# *IN SILICO, IN VITRO* AND *IN VIVO* HEPATOPROTECTIVE ACTIVITY OF *Caryota urens* L.FLOWERS AGAINST ETHANOL INDUCED LIVERTOXICITY USING RATS

**A Dissertation submitted to**
**THE TAMIL NADU 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**
**BRANCH - IV - PHARMACOLOGY**


Submitted by
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**COLLEGE OF PHARMACY**
**SRI RAMAKRISHNA INSTITUTE OF PARAMEDICAL SCIENCES**
**COIMBATORE – 641 044.**


**March 2020**

# Certificates

# CERTIFICATE

This is to certify that the M. Pharm., dissertation entitled **"*IN SILICO, IN VITRO AND IN VIVO* HEPATOPROTECTIVE ACTIVITY OF *CARYOTA URENS* L.FLOWERS AGAINST ETHANOL INDUCED LIVERTOXICITY USING RATS"** being submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment of **Master of Pharmacy** programme in Pharmacology, was carried out by **Ms. Dhanya R** Register No. **261825102** in the Department of Pharmacology, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, under my direct supervision and guidance to my full satisfaction.

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This is to certify that the M. Pharm. dissertation entitled **"*IN SILICO, IN VITRO AND IN VIVO* HEPATOPROTECTIVE ACTIVITY OF C*aryota urens* L. FLOWERS AGAINST ETHANOL INDUCED LIVER TOXICITY USING RATS"** being submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment of **Master of Pharmacy** programme in Pharmacology, was carried out by Dhanya R ( Register No. **261825102**)**.,** in the Department of Pharmacology, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, under the direct supervision and guidance of **Dr. V. Subhadra Devi, M.Pharm.,Ph.D.,** Associate Professor, Department of Pharmacology, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore.

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# Introduction

# INTRODUCTION

The liver is one of the largest gland in the body [1]. The weight of liver in adult males 1.4 – 1.8 kg and in adult females 1.2-1.4kg [1]. Liver plays a vital role in secretion of bile for the process of digestion. Liver diseases are among the most serious ailments. They may be classified as acute or chronic hepatitis (inflammatory liver diseases), hepatitis (non-inflammatory disease) and cirrhosis (degenerative disorder resulting in fibrosis of the liver). All substance absorbed into the blood from the stomach and intestines are filtered through the liver, where some of them are stored and some toxic substances may be destroyed. Numerous other functions essential to the well-being of the individual are performed in the liver which is therefore, regarded as one of the vital organs [2]. It occupies a substantial part of the abdominal cavity. It lies under the diaphragm. It occupies most of the right hypochondrium, part of the epigastrum, and extending into the left hypochondriac region [1]. it is included amongest the accessory organs of the ailmentry system because it produces a secretion, called bile, which is poured in to the duodenum ( through the bile duct) and assists in digestive process. All substances absorbed into the blood from the stomach and intestines are filtered through the liver, where some of them are stored and some toxic substances may be destroyed. Numerous other functions essential to the wellbeing of the individual are performed in the liver which is therefore, regarded as one of the vital organs [2,3].

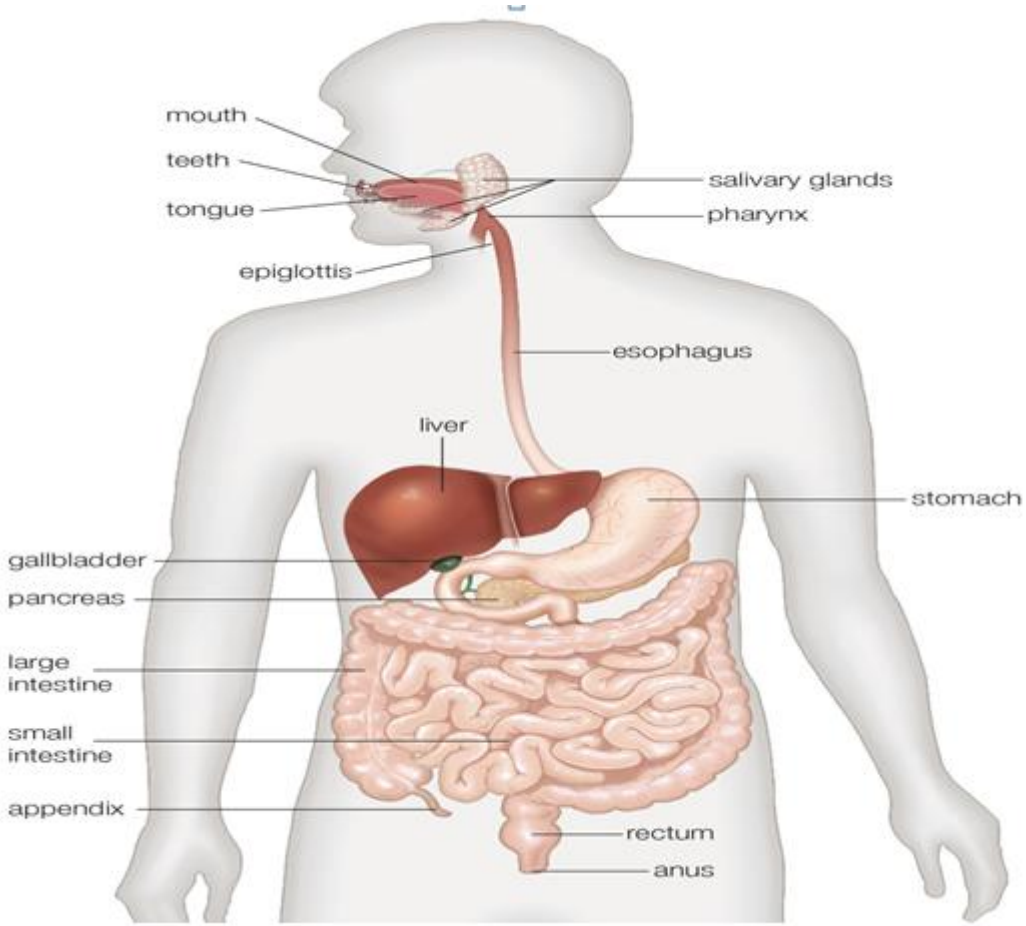


**Fig 1: Location of liver in human digestive system**

Liver is an extremely important organ and exhibits multiple functions. Liver detoxifies for blood cells by proper fixation of bile solution via chemical modification to form fewer toxic substances, example alteration of ammonia to urea. Liver damaged by various hazardous chemicals such as thioacetamide, carbon tetrachloride, chronic alcohol consumption, some drugs used to treat various diseases. Generation of free radicals leads to alteration in the normal physiological function of the liver. Currently available synthetic drugs used for the treatment of liver diseases are inadequate and known for various side effects. Scientific studies reported use of plant phenolics, reduces the risk of liver diseases by acting as antioxidant [3].

Many chemical substances are inactivated by liver through modification of chemical structure. Liver convert glucose to glycogen as a storage form of energy and it produces glucose from disaccharides and polysaccharides such as sugars, starches and protein molecules [3].

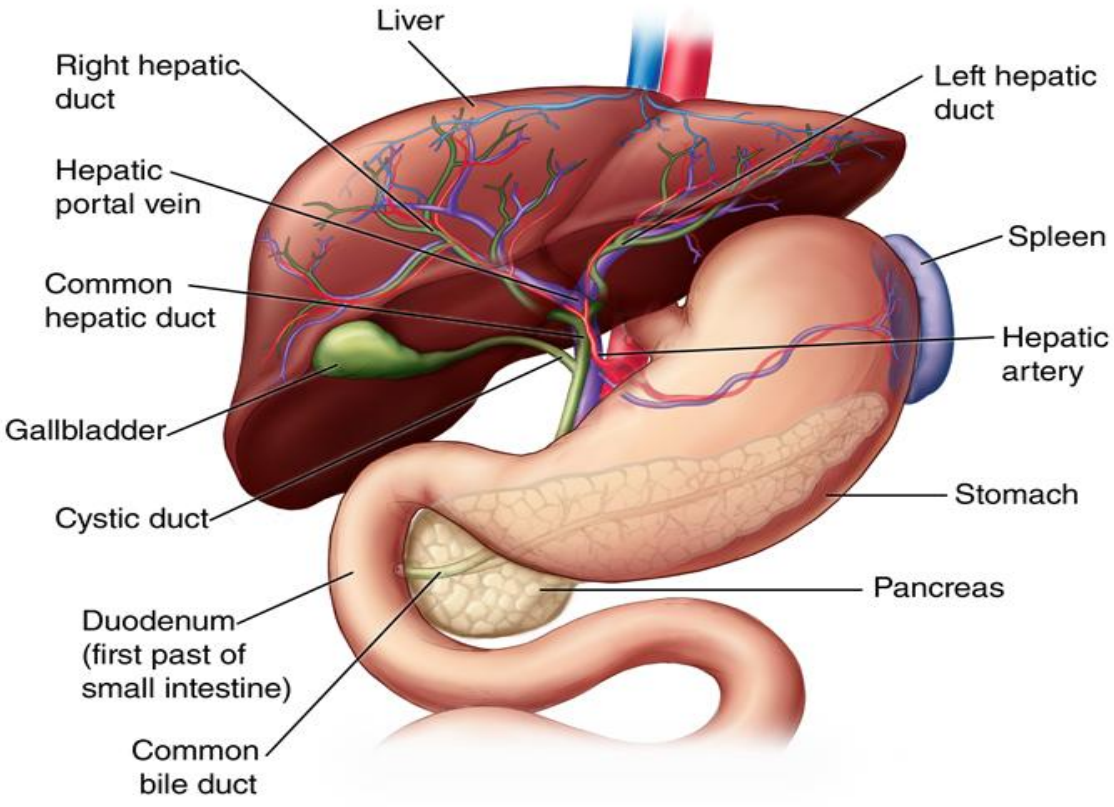


**Fig 2: Human liver and biliary system**

Hepatocytes are the functional cells of the liver which are arranged in pairs of columns radiating from a central vein. A wide range of metabolic, secretory and endocrine

functions are performed by hepatocytes. These are specialized epithelial cells with 5-12 sides that make-up about 80% of the volume of the liver. Hepatocytes form complex three dimensional arrangements called hepatic laminae and they are the sheet of hepatocytes one cell thick lined to either side by the endothelial-lining spaces called hepatic sinusoids. Grooves inside the cell surface between neighbouring hepatic cells which provide gaps for the canaliculi of hepatocytes that secrete bile. Bile is a yellow, brown, or olive-green colour type liquid which secreted from hepatic cells, which provide an excretory product and a digestive enzyme secretor. Bile canaliculi are narrow intercellular canals that collect bile secreted by hepatocytes. From bile canaliculi, bile passes into small bile ducts. These small ducts combined by form the higher right and left hepatic void that commonly connect and exit the liver via common hepatic duct. This common hepatic duct joins the cystic duct from the gallbladder to form the common bile duct. Bile enters the cystic duct and temporarily stored in the gallbladder. After a meal, various stimuli cause contraction of the gallbladder, which releases stored bile into the common bile duct. Hepatic sinusoids are freely permeable capillaries about sheets of liver cells that transports oxygenated blood via different branches of hepatic artery and higher amount of nutrient rich de-oxygenated blood from the branches of the hepatic portal vein system. The hepatic portal vein helped to take the venous blood via gastrointestinal organs and spleen into the liver. Blood flows from central veins into hepatic veins that drain into the inferior venacava. The sinusoids are partly lined with stellate reticuloendothelial (Kupffer's) cells that destroy worn-out WBC and RBC, bacteria and other foreign substance in blood. Bile duct have part in the hepatic artery, and branch of hepatic vein are referred to as portal triad [4].

**ANATOMY OF LIVER**

**Surface anatomy:**

**Peritoneal ligaments**: Apart from patch where it connects to the diaphragm, the liver is covered entirely by the visceral peritoneum (a thin double layered membrane) that reduces the friction against other organs. The peritoneum folds back on itself to form the flaciform ligament and the right and left triangular ligaments.

**Lobes and lobules**: The liver is divided into four lobes on the basis of surface features. The flaciform ligament divides the liver into left and right anatomical lobes. If the liver is looked from the visceral surface, there are two additional lobes between the right and left. These are the caudate lobe which is more superior, and below this the quadrate lobe. From behind, the lobes are divided by the ligamentum venosum and ligamentum teres. The transverse fissure or porta hepatis divides the caudate from the quadrate lobe, and the right sagittal fossa, where the inferior venacava seperates these two lobes from right lobe. The internal structure of the liver is made of around 1,00,000 small hexagonal functional units known as lobes[5].

**Functional anatomy**

The central area is the hilum or porta hepatis where the common bile duct, hepatic artery and hepatic portal vein enter the liver. The duct, artery and vein divide into right and left branches, and the respective positions of the liver supplied by these branches constitute

the functional right and left lobes. The functional lobes are separated by plane joining the gall bladder fossa to the inferior venacava. This separates the liver into the true left and right lobes[5,6].

