hypolipidemic and anti-obesity activity.

FORMULATION STUDIES

- The herbal raw materials were analyzed for their identity, quality and purity. The raw materials were standardized according to WHO Guidelines and Ayurvedic Pharmacopeia of India. Materials which complied with the specification were taken for further studies. The optimized herbal formulation was evaluated for various physiochemical and phytochemical parameters.

- The formulation was optimized for it quality measures and its batch consistency by making three different trials (Trial I, II, III).The trails were subjected to pre formulation parameters to confirm the uniformity and quality.

- The result concludes that the trial III was excellent in all parameters and the values were found within the standard limits. Quantitative estimation of phytoconstituents were done for flavonoid, saponin and phenolic compounds .

- Accelerated stability studies were carried out as per ICH Guidelines for a period of three month. The resultant stability data has shown that the formulation is stable under accelerated stability conditions.

- Acute toxicity studies were carried out and the herbal capsules were found to be very safe upto 2000 mg/Kg body weight by Acute toxicity study as per the OECD Guidelines 423.

PHARMACOLOGICAL STUDIES

Pharmacological studies were carried out for assessing the hypolipidemic and Anti-obesity of the plant *Caesalpinia sappan* Linn.,

- Acute toxicity studies of the HECS herbal capsule were carried out for a period of 14 days. It did not produce any behavioural changes or mortality up to the dose of 2000 mg/kg of body weight of rat.
- So the LD_{50} value in the range of 2000 to 5000 mg/kg of body weight .So the in vivo studies were carried out a dose of 200 mg/ kg and 400 mg /kg.

- The high fat induced obesity in rats model and cholesterol 400mg/kg was used to induce hyperlipidemia and obesity in rats.

- The in-vivo studies showed that the hydroalcoholic extract of *Caesalpinia sappan* (HECS) herbal capsule, especially at a higher dose, improved the lipid profiles of the animals.

- The total cholesterol and LDL cholestertol levels were decreased whereas the HDL cholesterol level showed an increase.

- The atherogenic index is an indicator of cardiovascular disease. A high atherogenic index indicates a higher risk of cardiovascular disease. The atherogenic index decreased in standard group and herbal capsule treated groups.

- The animals which were treated with high dose (400mg/kg) of the capsule showed a greater improvement in atherogenic index.

- Human studies have revealed that increased fat intake is associated with body weight gain, which can lead to obesity and other related metabolic diseases. This study proves that rats exposed to high fat diet for 60 days cause significant increase of animals body weight, thus verifying the rats obese. The animals which were treated with high dose of 400mg/kg of the HECS herbal capsule showed a decrease in animals body weight and internal organs weight.

  All the parameters reveal the potent hypolipidemic and anti- obesity activity of the HECS herbal capsule of *Caesalpinia sappan* Linn.,.

9. SUMMARY

Herbal medicines are found to be effective in treatment of various aliments but the major lacuna is lack of proper scientific validation .Hence the present study is aimed at investigating the selected plant *Caesalpinia sappan* Linn., for the hyperlipidemic and obesity problem.

The plant *Caesalpinia sappan* Linn., belong the family Caesalpiniaceae, is claimed to be useful for hypercholesteremia, but the claim has not been scientifically validated.

Authentication of the plant material plays a key role in pharmacognostical studies. The plant *Caesalpinia sappan* Linn., was collected from kulasekaram, kanyakumari district, and authenticated by Dr.V. Chelladurai, Research officer in siddha and ayurvedha.

The parameters studied were Macroscopy, microscopy, powder microscopy and physicochemical constants to establish data for proper authentication and detection of adulterants.

The qualitative and quantitative analysis were carried out to identify inorganic elements present in the plant.

The qualitative and quantitative analysis of toxic heavy metals like Cadmium, Arsenic, Lead and Mercury were within the WHO limits and ensure the safety of the drug .

In phytochemical analysis, extraction is the first step involved. The coarse powder was extracted by ethanol and water (1:1)by cold maceration method .

The preliminary phytochemical screening of various extract of the plant have revealed the presence of constituents like flavonoids , saponins, tannins , glycosides, carbohydrates, triterpenes ,and terpenoids .

HPLC analysis of hydroalcoholic extract was carried out to estimate the quantitative amount of flavonoids and phenolic content present in the extract.

TLC and HPTLC analysis of hydroalcoholic extract was carried out to identify phytoconstituents present.

The preparation of herbal capsule on heartwood of *Caesalpinia sappan(*HECS*)* for clinical trial in rats and to investigate the hypolipidemic and anti-obesity potency of the formulation.

The herbal raw materials were analyzed for their identity, quality and purity. The raw materials were standardized according to WHO Guidelines and Ayurvedic Pharmacopeia of India. Materials which complied with the specification were taken for further studies. The optimized herbal formlations was evaluated for various physiochemical and phytochemical parameters.

The physiochemical constants like Loss on drying, pH, Ash values and Extractive value were determined, which will help in preventing variation in quality of the drugs.

The heavy metal analysis and the microbial load analysis were carried out as per the WHO Guidelines to check its limit. These were found to be within the limits.

Accelerated stability studies were carried out as per ICH Guidelines for a period of three month. The resultant stability data has shown that the formulation is stable under accelerated stability conditions.

The quantitative analysis of phytoconstituents especially of flavonoids and phenolic content in extract and formulated herbal capsule was estimated by HPLC method showed the presence of rutin, quercetin, gallic acid, ascorbic acid and tannic acid.

Hypolipidemic and anti-obesity activity was assessed by method of high fat induced obesity in rats.The parameters studied were increase in body weight changes and organs weight, lipid profile parameters, liver function test and histopathological studies.

Hydroalcoholic extract of *Caesalpinia sappan* (HECS) herbal capsule was evaluated for acute toxicity studies in albino *wistar rats* and showed no toxicity upto 2000mg/kg.

In-vivo evaluation of high fat diet (HFD) induced obesity in rats was carried out for HECS herbal capsule .The studies showed HECS herbal capsule(250mg/kg and 500mg/kg) significantly reduced the elevated levels of body weight, total cholesterol, triglycerides, LDL-cholesterol, VLDL-cholesterol, SGPT and SGOT and elevate the decrease level of HDL-cholesterol.

Histopathological studies of aorta and liver of all treated group shows that they are normal and architecture is preserved.

These results suggest that, HECS capsule possess good hypolipidemic and anti-obesity activity, which may be due to its flavonoid, saponin and phenolic content.

CONCLUSION

Sappan wood with high therapheutic effect and vast folklore uses is a rich natural resource of lead compounds for drug development. Based on literature review, *Caesalpinia sappan* heartwood has high potential for therapeutic and colouring use.

Brazelien has potential pharmacological activity such as anti-tumour, anti-inflammatory, anti-diabetic ,immunostimulant properties and also anti thirst, blood purifying action and healing properties in Aurvedha and Unani beneficial to develop into a drug , neutraceuticals and cosmetics.

Future studies can be directed towards the Isolation, Characterization of individual compounds responsible for the hypolipidemic and Anti-obesity activity and mechanism of action responsible for this activity, so as to explore this plant for therapeutic purposes.

Further Scope: The developed herbal formulation may be taken up for clinical trials in the treatment of hyperlipidemic and obesity problems.

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REVIEW ARTICLE

A Review on Sappan Wood - A Therapeutic Dye Yielding Tree

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ABSTRACT:
The plant *Caesalpinia sappan* is a small leguminous tree upto 10m in height belonging to the Caesalpiniaceae family,with an orange-red hard Heartwood with spiny trunk ,which besides being useful in turnery gives a red dye. It is commonly known as Brazil or Sappan wood, native to tropical Asia, also grown as a hedge plant. The plant is being used worldwide for a large number of traditional medicinal purposes including anti-tumour, anti-inflammatory, immunosuppressive, anti-diabetic, anti-allergic, cardioactive, neurotoxicity, anti-bacterial, anti-acne , anemia, tuberculosis and some other activities. The main active constituents is Brazilin, and together triterpenoids, flavanoids, lipids, steroids, aminoacids etc are found. Leaves yield volatile oil, 0.16 to 0.25%; pods contain 40% tannin; seeds yield 32.1% and 34.4% mucilage and straw –yellow , edible oil(7.5%) having a characteristic smell; roots contains caesalpin-type diterpenoids along with sitosterol. In folklore medicine it is used as a herbal drinking water for its blood purifying, anti- thirst , anti-tumour and to improve complexion in Kerela. It has the potential to hit the market as a safe natural colouring agent with good medicinal value for pharmaceuticals, food products and beverages. The plant is one of the ingredients of an indigenous drug 'Lukol' which is administered orally for the treatment of non-specific leucorrhoea. The present article summarizes review on the plant, its phytochemistry and its pharmacological activity which have been reported.

KEYWORDS: *Caesalpinia sappan,* phytochemicals, Anti-tumour, Immunosuppressive, Cardioactive.

INTRODUCTION:
Caesalpinia sappan with highly interesting biological effects and vast folklore uses is worth studying more that might provide a rich natural resource of lead compounds for drug development. This plant enjoys the open altitude of 1000m above sea level and grows well in mountaineous areas that are not too cold (Fig 1). It is cultivated wild in west Bengal, Madhya Pradesh, orissa, These plant belongs to family Caesalpiniaceae, shaped small tree, 5-10m high; 15-25cm diam, spiny trunk and branches rufous-pubescent, stem round brownish green colour (Fig 2).

Propagation by seed or stem cuttings. Leaves 20-38cm long, pinnae 8-12 pairs, 10-15cm. Long , subsessile, with small prickles at the base. Leaflets 10-18pairs, subsessile, close, oblong, rounded at the apex, attached at the lower cornor, very in equilateral, glaborous above, more or less puberulous beneath (Fig 3)

Flowers in panicles, which are terminal and in the axils of the upper leaves, 30-40cm long; pedicles 1.3-1.5cm long, bracts lanceolate, 8mm long, caducous. Calyx 11mm, long , leathery, glaborous, corolla 2cm across; petals orbicular subequal, yellow, the upper with a red spot at the base, stamens delicate, waxy-white, filaments densely woolly at the base (Fig 3).Pods 7-10 by 3.8-5cm,woody, obliquely oblong, sub compressed, polished, indehiscent with a hard returned short beak at the upper angle of the obtuse apex (Fig 4) Seeds 3-4. wood has outer sapwood which is white pale buff colour and inner orange red heartwood[1-2] (Fig 5). The wood was formely used in calico printing of cotton, wool and silk. It is now however now replaced by synthetic dyes[3].

Fig 1 *Caesalpinia sappan*

Fig 2 Spiny trunk

Fig 3 Leaves and Flowers

Fig 4 Pods with Seeds

Fig 5 Heartwood

TAXONOMIC CLASSIFICATION[4]

Kingdom	: Plantae
Sub kingdom	: Viridiplantae
Infra kingdom	:Streptophyta
Super division	: Embryophyta
Division	:Tracheophyta
Sub division	: Spermatophytina
Class	:Magnoliopsida
Super order	:Rosanae
Order	:Fabales
Family	:Caesalpiniaceae/Fabaceae
Genus	:*Caesalpinia* Linn.
Species	:*Caesalpinia sappan* Linn– Sappan wood

PLANT CHEMICALS:

A great deal of chemical investigation has been carried out in this plant and presence of compounds, viz: triterpenoids, flavanoids, steroids, oxygen heterocycles, and aminoacids has been reported in the heartwood and seeds of this plant[3,5]. Brazilin, the main constituent of plant is oxidised to produce Brazilein by air and light[3]. Two new aromatic compounds structurally related to brazilin were isolated and identified from the heartwood of sappanwood. They possessed anti-hypercholesteremic activity[6]. The wood is reported to containing β-amyrin and glucose, and the free aminoacids: alanine, aspartic acid, glycine, proline, valine, leucine, threonine; free sugars: lactose, galactose, sorbose, 4-methyl galactose, 3,4-dimethyl galactose;2-deoxyribose and glucose are also present[7]. The petroleum ether extract of the heartwood gave 1.15% of a fixed oil(sap val,204.2; and iod val, 28.8) having the following fatty acid

composition: palmitic 27.6; stearic , 44.2; linoleic, 25.9; and oleic, 2.23%.The heartwood contains flavanoids and homoisoflavanoids. It also contain ns sappanol, episappanol, 3'-o-methylepisappanol, 3'-deoxysappanol, 3'-o-methyisappanol, 3'-o-methylbrazilin, 4-methylsappanol, quercetin, rhamnetin and ombuin is also reported[7,8]. Two new phenolic compounds, epicaesalpin j and 7,10,11-trihydroxydracaenone, were isolated from the heartwood of sappan[9]. Tannin is found in the leaves 19%, bark 44%, and pods 40%. Leaves yield volatile oil, 0.16 to 0.25%; d-α-phellandrene, terpene and methyl alcohol[10]

TRADITIONAL USES:

There are innumerable traditional medicinal uses of this plant. The plant is one of the ingredients of an indegenous drug 'Lukol' which is administered orally for the treatment of non-specific leucorrhoea (post-I.U.D), and gave encouraging results for bleeding following I.U.D-insertion[11].The decoction of the wood is considered as a powerful emmenagogue, astringent, sedative, stomachic, postpartum tonic, vulnery, tuberculosis, anemia, diuretic, used by women as tonic after confinement and also for vomiting of blood [12] in Ayurvedha and Unani. The wood is suitable for making cabinet-making, walking-sticks, and small ornamental turnery articles like dagger sheaths and hilts. Brazilin dye is reported to have anti-inflammatory activity[13]. The plant pigments find use in manufacture of facials which are resistant to light, heat and water, and non-irritating. The dyeing properties of wood is used in colouring food products, icecreams, bakery, confectionaries, beverages etc. The wood is the component of 'vicco vajaradanti'[TM] a famous tooth powder and paste of India. Its folkloric uses includes- in kerela , decoction of heartwood used for blood purifying, anti-thirst, anti-diabetic, and to improve complexion. In Ayurveda, useful in vitiated conditions of Pitta, burning sensations, wounds, ulcers, leprosy, skin diseases, diarrhoea, dysentery, and diabetes. In Thailand , used for arthritis, cancer, and inflammatory complaints. In Indo-china, seeds used as stomachic.[2]

PHARMACOLOGICAL ACTIVITY

ANTI-INFLAMMATORY ACTIVITY:

The methanolic extract of the heartwood showed anti inflammatory activity. The active constituent Haematin isolated from the heartwood used in oriental medicine act both as an analgesic and anti inflammatory agent. In the test conducted for inhibition of hyaluronidase activity, among 130 herbal medicines , sappan Lignum one of the six acive plants, its methanol extract at 5mg/ml concenteration showed more than 50% inhibition of hyaluronidase activity. Study evaluated the effects of an ethanolic extract on human chondrocytes and macrophages. Results showed an anti-inflammatory effect in an invitro cell model of joint inflammation[14].