**Blood supply to the liver**

Blood supply to the liver is unusual in that it arrives by two separates routes the hepatic artery (30%) and the hepatic portal vein (70%). The total blood flow is about 25% of the cardiac ouput at rest. The portal vein is formed by the union of the superior mesenteric vein and it drains blood from the major part of the gastrointestinal tract and the splenic vein which is the venous drainage of the spleen, pancreas, gall bladder and stomach. The hepatic artery, a branch of coeloic artery delivers blood both directly to the peripheral and midzone areas of hepatic lobule and via peribiliary plexus. The liver has a large blood supply and it receives about 1000-1100ml/min through portal veins and 300-400ml through the hepatic artery [6,1].

**Functions of the liver**

The liver has well over 500 functions and is known as the laboratory of human body. The liver is tied to almost all the bodily processes as it is responsible for filtration of all incoming foods and fluids [7].

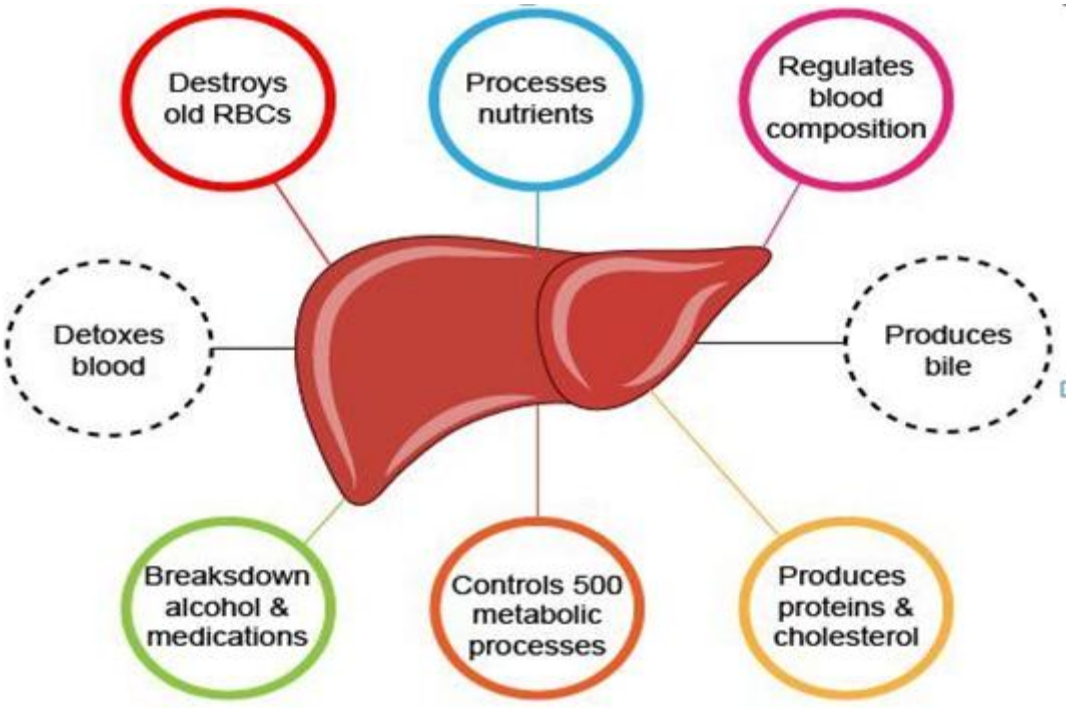


**Fig 3: Functions of the liver**

**Storage function**

The cells of liver act as a storage facility to house many crucial substances. Glucose is transported into hepatocytes under the influence of hormone insulin and stored as polysaccharide glycogen. Hepatocytes also absorb and store fatty acids from digested triglycerides. This allows the liver to maintain the homeostasis of blood glucose. Water soluble vitamins like vitamin $B_{12}$, folic acid and fat soluble vitamins like vitamin A, D and K are stored in the liver. Iron is stored in the form of ferritin and haemosiderin. Liver also stores copper and excess of copper is excreted into caeruloplasmin thereby preventing accumulation of excess of copper [7,8].

**Metabolic functions**

Liver is the central organ for metabolic functions. Hepatocytes are the metabolic overachievers in the body. They play critical role in synthesising molecules that are utilized elsewhere to support homeostasis, in converting molecules of one type to another and in regulating energy balances [8].

a) Carbohydrate metabolism

The liver performs the following functions in the carbohydrate metabolism.

- Conversion of glucose to glycogen allows the liver to remove excess glucose from the blood. This is known as glucose buffer function of liver (Glycogenesis)
- Depolymerisation of glycogen (Glycogenolosis)
- Conversion of galactose and fructose to glucose (Gluconeogenesis) [8].

b) Fat metabolism

In fat metabolism, the specific functions of liver include

- Conversion of fatty acids via oxidation to supply energy for other body functions.
- Synthesis of phospholipids, cholesterol and lipoproteins [8].

c) Protein metabolism

The liver plays important functions in the protein metabolism which includes

- Amino acid deamination.
- Amino acid transamination.
- Synthesis of urea for removal of ammonia from body.
- Synthesis of plasma proteins.
- Synthesis of amino acids.
- Interconversions of amino acids and formation of other compounds from amino acids[8].

d) Metabolism of hormones

A number of hormones are inactivated or modified by liver. Examples include de-iodination of thyroxin, degradation of insulin and glucagon etc. The various other hormones metabolized by liver include parathormone, growth hormone, oestrogens, glucocorticoids and testosterone [9].

**Detoxification**

Detoxification involves the conversion of fat soluble substances to water soluble so that these substances can be excreted in urine or bile. This is achieved with the help of enzyme systems including cytochrome P-450 which is present in the smooth endoplasmic reticulum of the liver. Phase1 metabolism includes oxidation, reduction, acetylation, deamination, ethylation etc. In phase2 metabolism, the formed products are usually conjugated with glucuronide, glutathione or sulphate and excreted in urine or bile. Ingested chemicals like alcohol, acetaminophen and various other drugs can be detoxified in the liver[8].

**Reservoir of blood**

The liver contains approximately 500ml of blood which include veins and sinusoids. Blood can be diverted from the liver, or more blood can be accommodated in the liver, depending up on the needs of body. When the amount of blood is reduced, a part of the blood is made available to the peripheral circulation. In case of systemic venous congestion, or when there is an increase in the blood volume, an additional 1litre of blood is pooled in the liver [8].

**Secretion of bile**

Cholesterol, bile pigments and bile salts are the important constituents of bile. Bile salts, formed from cholesterol in the liver helps in the absorption of fat. 80% of the bile salts are recycled in the liver. Bile also plays an important role for the elimination of break down products of red blood cells. The haeme portion of the haemoglobin released by the destruction of aged and fragile RBCs in the spleen is converted into bilirubin, which is then transported by blood to the liver. Hepatocytes extract the bilirubin and excrete it into bile. Liver qualifies as an exocrine gland because it secretes bile into ducts [9].

**Excretion of bile pigments**

The bile pigments are formed by the destruction of haemoglobin and are excreted by the liver into the bile. Jaundice occurs as a result of accumulation of bile pigments. This may be due to increased formation of bile pigments with the liver not being able to excrete them as rapidly as they are formed (Haemolytic jaundice). It may also occur due to biliary obstruction in which the pigments are prevented from passing into the intestine (Obstructive jaundice) [9].

**Blood coagulation**

A large proportion of blood substances used in coagulation are formed in the liver. The factors formed in the liver which aids in blood coagulation include Factor VII, prothrombin, fibrinogen, accelerator globulin and various other important factors. Vitamin K is required for the formation of Factors VII, IX, X and prothrombin [9].

**Synthesis of vitamin A from carotene**

Carotene is the pro-vitamin found in some plants for example carrots and green leaves of vegetables [10].

**Production of heat**

Liver able to create amount of energy which has a high metabolic rate and produces considerable amount of heat. It is an important heat producing organ of body [11].

**Phagocytosis**

The stellate reticuloendothelial (Kupffer's) cells of the liver phagocytize worn-out red and white blood cells and some type of the bacteria [7].

**Activation of vitamin D**

The cutaneous layer of skin, liver and kidneys essential for activation vitamin$D_1$ [7].

**ENZYMES INVOLVED WITH THE LIVER**

**Alanine transaminase**

Alanine transaminase or ALT is a transaminase, serum glutamic– pyruvic transaminase (SGPT or also known as alanine aminotransferase (ALAT)) commonly observed in many tissues and body fluids principally in liver. ALT is released into serum as a result of tissue injury [11,30].

**i. Function**

ALT catalyses the reversible transfer of an amino groups in the L-alanine enzyme to α-ketoglutarate proteins forms such as pyruvate and L-glutamate.

**L-Glutamate + Pyruvate □ □-Ketoglutarate + L-Alanine**

**ii. Clinical significance**

It is commonly estimated clinically as a parameter of diagnostic evaluation of hepatocellular injury in order to determine liver health. ALT has actually measured by international units/liter (IU/L) used in diagnosis. 10-40 IU/L are the standard reported range of experimental studies [13,31].

**iii. Elevated levels**

Abnormal range of alanine transaminase (ALT) often suggest the abnormality of conditions including viral hepatitis, diabetes mellitus induced cell necrosis, heart failure, liver injury, infectious mononucleosis, bile duct problems and myopathy. Because of these reasons, ALT is one of the important parameters used for screening of liver diseases. Dietary choline deficiency shows marked elevation in ALT levels. These enzyme variation levels of ALT do not have

significance of that medical problem is present. Fluctuation of ALT level is normal during course of day and ALT levels can increase in response to strenuous physical exercise. When elevated ALT levels are found in blood concentration subsequently narrowed down by measuring other enzyme concentration (example liver- cell damage usually distinguished from biliary duct problems by measuring increased ALP). Myopathy-related ALT levels can be found out by measuring the creatine kinase enzymes. Several drugs elevate ALT levels, for example, Zileuton. For years, American Red Cross society used for ALT testing as part of the key enzyme of tests to ensure the safety of its blood pumping by deferring donors with elevated ALT levels [14].

**Aspartate transaminase**

Aspartate Transaminase (AST) also called aspartate aminotransferase (ASAT/AAT/AspAT) or Serum Glutamic Oxaloacetic Transaminase (SGOT), is a transaminase enzyme containing pyridoxal phosphate. AST catalyses reversible transfer of αamino group between aspartate and glutamate. It is a key enzyme required for amino acid metabolism in human. It commonly present in liver, heart, skeletal muscle, kidneys, brain and red blood cells and AST is commonly measured clinically as a marker for liver health. It is also associated with liver parenchymal cell metabolism. The ratio of AST/ALT is may be useful for differentiation between etiology of liver damage [15]. The reference range is 6-40IU/L [16].

i. Function

AST catalyses the interconversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate.

**Aspartate (Asp) + □- Ketoglutarate □ Oxaloacetate + Glutamate (Glu)**

As prototypical transaminase AST relies on PLP as a cofactor to transfer amino group from aspartate or glutamate to corresponding ketoacid [17].

**ii. Isoenzymes**

Two isoenzymes are present in wide variety of eukaryotes. In humans,

i. **GOT1 / c AST,** the cytosolic iso enzyme synthesized mainly from red blood cells and heart.
ii. **GOT2 / m AST,** the mitochondrial isoenzyme presents predominantly in liver. These isoenzymes are considered to be evolved from a common gene duplication and subsequent synthesis [18].

**iii Clinical significance**

It is raised in liver inflammation. It is also elevated in diseases such as myocardial infarction, acute pancreatitis, nephrotoxicity, hemotoxicity, musculoskeletal diseases and trauma. AST was used initially biochemical marker for diagnosis of acute myocardial infarction but now redundant and has been superseded by the cardiac troponins.

AST is commonly measured clinically as a part of diagnostic liver function test in order to determine liver health [19].

**Reference range-** Male 8-40IU/L Female 6-34IU/L

**Alkaline phosphatase**

Alkaline phosphatase (ALP) has functioning towards removing phosphate group containing molecules such as nucleotides, proteins, and alkaloids. Process of removing phosphate group is called de-phosphorylation. It is sometimes used similarly as basic phosphatase. ALP is mainly present in cells lining of the biliary ducts.

**i. Elevated levels**

ALP levels in plasma increase due to large bile duct constriction, intrahepatic cholestasis. ALP is found also in bone and placental tissue and hence higher in growing children and elderly patients having Paget's disease. In third trimester of pregnancy ALP is two to three times higher than the normal range [20].

**Reference range**: 30-120 IU/L

Liver (ALP): Cholestasis, cholecystitis, cholangitis, cirrhosis, hepatitis, fatty liver, sarcoidosis, liver tumour, liver metastases, drug intoxication[21]. Placental ALP is elevated in seminomas [22] and active form of rickets as well as in following diseases[23].

- Biliary construction
- Bone conditions
- Osteoblastic bone cancer
- Osteomalacia
- Liver disorder/ hepatitis
- Leukemia
- Lymphoma
- Paget's disease
- Sarcoidosis
- Hyperparathyroidism

**ii. Lowered levels**

Following diseases may lead to decreased levels of alkaline phosphatase;

- Hypophosphatasia (autosomal recessive disease)
- Postmenopausal women undertaking estrogen therapy due to osteoporosis

- ▪ Hypothyroidism or severe anaemia
- ▪ Children affected with achondroplasia and cretinism
- ▪ Children who are victims of severe episode of enteritis
- ▪ Pernicious and aplastic anaemia
- ▪ Myelogenous leukemia
- • Wilson's disease [24]

**Total protein**

Total protein includes total amount of two classes of proteins present in fluid portion of blood. These include albumin and globulin. Total protein tests measures amount of albumin and globulin which are major groups of protein in blood. A low total protein level due to liver disorder, kidney disorder or protein is not digested or absorbed properly [25].

i. **Normal Range:** 6.0 - 8.3gm/dl

ii. **Higher -than –normal levels may be due to:**
- Chronic inflammation or infection (HIV, Hepatitis B or C).
- Bone marrow disorders (Multiple myeloma, Waldenstroms disease).

iii. **Lower-than-normal levels may be due to:**
- Bleeding (Haemorrhage)
- Burns (extensive)
- Liver disease
- Glomerulonephritis and nephritic syndrome
- Malabsorption
- Malnutrition [25]

## Liver diseases

**Liver cirrhosis**

Cirrhosis is a widespread and progressive chronic liver condition in which hepatocyte activity is depressed due to excessive amounts of fibrous scar tissue inhibiting blood flow. This blood flow obstruction can cause portal hypertension, which leads to additional complications, including shunting of veins around the liver. Potential complications of cirrhosis include bleeding problems, kidney disorders, osteoporosis and liver cancer. Any chronic liver disease can eventually lead to cirrhosis, which is believed to be irreversible. The only treatment options are to treat the cirrhosis condition is liver transplantation. Pathologic features reflect irreversible chronic injury of the hepatic parenchyma and include extensive fibrosis in association with formation of regenerative nodules. Cirrhosis may be usefully classified by a mixture of etiologically and morphologically defined entities as, alcoholic, eryptogenic and postural or post necrotic, biliary, cardiac, metabolic and inherited and drug related [26].

**Hepatitis**

Hepatitis refers to inflammation of the liver. Hepatitis can have several causes, the most common being viruses or alcoholism. Viral hepatitis comes in several forms, the most 9 common being hepatitis B (40%), hepatitis A (32%) and hepatitis C. Hepatitis virus B and hepatitis virus C are spread by the blood and can become chronic conditions, which can lead to cirrhosis [7].

**Alcoholic Liver Disease (ALD)**

Alcoholic Liver Disease (ALD) comes in 3 major varieties: alcoholic fatty liver, alcoholic hepatitis and alcoholic cirrhosis. All 3 can occur alone or even together in the same patient. The primary form of treatment is abstinence from drinking alcohol [26].

Fatty liver is the most common and the least harmful. It can occur within days of moderate to heavy drinking. Fat accumulates in the cytoplasm of liver cells, causing the liver to swell, sometimes to large proportions [7].

Alcoholic hepatitis is inflammation of the liver and can exist as either acute or chronic conditions. Symptoms can vary greatly, from asymptomatic to severe fever, nausea and abdominal pain. Acute hepatitis can often cause death and the chronic form often leads to cirrhosis. The disease is characterized by regenerative nodules of hepatic tissue completely surrounded by fibrous scar tissue. The scar tissue grows faster than liver cells can regenerate and the growing network of scar tissue inhibits blood flow. Once cirrhosis develops, the risk of liver cancer elevates substantially, even if the patient abstains from drinking for several years[27].

**Portal hypertension**

Excessive resistance to portal blood flow can be divided into intrahepatic, pre-hepatic, and post hepatic causes. The pre-hepatic conditions include narrowing of portal vein, obstructive thrombosis, and massive splenomegaly which may shunt excessive blood into the splenic vein. Right side heart failure, outflow obstruction of hepatic vein and constrictive pericarditis constitutes the main post hepatic causes. In cirrhosis, portal hypertension results from excessive resistance to portal flow at the level of sinusoids and also from the compression of terminal hepatic veins by parenchymal nodules. Nitric inhibition can be a major cause [28].

**Ascites and oedema**

Chronic hepatic failure due to cirrhosis may leads to ascites and portal hypertension. Development of oedema and ascites in patients results from reduced synthesis of albumin by the hepatocytes causing hypoproteinaemia, increased hydrostatic pressure due to portal hypertension, reduced plasma osmotic pressure, and secondary hyperaldosteronism. The pathogenesis of ascites include the following mechanisms.

- Sinusoidal hypertension.
- Fluid leakage in the intestine.
- Percolation of the hepatic lymph into the peritoneal cavity.
- Retention of sodium and water in kidney due to hyperaldosteronism[28,29].

**Liver cancer**

The liver can be affected by primary liver cancer that originates in the liver or by cancer which forms in other parts of the body and then spreads to the liver. The most common cause of liver cancer is cirrhosis which occurs due to hepatitis B, hepatitis C, or alcohol. The viruses induce malignant changes in the cells by affecting gene expression, altering gene methylation and repressing or promoting various cellular signal transduction pathways. Secondary liver cancer occurs as a result of metastasis of cancer from different parts of the body (intestine or pancreas) that drain into the liver via the portal vein or from other cancers. Liver cancer can also form from various other structures within the liver such as the blood vessels, bile duct and the immune cells. Many cancers found in the liver are not true liver cancers, but are cancers from other sites in the body that have spread to the liver

Liver cancers include:

- Hepatocellular carcinoma (HCC)
- Cholangiocarcinoma [28]

**Hepatic failure**

Hepatic failure may result from severe acute and fulminant injury to the liver along with massive necrosis of hepatocytes (acute liver failure) and also from various chronic liver diseases (chronic liver failure). 80 to 90% of functional capacity of hepatocytes must be eroded before hepatic failure ensues. The inter current diseases which place demands on the liver include systemic infection, gastrointestinal bleeding, severe stress like major surgery or heart failure, electrolyte disturbances etc. In most cases of severe liver failure, transplantation of liver is the only hope of survival [29].

**Treatment**

These are various sources for hepatotoxicity treatment mode these selected by consultation by physician about the treatment or change in treatment regimen. Treatment of hepatotoxicity has dependent upon causative agent, degree of liver dysfunction and age and general health of patient.

Treatments for hepatotoxicity include:

- Withdrawal of causative medication or removal from exposure to causative agent
- Regular monitoring of patient and review of liver function – where liver dysfunction is mild to moderate and liver function is improving
- Complete avoidance of alcohol and medication that may contribute to further liver damage.

Management of symptoms of liver damage

- Nutrition – with vitamin supplementation as required
- Regular exercise in order to maintain muscle mass
- Ursodeoxycholic acid

Management of pruritus

- Cholestyramine
- Antihistamines

Management of ascites

- Low sodium diet
- Diuretics – furosemide, spironolactone
- Removal of fluid via a needle in the abdomen – Paracentesis
- Portosystemic shunting

Management of portal hypertension

- Beta - blockers
- Oesophageal variceal banding
- Portocaval shunt

Management of acute liver failure due to hepatotoxicity

- Supportive care always in intensive care unit – airway protection, fluid and electrolyte management.
- Management of complications such as bleeding problems and hepatic encephalopathy.
- Liver transplantation – for acute fulminant liver failure or end stage cirrhosis [32,33,34].

**HEPATOPROTECTION**

The complexity of pathological features and insufficient knowledge of pathogenic mechanisms are responsible for improper development of treatment strategies in liver diseases. The allopathic therapies available for acute and chronic viral hepatitis are as follows.

At least 5 etiologically distinct forms of viral hepatitis as given below have been recognized.

1. Hepatitis A (HA) or infectious hepatitis.
2. Hepatitis B (HB) or serum hepatitis.
3. Hepatitis C (HC) or RNA virus that has homology with Flaviviridae
4. Hepatitis D (HD) of RNA virus which requires presence of HBV for successful infection.
5. Hepatitis E (HE) or enterically transmitted Non-A, Non-B. In addition, there is a theoretical possibility of another parenteral Non-A, Non-B agent.
6. Hepatitis F.

Modern medicines have been used for the treatment of hepatic ailments but these drugs possess harmful side effects such as insomnia, vomiting, constipation and depression. Therefore, search for a drug to treat liver diseases without side effects continues in the present scenario.

Medicinal herbs are widely used in the treatment of liver diseases like hepatitis and cirrhosis. The traditional medicine is being relied about 80% of the world population which is predominately based on plant material. Several plants were reported as hepatoprotective against hepatotoxicity in animals during the last decade [35].

Only few plants are really very promising hepatoprotective agents based on available data these include: *Silybum marianum, Picrorhiza kurrao (kutkin), Curcuma longa (Turmeric), Camellia sinensis, Androgaphis paniculata, Phyllanthus amarus, Chelidonium majus, Glycyrrhiza glabra* and *Allium sativum*. The active constituents elucidated to date

involve a wide range of components including terpenoids, curcuminoids, flavonoids, glycosides etc [36,40]

Commonly used plants in herbal formulation in India are *Andrographis paniculata, Boerhaavia diffusa, Eclipata alba, Picrorrhiza kurroa, Olednlandia corymbosa, Asteracantha longifolia, Apium graveolens, Cassia occidentalis, Cichorium intybus, Embelia ribes* and *Tinospora cordifolia*.

Some of the poly herbal formulations tested for their hepatoprotective activity are Liv.52, Liv.42, Liver cure, Livol, B. Liv, Hepatomed, Jigrine, Tefroli, Stimuliv, Koflet and Lacterine [37].

**Role of hepatoxicants in liver damage**

Chemicals which are administered to induce or cause liver injury are called hepatotoxic agents. Although a wide variety of industrial chemicals, solvents or therapeutic drugs can produce liver injury, it is apparent that their pharmacological effects on the liver differ in many ways [1].

Hepatotoxic agents are generally divided into two groups. The first group includes those hepatotoxic chemical or agents which produce hepatotoxicity or liver damage when get metabolized in liver. While the other group of chemicals do not require metabolism to produce hepatotoxicity.

Based on properties of hepatotoxic agents, the mechanism of liver damage or injury has been proposed to involve two classic division of drug reactions,

1) Drugs that directly affect the liver and

2) Drugs that mediate an immune response and then produces liver injury.

Drug induced liver injury is one of the most common causative factors that poses a major clinical and regulatory challenge. Various chemicals and drugs used to induce hepatotoxicity which includes ethanol, $CCl_4$, d-galactosamine etc and drugs include cadmium, paracetamol, isoniazid, rifampicin etc. Here ethanol mediated hepatotoxicity was chosen as the experimental model [1].

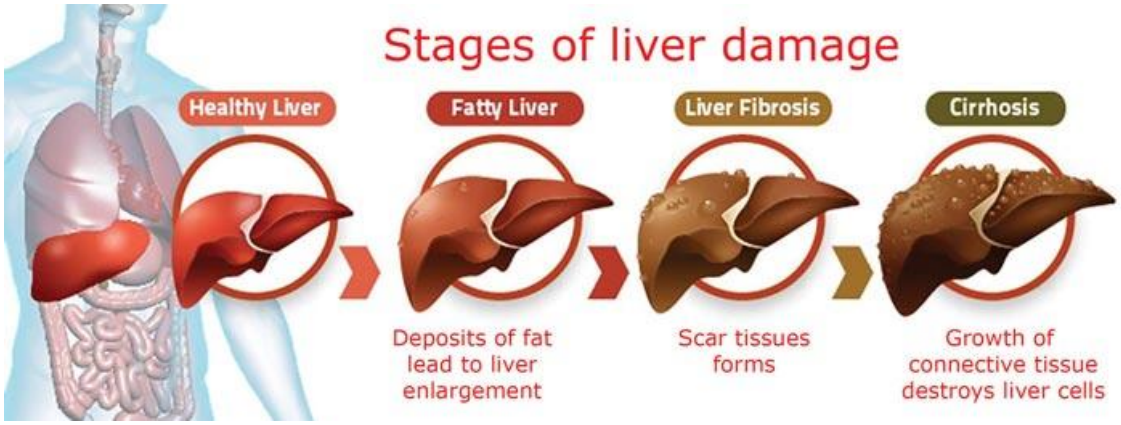


**Fig 4: Stages of liver damage**

**Mechanism of action of ethanol**

Ethanol is metabolised in the liver through alcohol dehydrogenase and microsomal ethanol oxidizing system (MEOS) pathway. Ethanol metabolised in to the highly toxic acetaldehyde is then oxidized to acetate by acetaldehyde oxidase giving rise to ROS via cytochrome P4502E1. Prolonged consumption of alcohol increases nitric oxide level which leads to formation of toxic oxidant peroxynitrite. Low capacity of antioxidant in this situation leads to damage of the cells of the hepatic cells and the cell organelles with the release of reactive aldehydes and ROS [38].