CYTOTOXICITY AND ANTI-TUMOUR ACTIVITY:

The anti cancer activity of brazilein, a compound isolated from *Caesalpinia sappan* was investigated. MTT assay showed that the IC50 value of brazilein against human breast cancer MCF-7 cells was 7.23±0.24 umol/L. Western blot, RT-PCR assay, and RNA interference assay illustrated that brazilein induced growth inhibition of breast cancer cells and down regulation of GSK-3β/β-catenin pathway was involved in its mechanism.[15] The chloroform extract induces cell death in head and neck cancer cells lines. It resulted in increases in the HNSCC4 and HNSCC31 cells, which is linked to increased cellular levels of p21 $^{WAF}1/^{CIP}1$. Sappan wood act as a anti tumour agent in oriental medicine[16]. Methanol and water extracts exhibited marked cytotoxic activity against human cancer cells lines such as HeLa, MDA MB, A 549, and HCT-15 in the MTT assay. The water extract obtained from the heartwood of *caesalpinia sappan* has shown promising cytotoxic and apoptotic potential. The in vivo study in albino mice using Ehrlich carcinoma model resulted in an increase in the life span[17].

HEPATOPROTECTIVE ACTIVITY:

Anti hepatotoxic effect of methanolic extract was observed in freshly isolated rat hepatocytes at concenteations 1000-800 micro g/ml and was found to be similar to that of standard drug silymarin. Both extracts was abel to restore the biochemical levels to normal which were altered due to ccl_4 intoxification in freshly isolated rat hepatocytes and also in animals.[18] The methanol and aqueous extracts of heartwood of *Caesalpinia Sappan* for its hepatoprotective activity against ccl_4 induced toxicity in freshly isolated rat hepatocytes and animals[19].

IMMUNOSUPPRESSIVE ACTIVITY:

Brazilein an important immunosuppressive component of sappan showed inhibition of T cells proliferation and suppress mice humoral immune response. Ethanol extract of sappan heartwood suppress the immune competence of lymphocytes , water extract affect T cells function and its ester extract can affect immune competence and NK cells. Ehanol extract has the strongest immunosuppressive function[20].

ANTI OXIDANT ACTIVITY:

Brazelien showed highest DPPH radical scavenging activity and ferric reduction activity as compared to standard vitamin E and other compound isolate from sappan lignum including brazilein, sappanchalcone, protosappanin B and C. It also showed dose dependant inhibition of peroxide formation in linoleic acid emulsion during incubation at 50^0 c for 250h[21].

ANTI ALLERGIC ACTIVITY:

The dichloromethane extract of the roots and heartwood of Sappan lignum exhibit potent inhibitory activity against β- hexosaminidase release as marker of degranulation in rat basophilic leukemic cells, with inhibition of 98.7% and 87.5% at concenteration of 100ug/ml, respectively. Sappanchalcone possessed the most potent effect against allergic reaction in rat basophilic leukemic (RBL 2H3) cells with an inhibitory concenteration (IC50) value of 7.6um[22].

CARDIOACTIVE EFFECTS OF BRAZILEIN:

Ethanol extract of Sappan lignum obtain brazilein. In isolated cardiac tissues, brazilein exhibited a +ve inotropic action in a concenteration dependant manner with little effect on heart rate and coronary perfusion. Albino guinea pig erythrocytes enriched with Na+ K+, ATPase isoforms were utilized to compare the inhibition promoted by brazilein with that of classical inhibitors such as cardiac glycosides deslanoside. The extent of maximum inhibition rate was about 50%. Brazelien produced its positive inotropic effect through inhibiting Na+ K+-ATPase .[23]

HYPOGLYCEMIC ACTIVITY:

Brazilin, the principle component of sappan lignum has been found to exhibit hypoglycaemic properties and to increase glucose metabolism in diabetics rats. It improved glucose metabolism in cultured rat hepatocytes, also increased basal glucose transport in 3T 3L1 fibroblasts and adipocytes , but insulin stimulated glucose transport was not influenced.[24]

ANTI ACNE ACTIVITY:

Brazilin isolated from Sappan lignum possessed antibacterial activity against propionibacterium acnes with MIC and MBC values of 15.6 and 31.2ug/ml, respectively. Brazilin rich extract considered as a potential coloring agent with anti bacterial and acne activity which is used for pharmaceutical, cosmetic, and neutraceutical applications.[25]

MELANOGENESIS INHIBITORY ACTIVITY:

Homoisoflavanone, sappanone A, was isolated from *Caesalpinia sappan* and proven to dose-dependently inhibit both melanogenesis and cellular tyrosinase activity via repressing tyrosinase gene expression in mouse B16 melanoma cells. sappanone A is the first homoisoflavanone to be discovered with melanogenesis inhibitory activity. Results gave a new impetus to the future search for other homoisoflavanone melanogenesis inhibitors[26].

OTHER ACTIVITIES:

The *Caesalpinia sappan* possessed various reported activities such as anti osteoporotic activity[27], suppression of melanin synthesis[28], anti helmintic[29], anti bacterial[30], anti influenza virus[31] , vasorelaxant[32] were also reported in this plant. Toxicity evaluation of Sappan wood extract showed it did not produce any acute or sub acute toxicity upto 5000 mg/kg in rats[33].

CONCLUSION:

Sappan wood with high therapheutic effect and vast folklore uses is worth studying more that might provide a rich natural resource of lead compounds for drug development. Medicinally the wood is recommended as a substitute for logwood. It has vast uses in ayurveda and yunani. Based on literature review, *Caesalpinia sappan* heartwood has high potential for therapeutic and colouring use. Dyeing properties which has a use in colouring foods such as hard cheese, butter, dairy products, fish products, beverages, bakery, ice creams etc. Brazelien has potential pharmacological activity such as anti-tumour, anti inflammatory, anti-diabetic, immunostimulant properties and also anti thirst, blood purifying action and healing properties in Aurvedha and Unani beneficial to develop into a drug , neutraceuticals and cosmetics.

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PHARMACOGNOSTICAL AND PHYTOCHEMICAL STUDIES ON HEARTWOOD OF *Caesalpinia sappan* Linn

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ABSTRACT

The present work was undertaken to establish the pharmacognostic and phytochemical standards of Heartwood of Caesalpinia sappan L. It is a highly apparent plant in ayurvedic system of medicine for the treatment of various ailments. The plant is traditionally used for the treatment of dysentery, cancer, worm infestation, anemia, to improve complexion and ulcer . In the present study, pharmacognostical studies of the heartwood were carried out. The parameters studied are macroscopy, microscopy of heartwood, powder microscopy, heavy metals analysis and HPLC. HPLC analysis of hydroalcoholic extract of heartwood showed presence of rutin, quercetin, gallic acid, ellagic acid and ascorbic acid in variable amount. All the parameters were studied according to the WHO & Pharmacopoeial guidelines. This parameters will help for correct identification of this plant for the future references.

KEYWORDS: *Caesalpinia sappan*, Pharmacognostical, phytochemical, Heavy metal Analysis, HPLC.