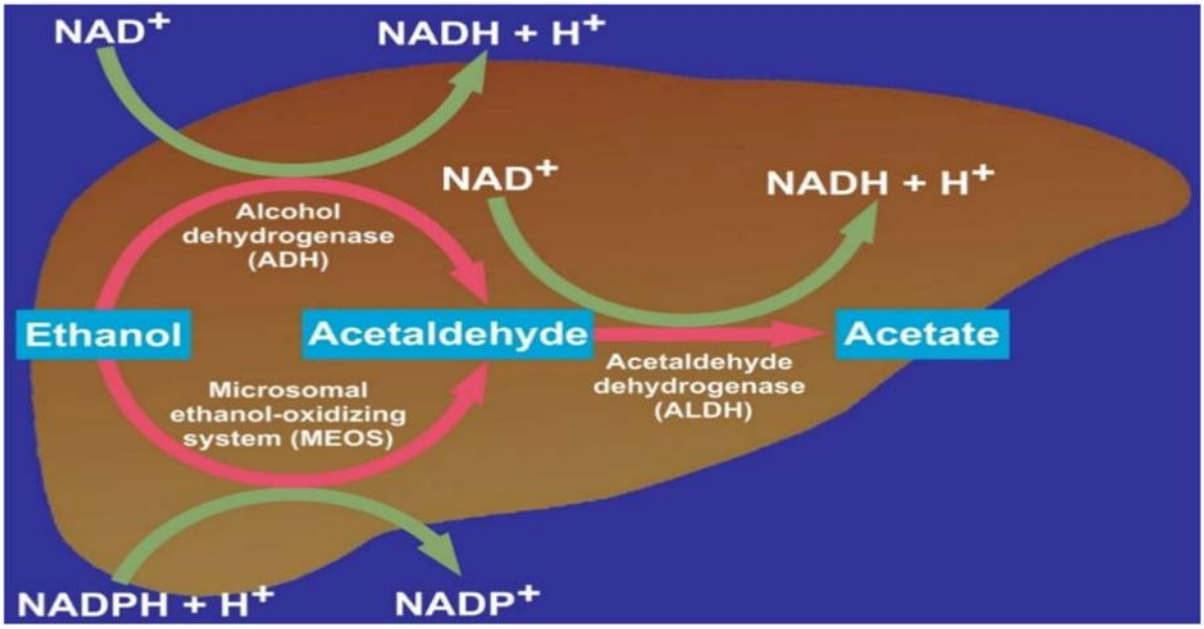


**Fig 5: Mechanism of action of ethanol in liver cells**

**Standard liver treatments**

Hepatoprotectives are a class of therapeutic agents that includes synthetic as well as natural products which offer protection to liver from damage or help in regeneration of hepatic cells Although a number of remedies for hepatic dysfunctions are available none of them is proved to be completely safe and effective. There are however, members of drugs employed in traditional system of medicine for liver dysfunction. For the management of hepatic disturbances, the preparations containing herbal extracts are widely available but are not sufficient to provide complete protection from numerous infectious agents. The management of liver disorders is offered by Indian traditional system and some of the plants have already been accounted to hold significant antioxidant activity [39]. Some common hepatoprotective agents are Silymarin [40],Lecithin [41],L-Ornithin and L-Aspartate [42] and Ursodeoxycholic acid [81].

# PROFILE OF THE PLANT

**Taxonomical classification of plant**

Kingdom : Plantae

Phylum : Spermatophyte

Subphylum : Angiospermae

Class : Monocotyledonae

Order : Arecales

Family : Arecaceae

Genus : Caryota

Species : *Caryota urens*.L

**Synonyms of *Caryota urens***

Tamil : Kondapanei

Malayalam : chuntappana

Sanskrit : Moha-karin

Hindi : Mari

Gujarati : Shivjata

English : Fishtail palm

**Distribution of plant**

It is mainly distributed in the low land forest of tropical Asia including, India, Malaysia, Indonesia, Burma, Philippine, and Srilanka.

**Description**

It is an evergreen tall handsome plant, growing up to 12-20m height and 30cm width. This is a solitary trunk palm covered with leaf scar rings. Leaves: Large, compound, bipinnate, bright to deep green colour, 24-28 cm × 6-8 cm, and held 60cm long petioles. Leaflet triangular, irregularly cut, wedge – shaped, obliquely truncate, apex praemorse, the margin prolonged into a tail like a process which resembles with the lower fin of fish so called fishtail palm. Flowers: White and unisexual flower. Inflorescence is spadix, 3m long and freely hanging around the palm and flower remains open on each inflorescence for six weeks. Fruits: Fruits are round and yellow in colour, matures become red drupe about 1cm wide and has a single seed. Seeds are spread by bats and palm civets. Trunk and bark: Trunk

is smooth and contains annular leaf scar. The mature wood is strong, heavy and sustainable. It used in construction purposes and food [44,45,46].

Fig 6a

Fig 6b

**Fig 6a: Immature fruits, leaf, mature fruits of the *Caryota urens* L.**

**Fig 6b: Immature fruits of the Caryota urens L.**

**Chemical constituents of flowers of *Caryota urens* L.**

Cyclopentanone, stearic acid, Oleic acid, palmitic acid, hendecynoic acid, 3-butenoic acid, phthalic acid, cyclopentaneundecanoic acid, 1,5- heptadiene, (2E,6E)-farnesol, pyroglutamic acid, decylenic alcohol, 11-octadecenoic acid, pyrogallol 1,3-dimethyl ether, 2-methoxy-4-vinylphenol, phenol, acidecarbolique, 1,8-nonadien-4-ol[45,46,47].

**Medicinal uses of *Caryota urens L.***

Hair growth, gastric ulcer, migraine headachae, snakebite poisoning and also rheumatic swellings, seminal weakness and urinary disorders, boil, gastric ulcer, tooth ailments and Jaundice [45,46,48,49].

**Reported activities of the *Caryota urens L.***

The plant has been reported to possess antioxidant activity, antidiabetic activity, & antimicrobial activity [48,49].

# Review of literature

# REVIEW OF LITERATURE

**Review based on the plant used in the study**

Ananth *et al.,* 2013 carried out the antimicrobial potential effect of *Caryota urens L*. antimicrobial activity was determined by disc diffusion method. GC-MS analysis showed the presence of fatty acids, aliphatic, aromatic and phenolic acids. The fruit skin and immature fruit of *Caryota urens* exhibited strong antibacterial activity against the tested pathogens (*Escheria coli, vibrio cholera, salmonella typhi, Staphylococcus aureus and Shigella flexneri*) when compared to leaf. *Caryota urens* extracts have strong antioxidant and antimicrobial potential property [46].

Uddin *et al.,* 2015 *In vitro* determined the of antioxidant property of the *Caryota urens* fruits extracts, DPPH (2, 2 diphenyl 2 picrylhydrazyl) radical scavenging assay was performed. CLF showed the highest antioxidant activity (61.58 % scavenging) at 400 µg/ml concentration. They are used for prevention of free radicals facilitated oxidative damages of the biomolecules. Hence, *Caryota urens* fruits had good antioxidant activity [48].

Wimalasiri *et al.,* 2016 done her study with the methanolic extract of *Caryota urens* showed antidiabetic activity. Anti-diabetic properties were estimated using Alpha amylase and Alpha glucosidase enzyme inhibition assays. They dried methanolic extracts of both boiled and raw samples were used in all assays. *Caryota urens* had very less antidiabetic activity. The study showed antioxidants activity of *Caryota urens* flour, estimated as free radical scavenging activity, metal ion chelating capacity, electron donating reducing power and oxygen radical absorbance capacity providing some scientific information for traditionally claimed health benefits [47].

Ranasinghe *et al* conducted a study on antioxidant activity of *Caryota urens* L. sap on 2012. The antioxidant activity of *Caryota urens* sap, as determined by radical scavenging activity, electron donating reducing power and metal ion chelating capacity. *Caryota urens* sap showed good antioxidant activity [49].

**Review of *in silico* hepatoprotective activity**

Burle *et al*., 2017 carried out the study on the hepatoprotective aspects of hybrid taurine amino acid peptides analogues before the synthesis carried out the *in silico* designing of molecules and from the results of this *in silico* study carried out forwarded the synthesis of hybrid compound followed by their *in vitro* and *in vivo* studies the binding affinity of the designed compound towards CYP2E1 (3GPH) was selected on the basis docking score. The compound SSSB-16 shows the maximum score having the docking score is -24.84 as compared with single taurine and other taurine hybrid compound. The compound SSSB 15 is second in the list of docking score with the docking score is -24.67. The reference ligand having the docking score is -11.90. All the compounds were screened for their *in vitro* antioxidant activity by employing DPPH, nitric oxide scavenging method. From the *in vitro* result of antioxidant activity those compound which had shown maximum activity till use for hepatoprotective activity. The compound SSSB3 which is the combination of Taurine- Glycine-Glycine shows the

maximum activity as compared to all other compounds. The result exhibited good activity for SSSB3 (Taurine-Gly-Gly) compound. It can be concluded that the amino acid hybrid with future being proof to be novel compound as hepatoprotective activity. It may be used as a supplement with the drugs to reduced hepatotoxicity [50].

**Review of *in vitro* hepatoprotective activity**

Beena *et al*., 2011 carried out to evaluate *in vitro* hepatoprotective activity of ethanolic extract of *Coldenia procumbens* Linn. Here antitubercular drugs as test drug and silymarin as standard drug by MTT assay[(3-(4,5 dimethylthiazole –2 yl)-2,5 diphenyl tetrazolium bromide) assay]. *Coldenia procumbens* Linn has been widely used for a number of medicinal purposes especially in Siddha medicine. *Coldenia procumbens* showed hepatoprotective activity against antitubercular drugs [51].

Bao *et al.,* in 2012 introduced Cytotoxicity and Apoptosis Induction in Human HepG2 Hepatoma Cells by Decabromodiphenyl Ethane. This is evaluated by the 3(4,5dimethylthiazol2yl)2,5diphenyl tetrazolium bromide and lactate dehydrogenase assays and nuclear morphological changes, DBDPE inhibited HepG2 viability in a time and dose dependent manner within a range of 12.5 mg/L to 100 mg/L and for 48 h and 72 h. Induction of apoptosis was detected at 12.5-100 mg/L at 48 h and 72 h by propidium iodide staining, accompanied with overproduction of reactive oxygen species (ROS). Furthermore, N- acetyl- L- cysteine, a widely used ROS scavenger, reduced DBDPE- induced ROS levels and increased HepG2 cells viability[52].

Pranati *et al.,* 2017 carried out the hepatoprotective property of organic green tea in change in liver cell line. To assess the hepatoprotective property of organic green tea in Chang liver cell line. Green tea is found to have anti-inflammatory, anti-oxidative, anti-mutagenic and anti-carcinogenic properties and also helps in preventing cardiac disorders, gives protection against solar UV rays, maintains body weight and prevents Intestinal Dysbiosis and infection. Green tea extract was evaluated for hepatoprotective property against $CCl_4$ induced toxicity. This is done by MTT assay and by evaluating the ALT and AST levels *invitro* study. This study proves that green tea extract can be used as a potent hepatoprotective drug against $CCl_4$ induced hepatotoxicity. MTT assay shows the cell viability is >80% even at higher concentration of 120µg/ml of green tea and hence considered to be nontoxic. Also from the results of *invitro* study, it has been proved that pre-treatment with green tea exact prevented Chang liver cells from $CCl_4$ induced hepatotoxicity. This can be appreciated by the reduced levels of ALT and AST in green tea extract treated Chang cell lines in contrast with untreated Chang cell lines upon $CCl_4$ induced toxicity. This study proves that green tea extract can be used as a potent hepatoprotective drug against $CCl_4$ induced hepatotoxicity[53].

**Review of *in vivo* hepatoprotective activity**

Sivaraj *et al.,* evaluated the hepatoprotective effect of aqueous leaf extract of *Andrographis paniculata* against ethanol induced liver toxicity in albino rats. The animals were divided in to four groups and duration of study was 45 days, ethanol (7.9g/kg) administered intragastrically. *Andrographis paniculata* belongs to the family Acanthaceaae, aqueous extract of *Andrographis*

*paniculata* exhibited hepatoprotective activity. The Oral administration of aqueous extract (250mg/kg) of *Andrographis paniculata* was done for 45 days. Here they used reference drug as silymarin (25mg/kg). On 46th day anaesthetized the animals by using anaesthetic agent and serum biochemical parameters were analysed. They included, ALT, AST and ALP, serum bilirubin, thiobarbituric acid, lipid hydroperoxide, super oxide dismutase, catalase, glutathione peroxidase and glutathione –s-transferase, total cholesterol, TG, free fatty acids, phospholipids. The plant extracts showed good hepatoprotective activity [54].

George, 2014 carried out her work using ethanol as inducing agent to induce liver damage in rats. The rats were divided into six groups, each group contain six rats and the duration of study was 30 days. Methanolic extract of *Ocimum basilicum* (120mg/kg) showed hepatoprotective activity against ethanol (5gm/kg) induced liver disease. At the end of the experiment, rats were sacrificed; blood and liver tissue were collected to conduct the bioassays. From the blood collected, thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) were estimated together with the liver markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). The results indicated that there were high difference between the experimental rats when compared with alcohol control in all the parameters checked and they were not different from the normal control. Again, the groups, treated with extracts only, did not show any negative effect, which clearly indicated that the extract had no toxic effects. A reduction in the levels of TBARS, ALT, and AST and high increase in the levels of GSH, CAT and SOD were noticed in experiment groups when compared with the disease control groups. Thus the results showed protective activity by these extracts against ethanol-induced hepatotoxicity [55].

Olanrewaju *et al.*, 2016 planned to evaluate hepatoprotective activity of methanolic extract of *Hibiscus sabdariffa* in ethanol-induced hepatotoxicity. Hepatotoxicity was induced on the rats using ethanol and the levels of serum enzymes such as alanine aminotransferase, aspartate aminotransferase, total and conjugated bilirubin was estimated. The levels of hepatic enzymes (AST, ALT and bilirubin) in the serum were significantly increased ($P < 0.05$) in rats treated with ethanol when compared with the control. In the post phase, administration of different doses of ethanol extract reduced the levels of increased hepatic serum enzymes when compared with the control. The Methanolic extract of *Hibiscus sabdariffa* showed good hepatoprotective effect on ethanol induced liver damage [56].