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INTRODUCTION

The plant *Caesalpinia sappan* is a small leguminous tree belonging to the Caesalpiniaceae family,with an orange-red hard Heartwood ,which besides being useful in turnery gives a red dye. It is commonly known as Brazil or Sappan wood, native to tropical Asia, also grown as a hedge plant. The plant is being used worldwide for a large number of traditional medicinal purposes including anti-tumour, anti-inflammatory, immunosuppressive, anti-diabetic, anti-allergic, cardioactive, neurotoxicity, anti-acne , anemia, tuberculosis and some other activities. The main active constituents is Brazilin, and together triterpenoids, flavanoids, lipids, steroids, aminoacids etc are found[1,2]. In folklore medicine it is used as a herbal drinking water for its blood purifying, anti- thirst , anti-tumour and to improve complexion in Kerela. The plant is one of the ingredients of an indigenous drug 'Lukol' which is administered orally for the treatment of non-specific leucorrhoea[3]. The wood was formely used in calico printing of cotton, wool and silk. It is now however now replaced by synthetic dyes[4].

MATERIALS AND METHODS

COLLECTION AND AUTHENTICATION OF THE PLANT MATERIAL:

The Heartwood of *Caesalpinia sappan* Linn., was collected from Kulesekaram in Kanyakumari district, Tamilnadu in June-2015. The plant material was authenticated by Dr.V.Chelladurai, Research officer- Botany, Central Council for Research in Ayurveda and Siddha, Tirunelveli. The heartwood were shade dried, Coarsely powdered and used for further studies. The macroscopical Characters of *Caesalpinia sappan* heart wood such as odour, taste, shape, texture and surface were observed .

PHARMACOGNOSTICAL STUDIES:

a) Morphology: Morphological studies such as shape, size, surface, colour, taste, texture of heartwood(Fig-1) were carried out and showed in table-1

Fig-1 Heartwood

b) Microscopy

The required sample of heartwood were cut and removed from the plant and fixed in FAA(Formalin-5 ml + Acetic acid-5 ml + 70% Ethyl alcohol 90 ml). The shade dried heartwood of *Caesalpinia sappan* were powdered well and then the powder was passed through sieve no:60 and used for powder analysis.An exhaustive pharmacognostical study was carried out using standard Methodology [(5-16)].

EXTRACTION

About 200 gm of coarsely pulverized heartwood was taken in a closed bottle and it was defatted with petroleum ether. The deffating was continued for 9-10 days with occasional shaking. The petroleum ether extract was filtered. The marc left after petroleum ether deffating was taken out and dried under shade to get a dry mass, then extracted with ethanol and water(50:50) by using cold maceration extraction. The extraction was continued for 9-10 days with occasional shaking. The hydroalcoholic extract was filtered, concentrated under reduced pressure to a semisolid mass and was made free from solvent. The final obtained extract was weighed; percentage yield was calculated and stored in a cool place.

PHYTOCHEMICAL STUDIES:

Phytochemicals are non-nutritive plant chemicals that contain protective and disease preventing compounds. They are involved in many process including ones that help to prevent cell damage, prevent cancer cell replication and decrease cholesterol levels. Preliminary Qualitative phytochemical evaluations were carried out on hydroalcoholic extracts and dried powder to identify the presence of various phytochemical constituents like flavonoids, phenolic compounds,

glycosides, saponins, tannins were detected[17]. Particularly flavonoids and phenolic compounds were detected by HPLC method.

QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

Estimation of flavonoids[18] :

Method : HPLC

Extraction solvent- Mixture of alcohol, water and HCl (50:20:8)

Mobile phase- A mixture of methanol, water and phosphoric acid (100:100:1)

Standard solutions- Accurately weighed quantities of USP quercetin RS, Rutin, Gallic acid, Thymoquinone, Gallangin to separate volumetric flasks, which is dissolved in methanol, and diluted quantitatively, and stepwise if necessary, with methanol to obtain standard solution 1 mg/ml, respectively.

Test solution – About 10g of hydroalcoholic extract were weighed to a 250 ml flask fitted with a reflux condenser. 78ml of extraction solvent added and refluxed on a hot water bath for 135 minutes. Then it was allowed to cooled at room temperature. Decanted to a 100 ml volumetric flask. Then 20ml of methanol added to 250 ml flask, sonicated for 30 minutes, filtered and the filterate was collected in 100 ml volumetric flask, the residue was washed on the filter with methanol. Then the washing was collected in the same 100 ml volumetric flask, diluted to volume and mixed.

Chromatographic system – The liquid chromatograph was equipped with a 270 nm detector and a 4.6mm x 25 cm column that contains packing L1. The flow rate was about 1.5ml per minute. Chromatograph standard solution 1 and the peak reponses was recorded.(Fig 12 & 13)

Procedure – Separately injected equal volumes(about 20 µL) of each of the standard solution and the test solution into the chromatograph, chromatographs was recorded and areas were measured for the major peaks. The quantity of each flavonoids in sample was calculated and given (Table 4).

Estimation of phenol[19]:

Method:HPLC

Buffer: phosphate buffer

Mobile phase: phosphate buffer with Acetonitrile

Detection: UV(260nm)

Column: C_{18}

Injection volume:20µl

Standard solutions: gallic acid, ascorbic acid, ellagic acid, coumaric acid and tannic acid.

From the peak responses of the relevant component obtained(Fig 14&15) the quantity of phenols in sample was calculated and given(Table 5).

RESULTS AND DISCUSSION:

Macroscopic features

TABLE-1

s.no	Parameters	Observation
1	Colour	Orange- red
2	Taste	Bitter
3	Odour	Slight
4	Surface	Grained
5	Texture	Fine and even
6	Shape	Straight

MICROSCOPICAL FEATURES

Transverse section (TS) of the wood:

Wood is porous (having the pores or vessels) The growth – rings are fairly distinct. The vessels are circular or ovate . They are mostly solitary or less frequently radial multiples of two to four vessels. The vessels have very thick walls which are lignified. Most of the vessels are open and some have gum deposits(Fig.2). The vessels are 100-200 µm wide.

Axial parenchyma :-

Parenchyma cells wich are vertically oriented are called axial parenchyma cells. The axial parenchyma is abundant . They occur as thin tangential layer along boundary of the growth ring.Such parenchyma cells are called paratracheal parenchyma . The parenchyma cells are thick walled, lignified and have wide lumen(Fig.3).

The xylem rays are seen in several closely running radial lines of parenchyma cells. The ray parenchyma cells are radially elongated, narrow lumened and thick walled. They run in straight lines. Xylem fibres form the ground tissue of the wood. The fibres are angular in sectional view. They are compact; they have very thick, lignified walls. The cell lumen is reduced to a narrow hole (Fig.4).

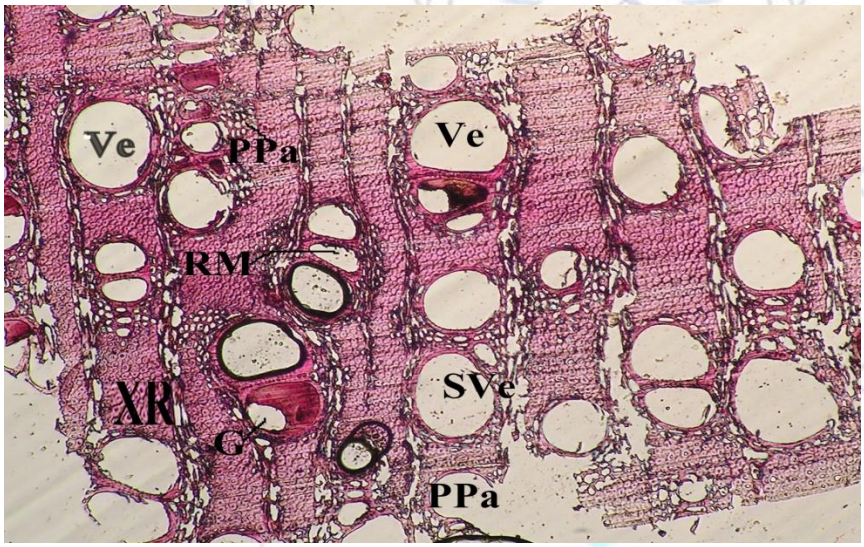

Fig 2 TS of wood showing vessel distributions.

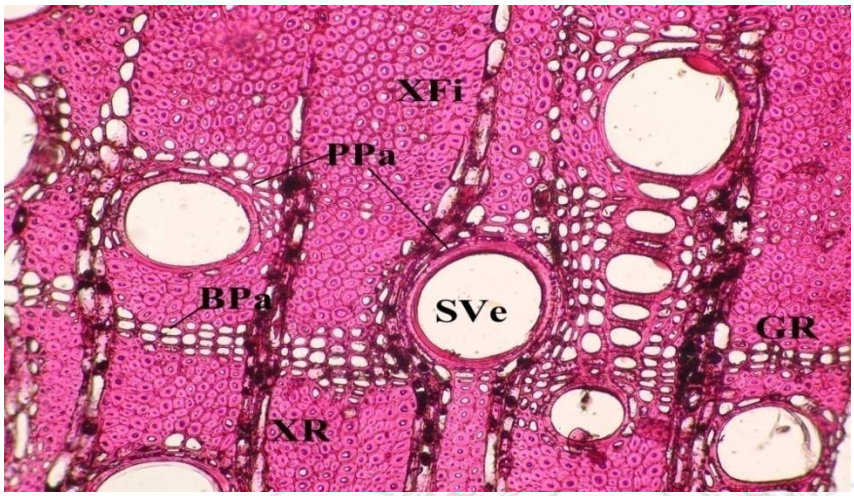

Fig 3 TS of wood showing solitary vessels, growth ring.

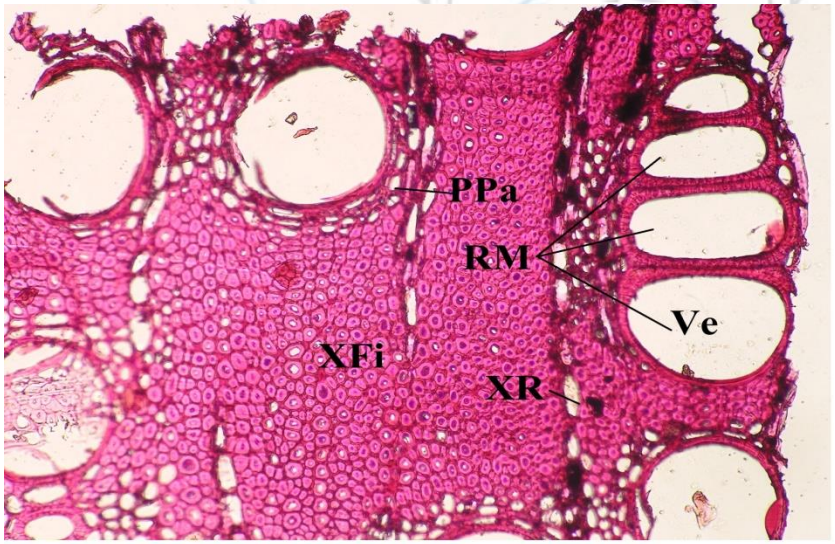

Fig 4 TS of wood showing radial multiples of vessels.

G- gum deposition on the crosswall of the vessels; **PPa**- paratracheal parenchyma; **BPa**-boundary parenchyma; **RM**- radial multiples of vessels; **Ve**- vessels ; **XF**- xylem fibres;
XP- xylem parenchyma; **XR**- xylem ray; **GR**-growth ring; **SVe**-solitary vessel.

Tangential longitudinal section(TLS view **)**

In TLS view, the xylem rays spindle shaped; the vessels, axial parenchyma and xylem fibres are seen in vertical alignment (Fig.5). The rays are random in vertical orientation. So the rays are uniseriate , biseriate or multiseriate . Thus the rays exhibit variations in seriation.The rays are either homocellular or heterocellular. The homocellular rays have uniform type of cells. The

heterocellular rays have two type cells: the cells in the middle part of the rays isodiametric; the cell at the ray are vertically elongated. The isodiametric cells in the middle part of the rays are called procumbet cells(Fig.6,7); those at the ends of the rays are 300-600 µm in height and 50-100 µm thick. The ray cells are narrow and thick walled.

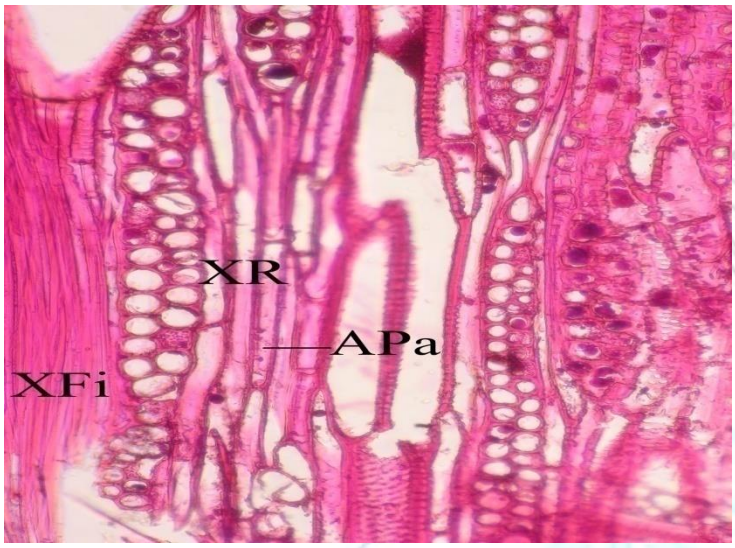

Fig 5 TLS of wood showing axial parenchyma

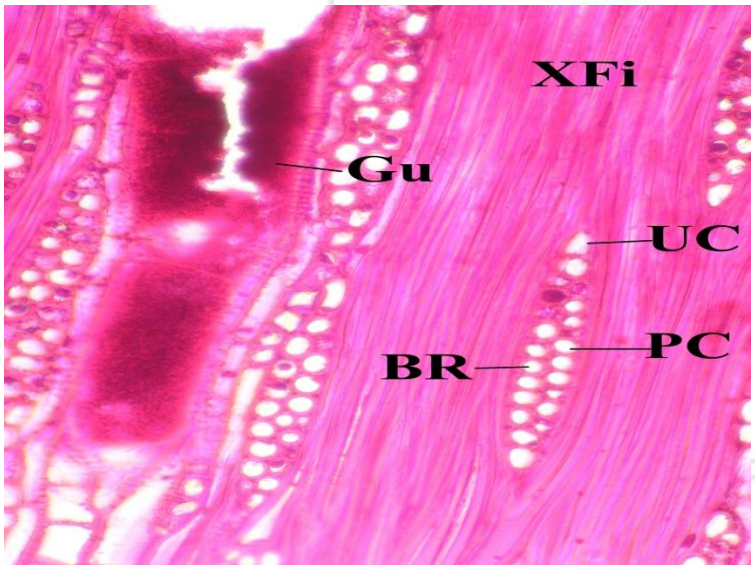

Fig 6 TLS of wood showing gum, biseriate xylem rays.