Qadrie *et al.*, 2019 investigated the hepatoprotective effects ethanolic leaf extract of *Callicarpa lanata* (EECL) in ethanol induced hepatotoxicity in wistar rats. The ethanol treated rats showed highly increase in the levels of serum enzyme activities, total bilirubin and reduction in total proteins reflecting the liver injury caused by ethanol. EECL, at a dose of 400 and 200mg/kg body weight (P.O) exhibited hepatoprotective effect by lowering the Alanine transaminase, Aspartate amino transferase, Alkaline Phosphate, Gama Glutamyl Transpeptidase (GGTP), total Bilirubin. Total protein level also increased in a dose dependent manner. The effects of EECL at both levels were comparable with standard drug Silymarin. The hepatoprotective activity was also supported by histopathological studies of liver tissue [57].

Rekha *et al*., 2013 conducted a study on protective effect of *Vitis vinifera* L seed extract against ethanol induced oxidative damage of liver in *wistar* rats. Here Silymarin was used as reference drug. Liver injury is induced by ethanol (5g/kg/day,20%w/v) for 4 weeks of experiment. At the end of experiment, the animals were sacrificed and the hepatoprotective activity assessed by using various biochemical parameters like AST, ALP, ALT, SOD, CAT and MDA. The ethanolic seed extract of *Vitis vinefra* showed protective effect against ethanol induced toxicity. It is due to the presence of bioactive compounds such as flavonoids, polyphenols, anthocyanins, proanthocyanidins, procyanidins and resveratrol [58].

Mani *et al*., in 2016 reported zingerone ameliorates hepatic and renal damage in alcohol-induced toxicity in experimental rats. ethanol-induced liver and kidney damage can be alleviated by the potential beneficial effects of zingerone as evidenced by the improvement in the liver function, hastening of alcohol metabolism, improvement of renal function and thereby the restoration of the liver and kidney architecture. At the end of the study sacrificed the animals and assessed hepatoprotective and renal protective activity. AST and ALT were assayed by the method of Reitman and Frankel. ALP was assayed using the diagnostic kit based on the method of Kind and King. GGT was assayed by the method of Rosalki and Rau. Serum total bilirubin was estimated by the method of Malloy and Evelyn. LDH by the method of King and total proteins by method of Lowry *et al.* Serum urea was determined by the method of Fawcett and Scott, serum uric acid by the method of Caraway and serum creatinine by Jaffe's method. The ethanol-induced liver and kidney damage can be alleviated by the potential beneficial effects of zingerone as evidenced by the improvement in the liver function, hastening of alcohol metabolism, improvement of renal function and thereby the restoration of the liver and kidney architecture [59].

Padmanabhan *et al.,* 2014 conducted the hepatoprotective activity of herbal preparation (hp-4) against alcohol induced hepatotoxicity in mice. Free radicals include both reactive oxygen Species (ROS) and reactive nitrogen species (RNS).When free radicals are produced in a regulated manner in a healthy human body it is scavenged efficiently by antioxidant defense system. But excess generation of pro-oxidants by continuous chain reaction in the form of ROS and RNS cause several human diseases. The shift of the balance in the favour of pro-oxidants results in a condition called "oxidative stress". Alcohol is primarily metabolized in the liver to generate ROS and RNS, leading to diseases such as cirrhosis, fatty liver and chronic hepatitis. Alcohol induced damage is associated with oxidative stress. The excess generation of pro-oxidants and reduced antioxidant levels provide an effective model of Hepatotoxicity which is noteworthy. Recent trend is to discover polyherbal formulation of medicinal plants which have hepatoprotective function. In the present study 80% alcoholic extract of leaves of *Aloevera, Bacopa monniera, Moringa oleifera* and rhizome of *Zingiber officinale* were utilized to prepare Herbal Preparation or HP-4. Further the hepatoprotective effects of HP-4 was tested in alcohol induced Hepatotoxicity in mice [60].

Kumar *et al*.,2013 investigated *In vitro* antioxidant activity and *in vivo* hepatoprotective activity of aqueous extract of *Allium cepa* bulb in ethanol induced liver damage in *Wistar* rats. The

*In vitro* antioxidant and *in vivo* hepatoprotective effects of aqueous extract of *Allium cepa* (A. cepa) bulb were evaluated in male rats against ethanol induced liver damage in preventive and curative models. The antioxidant activity of *Allium cepa* was assayed and activities were compared to standard antioxidant, ascorbic acid. The results revealed that the IC50 values of *A. cepa* bulb extract for DPPH, hydroxyl, superoxide radical scavenging activities were 195.2 ± 0.2, 374.7 ± 0.4 and 182.5 ± 1.7g/mL, respectively. Liver injury was induced by 40% ethanol administration (3.76 g/kg bw, orally) for 25 days. In two different sets of experiments, the *A. cepa* extracts (100, 300 and 600 mg/kg bw) and silymarin (100 mg/kgbw) were administered orally in preventive and curative models. The ethanol administration caused severe hepatic damage in rats as evidenced by elevated serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin levels. The *Allium cepa* and silymarin administration prevented the toxic effect of ethanol on the above serum parameters in both preventive and curative models [61].

# Aim & Objective

# AIM AND OBJECTIVE OF THE WORK

Liver is the most vital organ of the body, regulating the various physiological process of the body. Vital functions such as metabolism, secretion, storage and excretion of many endogenous and exogenous causing its injury or impairment. The liver has a great capacity to detoxify toxic substances and synthesize useful materials. Nowadays, liver diseases remain one of the serious health problem and traditional medicinal plants for liver disorders. Herbal drugs are prescribed because of their fewer side effects and relatively low cost. Anemia and muscle pain is due to the improper functioning of liver. Hepatotoxicity refers to liver dysfunction, liver injury due to overload of drugs, alcohol or xenobiotics. The chronic alcohol use is the most prevalent single cause of illness and death from liver disease. The liver susceptible to alcohol related injury because it is the primary site of metabolism. The alcohol produce highly reactive molecules called freeradicals cause damage the liver.

*Caryota urens* is one of most valuable medicinal plant species having nutritional as well as therapeutic and commonly known as fish tail palm. The inflorescence 3m long and freely hanging around the palm and flower remains open on each inflorescence for six weeks. This produces a cluster of the white and unisexual flower.

*Caryota urens* have antioxidant activity, which can prevent oxidative stress related liver disease. It has high nutritional and therapeutic potency and contains many phytoconstituents like alkaloids, glycosides, carbohydrate flavonoids, saponin, phenolics, phytosterols, fixed oil and fats etc. The traditional treatment of jaundice ulcer, migraine, burns, snake bite poisoning, rheumatic pain. There is no systematic pharmacological work has ever been carried out on disease caused by oxidative stress such as liver disorders and it is associated with symptoms like anemia and muscle spasm.

The objective of the work is to determine the hepatoprotective activity of flowers of *Caryota urens* by *in silico*, *in vitro*, and *in vivo* methods against ethanol induced liver toxicity in rats.

# Plan of work

# PLAN OF WORK

The aim of the work is to determine *in silico, in vitro* and *in vivo* hepatoprotective activity of flowers of *Caryota urens* L. against ethanol-induced liver toxicity using rats.

The plans of the research work are as follows

1. Review of literature

2. Collection and authentification of plant

3. Preparation of various extract (aqueous and ethanol) using the flowers of *Caryota urens* L.

4. Phytochemical screening of the various extracts (aqueous and ethanol) of flowers of *Caryota urens* L.

5. Study of *in silico* hepatoprotective activity using phytoconstituents of flowers of *Caryota urens* L. against CYP2E1 enzyme

6. Study of *in vitro* hepatoprotective activity of flowers of *Caryota urens* L. against ethanol-induced hepatotoxicity by MTT assay method.

7. Study of *in vivo* hepatoprotective activity of flowers of *Caryota urens* L. against ethanol-induced hepatotoxicity model.

8. Determination of biochemical parameters.

9. Estimation of total protein and malondialdehyde levels.

10. Estimation of liver enzymatic and non- enzymatic antioxidants.

11. Histopathological studies.

12. Statistical analysis involved in the work.

# Materials & Methods

# MATERIALS

| Chemicals | Manufacturer |
|---|---|
| Silymarin | Microlabs, Bangalore |
| AST | Agappe diagnostics Ltd, Kerala |
| ALT | Agappe diagnostics Ltd, Kerala |
| ALP | Agappe diagnostics Ltd, Kerala |
| Thiobarbituric acid | Agappe diagnostics Ltd, Kerala |
| Carboxyl methyl cellulose | Loba chemie |
| Ether anaesthetic | Hi-Pure Fine Chemical Industries, Chennai |
| Glucose | Qualigens Fine Chemicals, Mumbai |
| Nicotinamide adenine dinucleotide | S.D. Fine Chem Ltd, Mumbai |
| Lithium lactate | Hi Media Laboratories Pvt. Ltd, Mumbai |
| Sodium pyruvate | Hi Media Laboratories Pvt. Ltd, Mumbai |
| Reduced glutathione | Hi Media Laboratories Pvt. Ltd, Mumbai |
| Sodium hydroxide | SD Fine Chemicals Ltd, Mumbai |
| Sulphanilamide | Sisco Research Laboratory, Mumbai |
| Hydroxyl ammonium chloride | Qualigens Fine Chemicals, Mumbai |
| Triton X-100 | Hi Media Laboratories Pvt. Ltd, Mumbai |
| N-ethylene diamine dihydrochloride | Hi Media Laboratories Pvt. Ltd, Mumbai |

All other chemicals used were obtained commercially and were of analytical grade.

## INSTRUMENTS

| Instruments | Manufacturer |
|---|---|
| Centrifuge | Remi |
| Auto analyser | Mispa viva |
| pH meter | ELCO 1/27 pH meter |
| UV-spectrophotometer | Jasco v530 model |

# EXPERIMENTAL METHODS

## Experimental animals

Male *Wistar* rats weighing between 180 – 220 g of either sex would be used for the study. The animals were procured from College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur, Kerala. The animals were maintained under controlled conditions of temperature (23±2˚C), humidity (50±5%) and 12h light-dark cycles. All the animals were acclimatized for 7 days before the study. The animals were randomized to experimental, normal and control groups, housed individually in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free access to standard pellets as basal diet and drinking water ad libitum. Animals were habituated to laboratory conditions for 48h prior to experimental protocol to minimize if any of nonspecific stress. The animals used for this study were approved by institutional animal ethical committee (IAEC), College of pharmacy, SRIPMS, bearing the approval no.

## Plant material

The plant material used for this study consisted of dried flowers of *Caryota urens* L. belonging to the family Areaceae.

## Collection and authentification of the plant

The flowers of the *Caryota urens* L. were collected from Thalassery, kerala during July 2019. The flowers of *Caryota urens* (L) were identified and authenticated by Dr.C.Murugan, Scientist 'E'& Head of office, Botanical Survey of India, Southern Regional Centre, Tamilnadu Agriculture University Campus, Coimbatore, India. The voucher specimen has been (BSI/SRC/5/23/2019/TECH/141) deposited in the institution.

## Preparation of various plant extract

The plant materials used for the study consist of dried flowers of *Caryota urens*.L. belongs to the family Areaceae were dried under shade at the room temperature and the dried flowers were powdered using mechanical grinder. The powdered plant materials were then passed through sieve No. 22 and stored in an air tight container until the time of use.

## Extraction procedure of *Caryota urens* L.

## Aqueous extraction procedure of *Caryota urens* L. (AECU)

The plants were washed with water and dried in shade for 3 weeks. Dried flowers of *Caryota urens* (500 g) powdered and refluxed at $80^0$ C with 1500ml of sterile distilled water for 2h. Then the mixture allowed to cool at room temperature and aqueous extraction collected by filtration. The filtrates were concentrated to dryness under controlled temperature and pressure. The percentage yield of the extract was calculated.

**Ethanol extraction procedure of *Caryota urens* L. (EECU)**

After the shade drying extraction of flowers of *Caryota urens* (500g) were to be carried out with 1500ml volume of ethanol for 48 h in a Soxhlet apparatus. The extract was filtered and concentrated under reduced pressure and low temperature (40-50°C) on a rotary evaporator. The filtrates were concentrated to dryness under controlled temperature and pressure. The percentage yield of the extract was calculated [46,62,63].

**Phytochemical screening of plant extract**

The plant extraction was subjected to qualitative test for the identification of various phytochemical constituents (alkaloids, flavonoids, glycosides, triterpenoids, phenolics, tannin and saponin) as per the standard procedure [64,65,66].

**Test for alkaloids**

a) Dragendorf's test:

To 2 ml of AECU and EECU was treated with Dragendorf's reagent (potassium bismuth iodide).The formation of orange red colour or precipitate which indicated for the presence of alkaloids.

b) Mayer's test:

To 2 ml of AECU and EECU was treated with few drops of Mayer's reagent (potassium mercuric iodide). The formation of a turbid or creamy-white precipitant formed indicated for the presence of alkaloids.

c) Hager's test:

To 2 ml of AECU and EECU was treated with few drops of Hager's reagent (Saturated aqueous solution of picric acid). The formation of crystalline yellow precipitate formed indicated for the presence of alkaloids.

d) Wagner's test:

To 2 ml of AECU and EECU was treated with few drops of Wagner's reagent (dilute iodine solution). The formation of reddish-brown precipitate formed indicated for the presence of alkaloids.