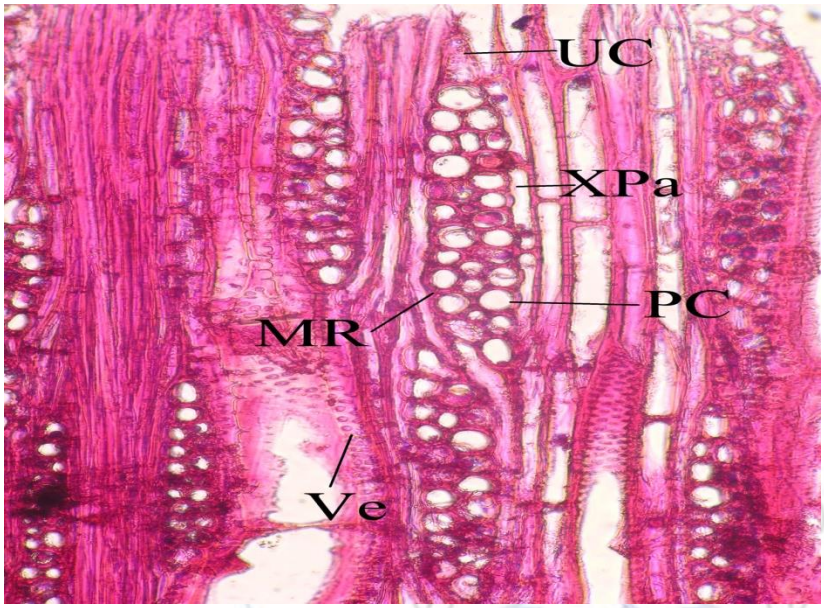

Fig 7 TLS of wood showing horizontal , ribbon shaped ray.

GPI- gum plate; **Ve**- vessel; **XFi**- xylem fibres; **XPa**- xylem parenchyma; **XR**- xylem rays **APa**- axial parenchyma; **BR**- biseriate ray; **Gu**-gum; **MR**- multiseriate ray; **Pc**- procumbent cells; **UC**- upright cells; **Ve**-vessel; **XFi**- xylem fibres; **XPa**- xylem parenchyma ; **XR**- xylem ray.

Radial longitudinal sections of the wood(RLS view)

In RLS view, the xylem rays appear as wide, and flat horizontal ribbon like bands. The xylem fibres, parenchyma and the vessels are at vertical rows at right angles to the rays. The procumbent cells of the ray are horizontally elongated, narrow and thick walled. The ray cells have dense simple pits (Fig .8). The upright cells are short, and squarish in shape.The axial parenchyma cells are long in vertical plane; they occur clusters of 2-4 rows (Fig .9) . The cells have minute circular pits.

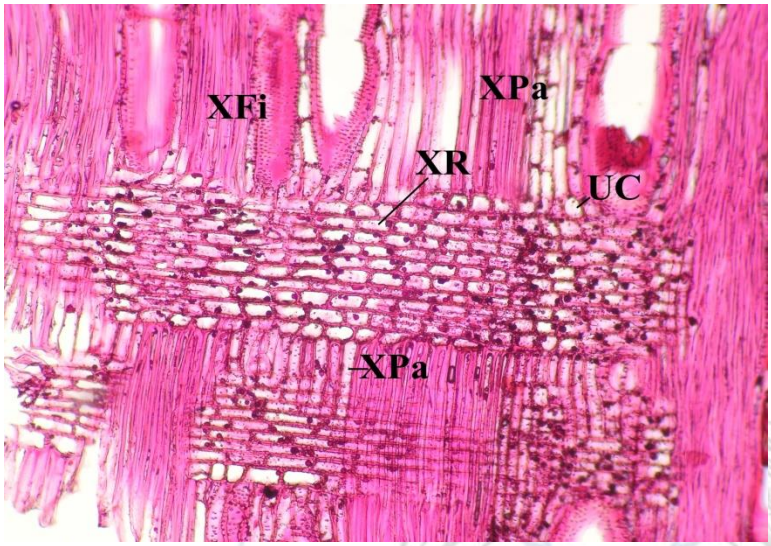

Fig 8 RLS of wood showing horizontal ribbon shaped rays

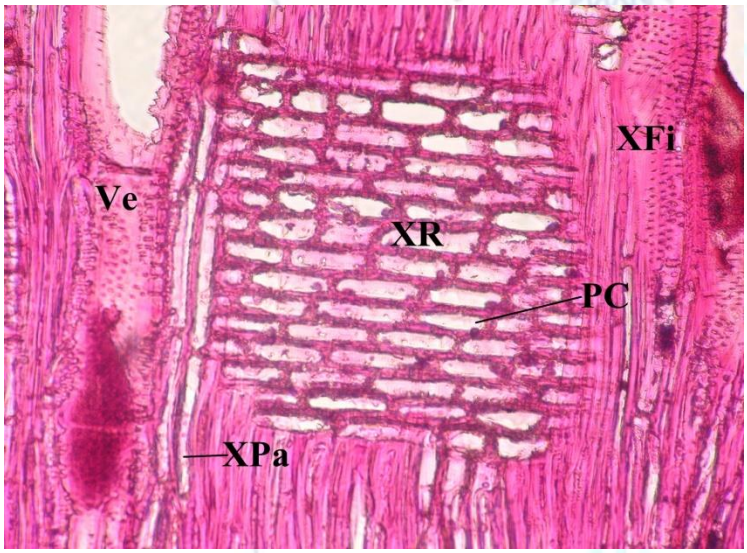

Fig 9 RLS of wood showing ray cells enlarged.

Pc- procumbent cells; **Ve**- vessel; **XFi**- xylem fibres; **XPa**- xylem parenchyma; **XR**- xylem ray ; **Uc** – upright cells

Powder microscopic observations

The powder preparations of the wood shows the following elements when observed under the microscope

(i) Fibres:-Long, thin needle shaped fibres are abundant in the powder. They have thick lignified walls and much reduced lumen. The fibres are 600- 800 µm long and 20µm thick(Fig.10).

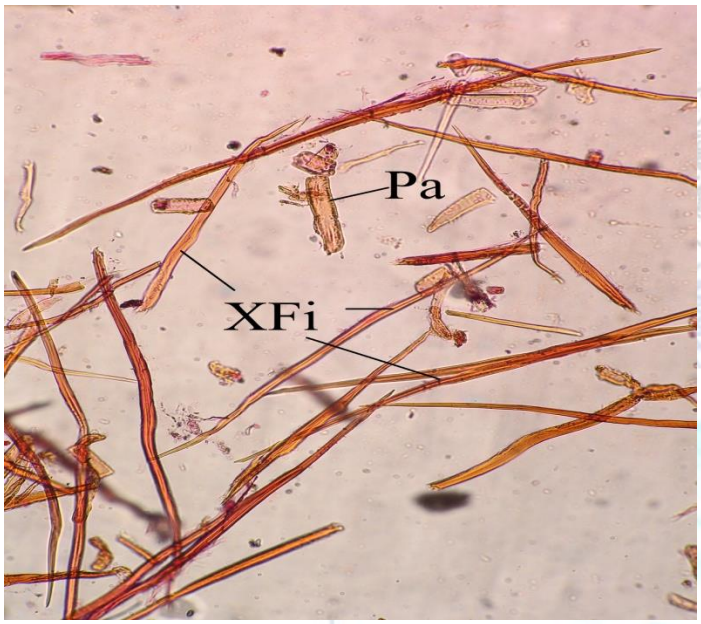

Fig 10 xylem fibres

Pa- parenchyma; **XFi-**xylem fibres.