**Test for flavonoids**

a) Ammonia test:

The filter paper was dipped in AECU and EECU and then exposed to ammonia vapour. The formation of yellow spot on filter paper indicated for the presence glycosides.

b) Concentrated sulphuric acid test:

To 2 ml of AECU and EECU was treated with concentrated sulphuric acid, which produced yellow colour. This indicated for the presence of flavonoids.

c) Aluminum chloride test:

To 2 ml AECU and EECU was treated with 1% aluminum chloride. The formation of yellow colour indicated for the presence of flavonoids

**Test for glycosides**

a) Baljet test:

To 2 ml of AECU and EECU when mixed with sodium picrate solution turned yellow to orange colour in presence of aglycones or glycosides.

b) Legal test:

To 2 ml AECU and EECU equal volume of water and 0.5 ml of strong lead acetate solution was added, mixed and filtered. The filtrate was extracted with equal volume of chloroform and evaporated to dryness. The residue was dissolved in 2 ml of pyridine and 2 ml of sodium nitroprusside was added followed by the addition of 2 ml sodium hydroxide solution to make alkaline in nature. The formation of pink colour indicated for the presence of glycosides or aglycone moiety.

**Test for Carbohydrates**

a) Molisch's test:

To 2 ml of AECU and EECU was mixed with few drops of Molisch reagent (α-naphthol) and concentrated sulphuric acid was added from sides of test tube. A purple colored ring formation at junction indicated for the presence of carbohydrates.

b) Fehling's test:

Equal volume of Fehling's A and Fehling's B were mixed (1 ml each) and 2 ml of AECU and EECU was added followed by boiling for 5-10 minutes on water bath (Fehling's A solution composed of 0.5% of copper sulphate and Fehling's B solution composed of sodium potassium tartrate).The formation of reddish brown colored precipitate (due to formation of cuprous oxide) indicated for the presence of reducing sugar.

c) Benedict's test:
It used for test reducing sugars and the Benedict's reagent composed mainly of copper sulphate and sodium hydroxide. To about 2 ml of AECU and EECU, 1ml of Benedict's solution was added and heated to boiling. Green, yellow, orange, red or brown colour formation occurred in order of increasing concentration of simple sugar in the test solution (due to formation of cuprous oxide).

**Test for steroids and triterpenoids**

a) Libermann Burchard's test:

To 2 ml of AECU and EECU was evaporated to dryness and extracted with chloroform. It was then added with few drops of acetic anhydride followed by concentrated sulphuric acid from the side wall of test tube. The formation of violet to blue colored ring at the junction of two liquids indicated for the presence of steroid moiety.

b) Salkovaski test:

Concentrated sulphuric acid was added to 2 ml of AECU and EECU dissolved in 1 ml of chloroform. A reddish blue colour exhibited by chloroform layer and green fluorescence by the acid layer suggested for the presence of steroids.

**Test for saponins**

a) Foam test:

To 2 ml AECU and EECU, 10-20 ml of water was added and shaken for few minutes. The formation of frothing which persists for 60-120 seconds indicated for the presence of saponins.

**Test for tannins and phenolics**

a) To 2 ml of AECU and EECU was treated with 5% ferric chloride which produced greenish black colour. This indicated for the presence of tannins.
b) To 2 ml of AECU and EECU was treated with 1 ml of 10% lead acetate solution in water. Formation of yellow colour precipitation gave a positive result.

**Test for proteins and amino acids**

a) Biuret test:

To 2 ml of AECU and EECU in hot water, few drops of Biuret reagent (potassium hydroxide, copper sulphate and sodium potassium tartrate) was added. The solution turned blue to violet color.

b) Millon's test:

Millon's test is usually positive for any compound containing a phenolic hydroxyl group. Millon reagent is a solution of mercuric and mercurous ions in nitric and nitrous acids. To 2 ml of AECU and EECU was taken in a test tube and few drops of Millon's reagent was added. The formation of white precipitate, which later turned red after heated for 5 minutes on water bath which indicated a positive reaction (due to formation of a mercury salt of nitrated amino acid).

c) Ninhydrin test:

This test is mainly used to detect the presence of α-amino acids and proteins containing free amino groups. To 2ml of AECU and EECU when heated with ninhydrin reagent gave characteristic deep blue or pale yellow colour due to formation of complex between two ninhydrin molecules and the nitrogen of free amino acid.

## *IN SILICO* HEPATOPROTECTIVE ACTIVTITY

Softwares and data bases used

➢ Accerlys discovery studio viewer 4.0.1

➢ Molinspiration ➢ RCSB protein data bank

➢ Online SMILES translator

➢ MGL tools-

- AutoDock 4.2
- Python 2.7 molecule viewer 1.5.6
- Vision 1.5.6
- Cygwin 64

➢ ChemSketch

➢ PreADMET

### *In silico* docking study

*In silico* docking studies were carried out using the enzyme CYP2E1. CYP2E1 which is an membrane protein of the cytochrome P450 expressed in high levels in the liver. In liver, most of the drugs undergo deactivation by CYP2E1 either directly or by facilitated excretion from the body [67,68,69]

**STEP I**

Ligand file format conversion

The flowers of *Caryota urens*.L. phytoconstituent structures were drawn using ChemSketch.

- Tools ⟶ clean structure.
- Tools ⟶ generate ⟶ SMILES notation.

d) Copied the smile notation and uploaded the smiles in online smile translator-cactus.nci.nih.gov/services/translate.

e) By choosing the required file format and save the file as pdb format.

**STEP II**

Protein structure refinement

The enzymes downloaded from RCSB (Research Co-laboratory for Structural Bioinformatics) Protein Data Bank and the protein was refined before use for docking.

- Opened Accelrys discovery studio viewer.
- File→open→RCSB PDB file.
- View →hierarchy click water molecules → select all water molecules →delete.
- Selected ligand, which was unnecessary and deleted.
- Saved the molecule in a desired location.

**STEP III**

**Docking with AutoDock 4.2**

- Opened the refined protein from the location in pdb format.
- Preparation of target and ligand in AutoDock 4.2

**STEP IV**

**Preparation of protein**

- AutoDock 4.2 →File →Read molecule →Choose refined enzyme file.

Elimination of water molecule carried out by:

- Select→ Select from string→Residue (*HOH*) →Add →Dismiss
- Edit→Hydrogen→Add → Polar only →Ok.
- Edit→Charges → Add kollmann charges →Ok.
- File → save →Write pdb →Browse →Save →Ok.
- Edit →Delete all molecules →Continue.

**STEP V**

**Preparation of ligand**

- Ligand →input →open.
- Ligand→ torsion tree → detect root.
- Ligand→ torsion tree →show root expansion.
- Ligand → torsion tree →choose torsions →done.
- Ligand→torsion tree →set number of torsions →dismiss.
- Ligand→torsion tree →hide root expansion.
- Ligand →torsion tree →show/hide root marker.
- Ligand→ output →save as pdbqt file.
- Edit →delete →delete all molecules →continue.

**Conversion of pdb files of protein in to pdbqt file**

- Grid → Macromolecule → Open → Save as pdbqt.

**Auto Grid calculation and creating "gpf" file**

- Grid → set map types → open ligand.
- Grid → grid box → set 60 points in XYZ.
- File → close saving current.
- Grid → output → save as gpf.
- Edit → delete → delete all molecules → continue.

**AUTODOCK calculation and creating 'dpf' file**

- Docking → macromolecule → set rigid file name → open.
- Docking → ligand → open → accept.
- Docking → search parameters → genetic algorithm → accept.
- Docking → docking parameters → accept.
- Docking → output → lamarckian genetic algorithm → save as dpf.

**Programming of 'Auto Grid' and 'Auto Dock' execution**

**Open Cygwin64 and type as given below:**

- **cd C:**
- **cd cygwin64**
- **cd usr**
- **cd local**
- **cd bin**

Program should list out the pdb, pdbqt, gpf and dpf files of an enzyme and ligand molecule.

Then type as:

- ./autogrid4.exe<space>–p<space>ligand.gpf<space> -l<space>ligand.glg

If a ligand gets into the spacing of the grid, then the execution of this command will be;

- ✓ 'Successful completion'

Then type as:

- ./autodock4.exe<space> –p<space>ligand.dpf<space> -l<space>ligand.dlg

If the ligand binds to the amino acids through 10 different conformations, then the execution of this command will be;

- ✓ 'Successful completion'

**STEP VI**

**Viewing docking results**

**Reading the docking log file.dlg**

- Toggle the AutoDock Tools button.
- Analyse → Docking.
- Analyse → Conformations → Load.

- Double click on the conformation for to view it.

**Visualizing docked conformations**

- Analyse →Dockings →Play.
- Load dlg file.
- Choose the suitable conformations.
- Analyse→ Docking→Show Interactions.

**Obtaining snap shots of docked pose**

- File→ Read Molecule.
- Analyse→Dockings →Open dlg file.
- Analyse → Macromolecule →Choose pdbqt file.
- Analyse Conformations Load.
- Double click the desired conformation.
- Analyse → Docking →Show Interactions

Proteins and ligand interaction displayed. It is then zoomed and increase the contrast by holding right key and ctrl. Rapid energy evaluation was attained by pre-calculating the atomic resemblance potentials for each atom in the selected compounds. In the Auto Grid process, the target was enclosed on a three-dimensional grid point and the energy of interface of each atom in the compounds were encountered. The following docking factors were chosen for the Lamarckian genetic algorithm as follows: population size of 150 individuals, 2.5 million energy evaluations, maximum of 27000 generations, and number of top individuals to automatically survive to next generation of 1, mutation rate of 0.02, crossover rate of 0.8, 10 docking runs, and random initial positions and conformations. The probability of performing local search on a single compound in the population was set to 0.06. AutoDock was run various times to obtain various docked conformations, and used to calculate the predicted binding energy.

***In vitro* hepatoprotective studies**

**Hepatoprotective activity**

HEP G2 (Human Hepatocellular Carcinoma) cell line was purchased from NCCS pune was maintained in Dulbecco's modified eagles media from National centre for cell science (NCCS), pune, India and maintained Dulbecco's modified Eagles medium (DMEM). The cell line was cultured in 25 cm tissue culture flask with DMEM. Cultured cell lines were kept at $37^0$ c in humidified 5% $CO_2$ incubator. The viability of cells were evaluated by direct observation of cells by inverted phase contrast microscope and followed by MTT assay method.

**Cell seeding in 96 well plate**

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium,100µl cell suspension (5×104 cells/well) was seeded in 96 well tissue culture plate and incubated at $37^0$ c in a humidified 5% $CO_2$ incubator.

Preparation of compound stock:

1) AECU was weighed and completely dissolved in 1ml DMEM using a cyclomixer. The extract solution was filtered through 0.22µm Millipore syringe filter to ensure the sterility. Ethanol (20Mm) was added to induce toxicity.

2) EECU was weighed and completely dissolved in 1ml DMEM using a cyclomixer. The extract solution was filtered through 0.22µm Millipore syringe filter to ensure the sterility. Ethanol (20Mm) was added to induce toxicity.

**Evaluation of cytotoxicity study**

After attaining sufficient growth, Ethanol (20mM) was added to induce toxicity and incubated for one hour, prepared extracts in 5% DMEM were five times serially diluted by two fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of 5% DMEM) and each concentration of 100µl were added in triplicates to respective wells and incubated at $37^0$ c in a humidified 5% $CO_2$ incubator.

**Cytotoxicity assay by direct microscopic observation:**

Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

**Cytotoxicity assay by MTT method**

Amount of 15 mg of MTT was reconstituted in 3ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at $37^0$ C in a humidified 5% CO2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT solubilisation solution (dimethyl sulfoxide was added) and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm[51,52,70,71].

***IN VIVO* HEPATOPROTECTIVE ACTIVITY**

**Acute toxicity studies**

The acute toxicity study of the flower extracts of *Caryota urens* L. has carried out as per the OECD guideline 420. It was noted that the extracts did not show any significant morbidity and mortality at the maximum dose tested i.e., 2000 mg/kg b.w. Hence the selection of dose levels for *in vivo* study as:

Low dose: 100 mg/kg

High dose: 200 mg/kg

**Ethanol-induced hepatotoxicity model**

Male *Wistar rats* weighing about180 – 220g were divided into seven groups, consisting of six animals each.

Group I - Control 0.5% CMC (10ml/kg b.w, p.o)

Group II - Negative control (40% v/v ethanol)

Group III - Aqueous extract of *Caryota urens* L. (100mg/kg, p.o)

Group IV - Aqueous extract of *Caryota urens* L. (200mg/kg, p.o)

Group V - Ethanolic extract of *Caryota urens* L. (100mg/kg, p.o)

Group VI - Ethanolic extract of *Caryota urens* L. (200mg/kg, p.o)

Group VII - Positive control - Silymarin at a dose. (100mg/kg, p.o)

The animals in group I were administered 0.5% CMC for the period of 42 days. The animals in group II-VII were administered 40%v/v orally for the period of 42 days. The animals in group III-VII were administered with respected drugs or extracts suspended in 0.5% CMC throughout 42 days. On the 43$^{rd}$ day, all the animals were anaesthetized and blood samples were collected through retro-orbital sinus puncture method and liver was removed.

**Estimation of serum bio-chemical parameters for both the extracts**

On day 43rd, all the rats were anaesthetized and blood samples were collected through retro-orbital sinus puncture under mild ether anaesthesia. The serum was used for assay of marker enzymes viz, ALT, AST, ALP, and total protein. On the same day, rats were sacrificed by cervical dislocation, liver were dissected out and homogenates (10%w/v) were prepared centrifuged. The enzyme levels were assayed using standard kits obtained from Agappe diagnostics Ltd., Kerala. The units were expressed as units per litre [54].

**ESTIMATION OF BIOCHEMICAL PARAMETERS**

**Determination of aspartate transaminase (AST) activity**

**Chemicals and Reagents**

Working reagent: Composition

Reagent 1 (R1)

Tris buffer (pH 7.8)

L-Aspartate

LDH

Reagent 2 (R2)

NADH

α- Ketoglutarate

Preparation

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

**Procedure:**

The working reagent was prepared by mixing 4 volume of Reagent1 [Tris buffer (88 mmol/L, pH 7.8), L-Aspartate (260 mmol/L), LDH (1500 U/L), MDH (900 U/L)] with 1 volume of Reagent2 [α- Ketoglutarate (12 mmol/L, NADH (0.24 mmol/L)] provided in the kit. About 100 µL of serum was added to 1000 µL of the working reagent. Mixed well and incubated for 1 minute at 37 □C. The change in absorbance was measured per minute for 3 minutes at 340 nm and the SGOT activity was expressed in U/L [72]. It was determined by using the formula:

AST activity (U/L) = Δ OD/ min ×1745

**Determination of alanine transaminase (ALT) activity**

**Chemicals and Reagents**

Working reagent: Composition:

Reagent 1 (R1)

Tris buffer (pH 7.5)

L-Alanine

LDH

Reagent 2 (R2)

NADH

α- Ketoglutarate

Preparation

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

**Procedure:**

The working reagent was prepared by mixing 4 volume of Reagent1 [Tris buffer (110 mmol/L, pH 7.5), L-Alanine (660 mmol/L), LDH (1500 U/L)] with 1 volume of Reagent2 [αKetoglutarate (16 mmol/L, NADH (0.24 mmol/L)] provided in the kit. About 100 µL of serum was added to 1000 µL of the working reagent. Mixed well and incubated for 1 minute

at 37 □C. The change in absorbance was measured per minute for 3 minutes at 340 nm and the SGPT activity was expressed in U/L [72]. It was determined by using the formula:

ALT activity (U/L) = Δ OD/ min ×1745

**Determination of serum alkaline phosphatase (ALP) activity**

**Chemicals and Reagents**

Working reagent: Composition

Reagent 1 (R1)

Citrate buffer (pH5.2)

Reagent 2 (R2)

Tablets

α-naphthyl phosphate

Fast red TR

Preparation

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

**Procedure:**

The working reagent was prepared by mixing 4 volume of Reagent1 [Diethanolamine buffer (125 mmol/L, pH 10.2), Magnesium Chloride (0.625 mmol/L)] with 1 volume of Reagent 2 [p-Nitrophenyl phosphate (50 mmol/L)] provided in the kit. About 20 µL of serum was added to 1000 µL of the working reagent. Mixed well and incubated for 1 minute at 37□C. The change in absorbance was measured per minute for 3 minutes at 405 nm and the ALP activity was expressed in U/L [72,73]. It was determined by using the formula:

ALP activity (U/L) = Δ OD/ min × 2750

**Determination of serum lactate dehydrogenase (LDH)**

**Procedure:**

The working reagent was prepared by mixing 4 volume of Reagent 1 [Tris buffer (pH 7.4, 80 mmol/L), Pyruvate (1.6 mmol/L), Sodium Chloride (200 mmol/L)] with 1 volume of reagent 2 [NADH (240 µ mol/L)] provided in the kit. About 10 µL of serum was added to 1000 µL of working reagent. Mixed well and incubated for 1 minute at 37□C. The change in absorbance was measured per minute for 3 minutes at 340 nm. The LDH activity was expressed in U/L [74].

**Preparation of liver homogenate**

The liver tissue excised was washed immediately with ice-cold saline to remove blood. A 10% w/v tissue homogenate was prepared in ice-cold potassium phosphate buffer (100 mM, pH 7.4) followed by centrifugation at 5000 rpm for 10 min. The supernatant was collected and used for the estimation of lipid peroxidation and enzymatic and non-enzymatic antioxidants.

**Biochemical parameters for liver tissue homogenate**

- Estimation of total protein (TP)

Estimation of lipid peroxidation

- Estimation of malondialdehyde (MDA)

Estimation of enzymatic antioxidants

- Assay of superoxide dismutase (SOD)
- Assay of catalase (CAT)
- Assay of glutathione peroxidase (GPx)
- Assay of glutathione reductase (GSSH)

Estimation of non-enzymatic antioxidants

- Assay of reduced glutathione (GSH)

**Estimation of total protein content:**

**Chemicals and reagents**

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

**Procedure:**

The amount of total protein in the tissue homogenate was estimated by the method of Lowry (1951) using bovine serum albumin as the standard. To 0.1ml of tissue homogenate, 4.0 ml of alkaline copper solution was added and allowed to stand for 10 min. Then, 0.4 ml of phenol reagent was added very rapidly, mixed quickly and incubated in room temperature for 30 min for colour development. Reading was taken against blank prepared with distilled water at 610 nm in UV-visible spectrophotometer. The protein content was calculated from standard curve prepared with bovine serum albumin and results were expressed as μg/mg wet tissue [75].

**ESTIMATION OF LIPID PEROXIDATION**

**Estimation of malondialdehyde (MDA):**

**Chemicals and reagents**

TBA-TCA-HCL reagent

**Procedure**

The level of lipid peroxidation in serum was measured as malondialdehyde (MDA) according to the method of Niehaus and Samuelson, 1986. About 0.1 ml of the tissue homogenate was combined with 2 ml of TCA-TBA-HCl reagent (1:1:1) (15% trichloro acetic acid (TCA) and 0.375% thiobarbituric acid (TBA) in 0.25 N HCl) and placed in water bath for 15 min, cooled and centrifuged at 100 rpm for 10 min. The precipitate was removed after cooling by centrifugation at 1000 rpm for 10 min. The absorbance of clear supernatant was measured against a reference blank at 535 nm. The levels of MDA were calculated using extinction coefficient calculation. The values are expressed as nmoles of MDA formed/min/mg protein [76].

**ESTIMATION OF ENZYMATIC ANTIOXIDANTS**

**Assay of superoxide dismutase (SOD):**

**Chemicals and reagents**

Carbonate buffer

Epinephrine

**Procedure**

The activity of SOD was determined according to the method of Kakkar, 1984. To 150 µL of liver homogenate, 1.8 ml of carbonate buffer (30 mM, pH 10.2), 0.7 ml of distilled water and 400 µL of epinephrine (45mM) were added and mixed well. The inhibition of autocatalyzed adrenochrome formation in the presence of liver tissue homogenate was measured at 480 nm using a spectrophotometer. Autoxidation of epinephrine to adrenochrome was performed in a control tube without the homogenate. SOD activity was expressed as nmoles/min/mg protein [77].

**Assay of catalase (CAT):**

**Chemicals and reagents**

Hydrogen peroxide

Dichromate-acetic acid reagent

**Procedure**

The assay of CAT was done by the method of Sinha, 1972. The reaction mixture contained 1.0 ml of 0.01 M phosphate buffer pH 7 and 0.1 ml of tissue homogenate and was incubated at 37□C for 15 min. The reaction was started by the addition of 0.4 ml of $H_2O_2$. The reaction is stopped by the addition of 2.0 mL dichromate acetic acid reagent (5% potassium dichromate and glacial acetic acid are mixed in 1:3). The absorbance was measured at 620 nm. CAT activity was expressed as the amount of enzyme using the decomposition of µmoles $H_2O_2$/min/mg protein [78].

**Assay of glutathione peroxidase (GPx):**

**Chemicals and reagents**

Tris-HCL buffer

Sodium azide

Hydrogen peroxide

Trichloro acetic acid

**Procedure**

GPx activity was measured by the procedure given by Paglia and Valentine, 1967. About 0.2 ml of the liver homogenate was mixed with 0.2 ml of 0.4 M Tris-buffer pH 7.0, 0.1 ml of

10 mM sodium azide, 0.1 ml of 0.042 % $H_2O_2$ and 0.2 mL of 200 mM glutathione and was incubated at 37 □C for 10 min. The reaction was stopped by the addition of 0.1 ml 10% trichloroacetic acid and the absorbance was measured at 340 nm. GPx activity was expressed as nmoles/min/mg protein [79].

**Assay of glutathione reductase (GSSH):**

**Chemicals and reagents**

Potassium phosphate buffer

Bovine serum albumin

**Procedure**

The activity of GSSH in the tissue was determined by the method of Racker, 1955. The reaction mixture contained 2.1 ml of 0.25 mM potassium phosphate buffer pH 7.6, 0.1 ml of 0.001 M NADPH, 0.2 ml of 0.0165 M oxidized glutathione and 0.1 ml of bovine serum albumin (10 mg/ml). The reaction was started by the addition of 0.02 ml of tissue homogenate with mixing and the decrease in absorbance at 340 nm was measured for 3 min against a blank. GSSH activity was expressed as nmoles/min/mg protein [80].

**ESTIMATION OF NON-ENZYMATIC ANTIOXIDANTS**

**Assay of reduced glutathione (GSH):**

The activity of GSH was determined by Ellman's method. About 1.0 ml of tissue homogenate was treated with 0.5 ml of Ellman's reagent (19.8 mg of 5, 5'- Dithiobis-(2-Nitro benzoic acid) [DTNB] in 100 ml of 0.1 % sodium citrate) and 3.0 mL of phosphate buffer (0.2 M, pH-8). The absorbance was read at 412 nm using a spectrophotometer. GSH activity was expressed as nmoles/min/mg protein [80].

**Histopathological evaluation of both the extracts against ethanol induced hepatotoxicity**

The liver specimens obtained from the control and treated groups of animals were fixed in 10% buffered formalin for 24 h. The formalin fixed liver samples were stained with haematoxylin eosin for photo microscopic observations of the liver histopathological architecture.

**Statistical analysis**

Quantitative data were expressed as mean ± SEM and all the comparisons were made of oneway analysis of variance (ANOVA) followed by Dunnett's test of $P < 0.05$ were considered as significant.

# Results

Table 1: Phytochemical screening of the various extracts of flowers of *Caryota urens* L.

The preliminary phytochemical screening of active constituents was carried out for various extracts of flowers of *Caryota urens* L.

| **Phytoconstituents** | **AECU** | **EECU** |
|---|---|---|
| A. Alkaloids | – | + |
| a. Mayer's test | – | + |
| b. Wagner's test | – | + |
| c. Dragendorf's test | – | + |
| d. Hager's test | | |
| B. Flavonoids | + | + |
| a. Shinoda test | + | + |
| b. Ferric Chloride test | + | + |
| c. Mineral Acid test | + | + |
| d. Lead-Acetate test | | |
| C. Glycosides | + | + |
| a. Modified Borntrager's test | + | + |
| b. Legal's test | + | + |
| c. Balget's test | | |
| D. Carbohydrates | + | + |

| | | |
|---|---|---|
| a. Molisch's test<br>b. Benedict's test | + | + |
| c. Fehling's test | + | + |
| d. Barford's test | + | + |
| E. Steroids and Terpenoids<br>a. Liberman-Burchard's test | + | – |
| b. Salkowski's test | + | – |
| F. Saponins<br>a. Foam test | – | + |
| G. Tannins & Phenolics<br>a. Ferric Chloride test | + | + |
| b. Lead Acetate test | + | + |
| c. Gelatin test | + | + |
| H. Proteins<br>a. Millions test | – | – |
| b. Biuret's test | – | – |
| c. Ninhydrin test | – | – |

**Table 2: *In vitro* hepatoprotective activity of various extract of flowers of *Caryota urens* L by MTT method**

| Treatment | Concentration µg/ml | | | | | Half maximal inhibitory concentration |
|---|---|---|---|---|---|---|
| | 6.25 | 12.5 | 25 | 50 | 100 | |
| **Silymarin** | 0.1784 ± 0.001401 | 0.2305± 0.001613 | 0.2767± 0.00312 | 0.358433± 0.006263 | 0.37303± 0.002095 | 9.133± 0.433 |
| **Percentage viability** | 42.43±0.44 | 55.69±0.6755 | 66.8±0.7506 | 86.57±1.532 | 90.09±0.51 | |
| **AECU** | 0.1643±0.0024 | 0.18926±0.0029 | 0.2879±0.008026 | 0.3306±0.0107 | 0.3602±0.0042 | 9.8± 0.4359 |
| **Percentage viability** | 39.14±0.78 | 45.72±0.72 | 69.55±1.94 | 79.87±2.58 | 87.02±1.01 | |
| **EECU** | 0.1587±0.0013 | 0.16623±0.00154 | 0.1741±0.00386 | 0.20126±0.00925 | 0.3033±0.00266 | 18.667± 3.283 |
| **Percentage viability** | 38.34±0.32 | 39.59±0.55 | 41.99±0.99 | 48.62±2.24 | 71.26±1.61 | |

All determinations were carried out in triplicate manner and values are expressed as the mean ± SEM (n=3)

*In vitro* hepatoprotective activity of various extract of flowers of *Caryota urens* L by MTT method

The treatment with ethanol showed loss of viability of cells as measured by this assay (47.84 ± 0.3118 %). The treatment with AECU showed increase in cell viability in a dose dependent manner. AECU at a concentration of 100 µg/ml showed highest protection (87.02±1.01 %) and showed least protection (39.14±0.78 %) at a concentration of 6.25 µg/ml. The EC50 value of the extract in cell viability was found to be as 9.8± 0.4359µg/ml. Treatment with EECU showed increased in cell viability in a dose dependent manner. EECU at a concentration of 100 µg/ml showed protection (71.26±1.61%) and showed least protection (38.34±0.32 %) at a concentration of 6.25 µg/ml. The EC50 value of the extract in cell viability was found to be as 18.667±3.283 µg/ml. Table 2.