(ii) vessel elements:-Vessel elements are fairly common in the powder . vessel elements of the powder shows long thick vessel elements with prominent bordered pits, short wide vessel element and wide, short barrel shaped vessel element were identified.. These vessels elements are 250 µm long and 140µm wide .These are aiso wide, very short and drum shaped vessel elements which are 130µm long and 250 µm wide. The vessel elements have dense multiseriate elliptical bordered pits(Fig.11).

(iii)parenchyma cells :-Rectangular , short parenchyma cells are occasionally seen. The cells are thick walled with dense simple pits. The cells are 90 µm long and 15 µm wide (Fig.11).

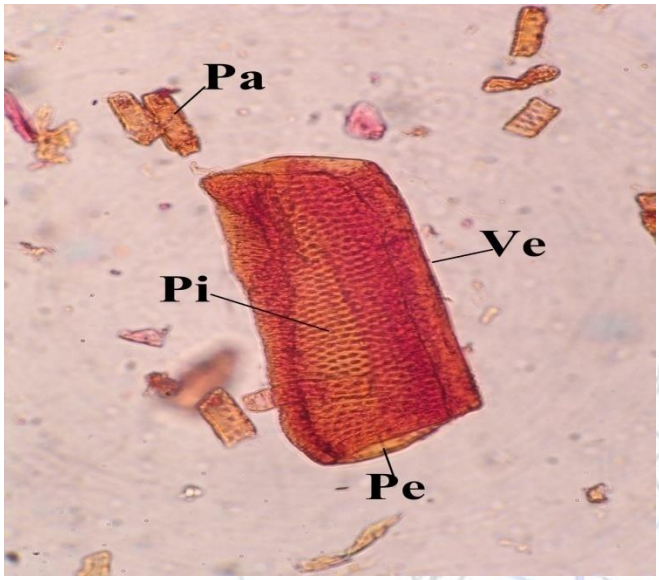

Fig. 11 pitted vessel

Pa – parenchyma cells; **Pe**- perforation; **Pi**- pits; **Ve**- vessel element.

TABLE-2 Preliminary phytochemical analysis of powder and extract of heartwood of *caesalpinia* sappan

S. NO	PHYTOCHEMICAL TESTS	POWDER	HYDROALCOHOLIC EXTRACT
1	Alkaloids	-	-
2	Carbohydrates	+	+
3	Flavonoids	+	+
4	Glycosides	+	+
5	Fixed oil and fats	+	-
6	Phytosterols	+	+
7	Phenolic compounds	+	+
8	Proteins,and aminoacids	+	-
9	Resins	+	-
10	Saponins	+	+
11	Terpenoids	+	+
12	Tannins	+	+
13	Gums and mucilage	+	-

+ve indicates presence, and -ve indicates absence.

Table 3: Quantitative estimation of Heavy Metals in heartwood powder of *Caesalpinia sappan*

S.NO	HEAVY METALS	OBSERVATION(PPM)	STANDARD LIMITS
1	Mercury	Not detected	Not more than 5ppm
2	Arsenic	Not detected	Not more than 5ppm
3	Lead	0.002	Not more than 10ppm
4	Cadmium	Not detected	Not more than 0.03ppm

QUANTITATIVE ESTIMATION

A) ESTIMATION OF FLAVONOIDS

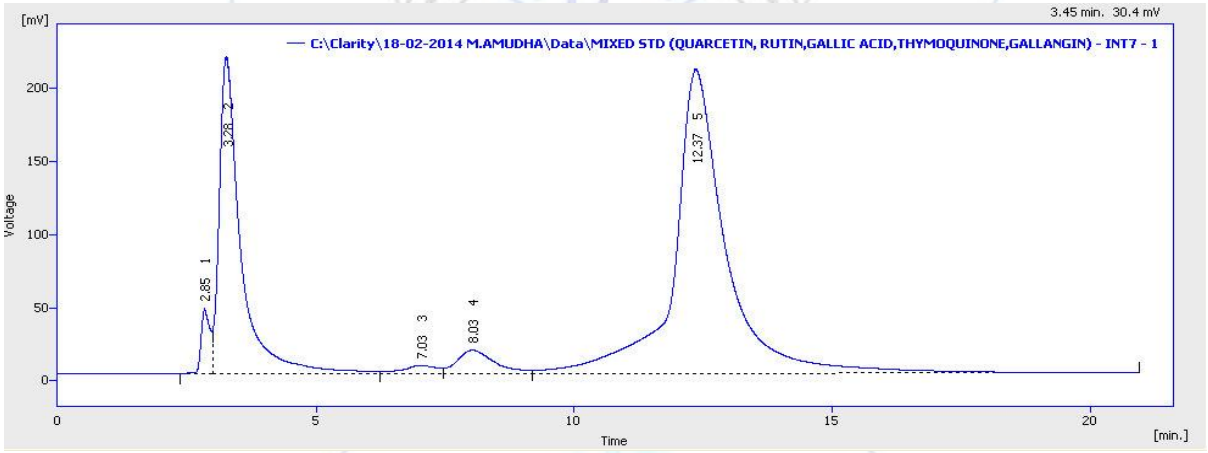

Fig 12 standard curves for Flovonoids

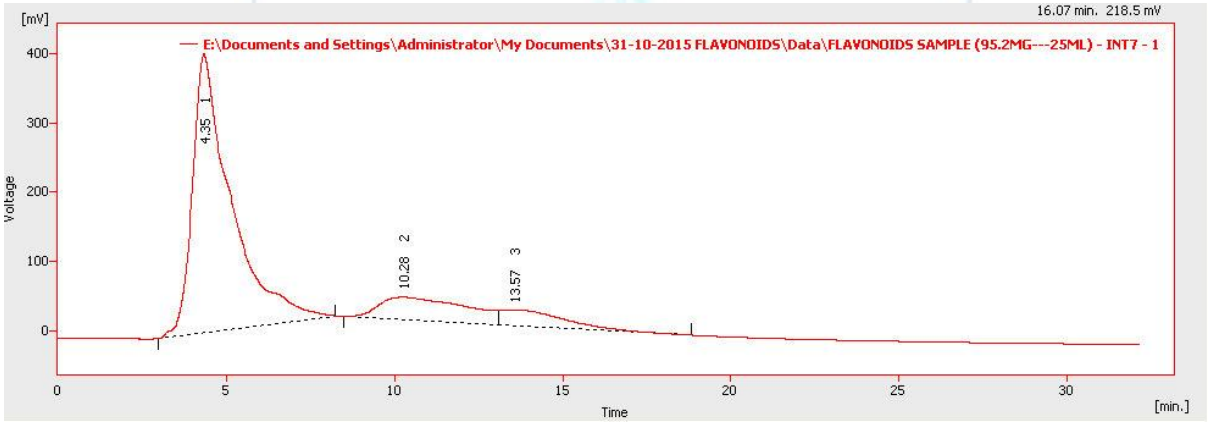

Fig 13 Hydroalcoholic extract sample curves for flavonoids.

TABLE-4(Flavonoids quantity)

FLAVONOIDS	SAPPANWOOD POWDER PER GM	SAPPAN WOOD ETRACT PER ML
Rutin	0.78mg	0.934mg
Quercetin	0.146mg	0.184mg
Gallangin	0.074mg	0.158mg

B) ESTIMATION OF PHENOLS

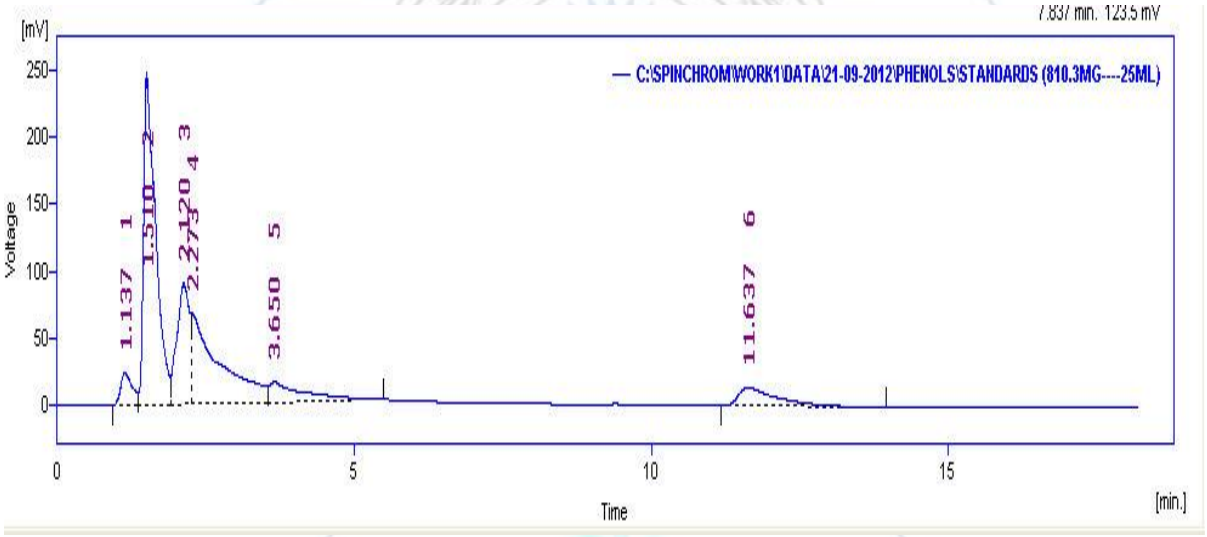

Fig 14 Standard curves for phenol

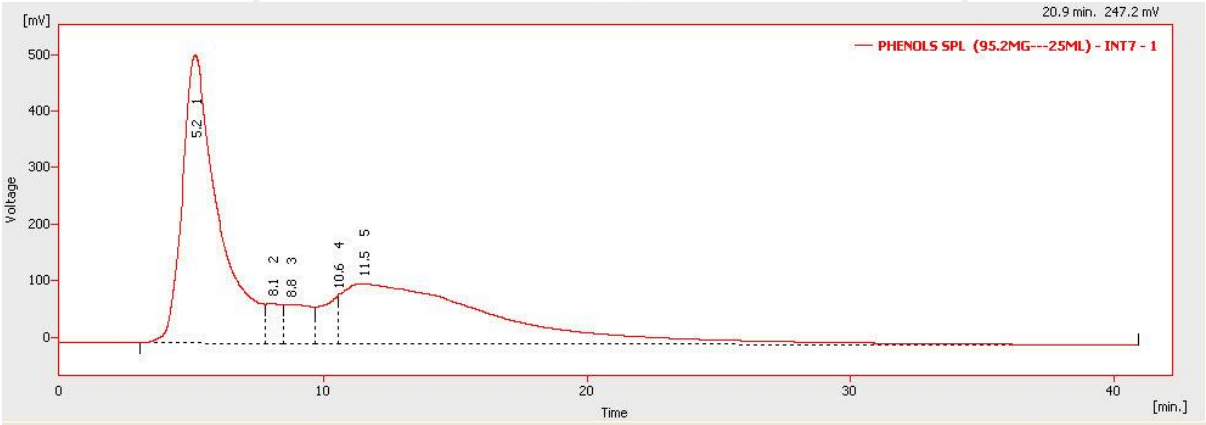

Fig 15 Sample curves for hydoalcoholic extract for phenol

TABLE-**5(phenols quantity)**

PHENOLS	SAPPAN POWDER PER ML	SAPPAN EXTRACT PER ML
Gallic acid	0.468mg	0.519mg
Tannic acid	0.029mg	0.083mg

Ellagic acid, coumaric acid, and ascorbic acid were also identified in smaller amount.

CONCLUSION:

A medicinally useful plants in the Indian system of medicine *Caesalpinia sappan* Linn., belonging to the family Caesalpiniaceae was selected. The pharmacognostical studies carried out provides a set of diagnostic macroscopic, microscopic features and physio chemical parameters that can be used in confirming the identity of these plants and to detect adulterants and their nature. Sappan wood with high therapeutic effect and vast folklore uses is worth studying more that might provide a rich natural resource of lead compounds for drug development. Medicinally the wood is recommended as a substitute for logwood. It has vast uses in ayurveda and yunani.

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HERBAL FORMULATION DEVELOPMENT ON HEARTWOOD OF *Caesalpinia sappan* FOR HYPOLIPIDEMIC AND ANTI-OBESITY ACTIVITY

K.MEKALA*, R.RADHA

Abstract

Cardiovascular disease (CVD), particularly coronary heart disease (CHD) and cerebro vascular accident (CVA) is the leading cause of medically certified death in both developed as well as developing countries. Hyperlipidemia and obesity is one of the greatest risk factors in the manifestation and development of CHD and CVA. Medicinal plants have been shown to play a major role in treating hyperlipidemia and obesity and considering this, the present study was designed to develop an herbal capsules formulation on heartwood of *Caesalpinia sappan* for hypolipidemic and anti-obesity activity. The hydro alcoholic extract (1:1) on heartwood of *Caesalpinia sappan* was prepared by cold maceration method and then it was formulated into a hard gelatin herbal capsule. The quantitative estimation of phytoconstituents especially of flavonoids and phenolic content in extract and formulated herbal capsule was estimated by HPLC method showed the presence of Rutin, Quercetin, Gallic acid, Ascorbic acid and tannic acid. Hydro alcoholic extract of *Caesalpinia sappan* (HECS) herbal capsule was evaluated for acute toxicity studies in albino wistar rats and showed no toxicity up to 2000mg/kg. *In-vivo* evaluation of high fat diet (HFD) induced obesity in rats were carried out for HECS herbal capsule .The studies showed HECS herbal capsule(250mg/kg and 500mg/kg) significantly reduced the elevated levels of body weight, total cholesterol, triglycerides, LDL-cholesterol, VLDL-cholesterol, SGPT and SGOT and elevated the decreased level of HDL-cholesterol. These results suggest that, HECS capsule possess good hypolipidemic and anti-obesity activity, which may be due to its flavonoid, saponin and phenolic content.