**Table 3: Effect of AECU and EECU on serum biochemical parameters in control and experimental animals**

| GROUPS | ALT (U/L) | AST (U/L) | ALP (U/L) | LDH ($\mu$ moles of pyruvate lib/min) |
|---|---|---|---|---|
| **Control** (0.5% CMC) | 1156.898±1.277 | 1174.36±0.5241 | 1127.72±6.018 | 362.8081±6.800 |
| **Negative control** 40% v/v ethanol (2ml/100g bw p.o.) | 3510.612±8.028# | 3528.774±0.4181# | 3861.22±16.59# | 860.9801±8.261# |
| **AECU- Low dose** (100mg/kg) | 1629.654±0.452** | 1647.455±4.520** | 1798.112±2.993** | 530.03588±6.817** |
| **AECU- High dose** (200mg/kg) | 1665.638±1.565** | 1984.064±10.369** | 1933.522±1.615** | 569.204±1.772** |
| **EECU - Low dose** (100mg/kg) | 2173.55±22.267** | 3151.4383±1.465** | 3090.36±9.461** | 639.82±1.018** |
| **EECU- High dose** (200mg/kg) | 1859.44±0.9813** | 2685.9883±3.430** | 2605.76±1.615** | 613.6±0.4137** |
| **STANDARD –** Silymarin (100mg/kg) | 1317.558±1.756** | 1321.4885±4.291** | 1388.10±1.845** | 446.53±2.229** |

Each value represents the Mean ± SEM, n=6

# P < 0.01 vs Control,

In treatment groups **P < 0.01 compared to negative control.

Data were analysed by One Way ANOVA followed by Dunnett's test.

**Effect of AECU and EECU of flowers of *Caryota urens* L. on serum biochemical parameters in control and experimental animals**

The flowers of AECU and EECU were evaluated for serum biochemical parameters in control and experimental animals. The serum enzymes levels (viz. ALT, AST and ALP) were significantly ($P<0.01$) increased in the ethanol treated group when compared to control. Pre-treatment with both the extracts for 42 days significantly ($P<0.01$) reduced the serum enzyme levels when compared to negative control. There was a dose dependent decrease in the normal enzyme levels. The activity produced by the plant extract was compared to that of standard silymarin treated group. Table 3.

**Table 4: Effect of various extracts of flowers of *Caryota urens* L. on tissue protein in control and experimental animals**

| **GROUPS** | **TP (μmol/ml)** | **MDA (μmol/g tissue protein)** |
|---|---|---|
| **Control** (0.5% CMC) | 562.772±11.73 | 2.4874± 0.0582 |
| **Negative control** 40% v/v ethanol (2ml/100g) | 231.646±3.098# | 41.0931±0.3508# |
| **AECU- Low dose** (100mg/kg) | 345.648±8.449 ** | 15.5158±0.3678** |
| **AECU- High dose** (200mg/kg) | 424.196±9.265** | 10.1398±0.1352** |
| **EECU- Low dose** (100mg/kg) | 263.52±4.910* | 25.0589±1.369** |
| **EECU- High dose** (200mg/kg) | 307.05±4.851** | 18.3472±0.3060** |
| **STANDARD** – Silymarin (100mg/kg) | 443.3998±4.601** | 5.3497±0.08911** |

Each value represents the Mean ± SEM, n=6

# P < 0.01 vs Control,

In treatment groups, **P < 0.01, *P < 0.05 compared to negative control.

Data were analysed by One Way ANOVA followed by Dunnett's test.

**Effect of AECU and EECU of flowers of *Caryota urens* L. on tissue protein in control and experimental animals**

Total protein level was significantly (P<0.01) reduced in ethanol induced group when compared to control. Pre-treatment of animals with the plant extracts AECU and EECU for 42 days significantly (P<0.01 and 0.05) elevated the protein level when compared to negative control. The protein level was significantly (P<0.01) elevated when animals were pre-treated with plant extracts at higher doses (200mg/kg) for 42 days when compared to negative control. The activity produced by the plant extract was compared to that of standard silymarin treated group. Table 4.

The MDA level was significantly (P<0.01) increased in ethanol treated group (Group II) than measured in the control group (Group I). Pre-treatment with plant extracts AECU and EECU decreased the elevated MDA levels. The MDA level for standard silymarin was also found to be significantly decreased. Table 4.

**Table 5: Effect of AESA and EESA on enzymatic and non-enzymatic antioxidants in control and experimental animals***

| GROUPS | CAT (units/mg liver protein) | SOD (units/mg liver protein) | GSSH | GPx (units/mg liver protein) | GSH (μmoles of GSH/g wet tissue) |
|---|---|---|---|---|---|
| **Control** (0.5% CMC) | 565.51± 24.09 | 1486.34±30.42 | 303.71±9.467 | 969.72±6.81 | 1663.17±18.77 |
| **Negative control** 40% v/v ethanol (2ml/100g) | 148.19±8.497# | 564.14±10.90# | 99.89±6.0.659# | 534.40±11.93# | 533.25±12.17# |
| **AECU- Low dose** (100mg/kg) | 279.16±13.471** | 1063.35±7.72** | 233.05±6.214** | 834.63±13.42** | 1059.27±33.92** |
| **AECU- High dose** (200mg/kg) | 381.75±212.492** | 1137.10±92.37** | 270.22±7.801** | 874.82±7.426** | 1284.24±37.39** |
| **EECU- Low dose** (100mg/kg) | 229.36±8.391** | 988.378±3.639** | 200.56±0.7060** | 631.54±8.723** | 766.22±20.63** |
| **EECU High dose** (200mg/kg) | 322.72±9.015** | 1012..266±6.930** | 209.97±3.368** | 682.65±15.41** | 861.34±16.06** |
| **STANDARD** – Silymarin (100mg/kg) | 492.63±22.41** | 1288.47±20** | 257.10±6.440** | 930.92±5.61** | 1384.81±27.82** |

Each value represents the Mean ± SEM, n=6

# P < 0.01 vs Control,

In treatment groups, **P < 0.01 compared to negative control

Data were analysed by One Way ANOVA followed by Dunnett's test

**Effect of AECU and EECU of flowers of *Caryota urens* L. on enzymatic and non-enzymatic antioxidants in control and experimental animals**

The levels of tissue enzymatic antioxidants namely, SOD, CAT, GPx, GSSH, and the non-enzymatic antioxidants GSH in ethanol treated group was found to be significantly ($P<0.01$) reduced when compared to control. The levels of tissue enzymatic and non-enzymatic antioxidants were significantly ($P<0.01$) increased in the groups treated with plant extracts for 42 days when compared to negative control. Pre-treatment of animals with the higher doses of AECU and EECU (200mg/kg) for 42days significantly ($P<0.01$) increased the enzymatic and non-enzymatic antioxidants levels when compared to negative control. The activity produced by the plant extracts as similar to the standard group. Table 5.

**Table 6: Molecular properties of phytoconstituents of flowers of *Caryota urens* L. by Lipinski parameters**

| Phytoconstituents | MiLogP | Mol.wt | nON(H-aceptors) | Nohnh(H-donors) | Violations |
|---|---|---|---|---|---|
| **Cyclopentanone** | 0.89 | 84.12 | 1 | 0 | 0 |
| **Phenol** | 1.46 | 94.11 | 1 | 1 | 0 |
| **(2E,6E)-Farnesol** | 5.05 | 222.37 | 1 | 1 | 0 |
| **Phthalic acid** | 1.03 | 166.13 | 4 | 2 | 0 |
| **Pyrogallol 1,3-dimethyl ether** | 1.34 | 154.16 | 3 | 1 | 0 |

Lipinski's rule is widely used to determine molecular properties that are important for drug's pharmacokinetic *in vivo*. According to Lipinski's rule of five (Ro5), a candidate molecule is more likely to be orally active if: a) the molecular weight is below 500, b) the calculated octanol/water partition coefficient (log P) is less than 5, c) there are not more than 5 hydrogen bond donors (OH and NH groups), d) there are not more than 10 hydrogen bond acceptors (notably N and O). It was found that among the 5 phytoconstituents used for docking studies, satisfied Lipinski parameters. Table 6.

**Table 7: Predicted bioactivity score of the phytoconstituents of flowers of *Caryota urens* L.**

| Phytoconstituents | GPCR ligand | Ion channel modulator | Kinase inhibitor | Nuclear receptor ligand | Protease inhibitor | Enzyme inhibitor |
|---|---|---|---|---|---|---|
| **Cyclopentanone** | -3.58 | -3.42 | -3.79 | -3.47 | -3.38 | -3.29 |
| **Phenol** | -3.47 | -3.16 | -3.51 | -3.25 | -3.56 | -3.26 |
| **(2E,6E)-Farnesol** | -0.13 | 0.22 | -0.60 | 0.20 | -0.43 | 0.42 |
| **Phthalic acid** | -0.74 | -0.25 | -0.92 | -0.68 | -0.87 | -0.30 |
| **Pyrogallol 1,3-dimethyl ether** | -1.04 | -0.45 | -0.97 | -1.10 | -1.25 | -0.48 |

The GPCR ligand activity phytoconstituents of flowers of *Caryota urens* L. were within the range -3.58to -0.13 indicate their moderate bioactivity score. The ion channel modulatory effects were found to be moderate within a range -3.42 to 0.22. Predicted kinase inhibitory values were found to be in the range between -3.79to -0.60, indicating their moderate bioactivity score. Biological nuclear receptor ligand activity was found to be in range between -3.47 to 0.20. Protease inhibitory (-3.56 to -0.43) and enzyme inhibitory (-3.29 to -0.42) actions predicted was found to be moderate. Table 7.

**Table 8: *In silico* docking of phytoconstituents of flowers of *Caryota urens* L. against CYP2E1 enzymes.**

| **CYCLOPENTANONE** | | | |
|---|---|---|---|
| **Enzyme** | **Binding energy (□G kcal/mol)** | **Inhibition constant (µM)** | **Intermolecular energy (kcal/ mol)** |
| CYP2E1 | -4.28 | 726.73 | -4.28 |
| **PHENOL** | | | |
| CYP2E1 | -4.28 | 727.64 | -4.58 |
| **(2E,6E)-FARNESOL** | | | |
| CYP2E1 | -6.07 | 35.8 | 8.45 |
| **PHTHALIC ACID** | | | |
| CYP2E1 | -5.79 | 57.33 | -6.98 |
| **PYROGALLOL 1,3-DIMETHYL ETHER** | | | |
| CYP2E1 | -4.9 | 256.83 | -5.76 |
| **SILYMARIN** | | | |
| CYP2E1 | -6.29 | 24.64 | -8.97 |

All phytoconstituents (2E,6E)-Farnesol shows excellent properties was found to possess excellent binding energy(ΔG) (-6.07), inhibition constant (35.8µM) and intermolecular energy (8.45 kcal/ mol). Based on the docking analysis, the flowers of *Caryota urens*. L have the potential for hepatoprotective activity by inhibiting CYP2E1. All the selected constituents had similar binding energy when compared to the standard silymarin (-6.29). This proves that constituents consist of potential activity against the selected enzyme CYP2E1 when compared to the standard silymarin. Table 8.

**Table 9: Docking orientations of selected phytoconstituents of flowers of *Caryota urens* L. with the enzyme CYP2E1**

| **Phytoconstituents** | **Binding interaction with amino acid residue** |
|---|---|
| **Cyclopentanone** | LEU363, GLN358, PRO429 |
| **Phenol** | PHE153,ASP190, TYR191, ALA157 |
| **(2E,6E)-Farnesol** | PHE430,CYS437,ARG126,ILE114, ARG435, VAL364,ASN387, LEU368, ARG100 |
| **Phthalic acid** | LYS123,ARG126,ARG127,LYS434 |
| **Pyrogallol 1,3-dimethyl ether** | PHE427,PHE430,ARG444,GLU440,LYS428,SER431 |
| **Silymarin** | THR362,PRO473,SER472,LEU471,PHE360,TYR398 |

**Binding sites of selected compounds with CYP2E1enzyme**

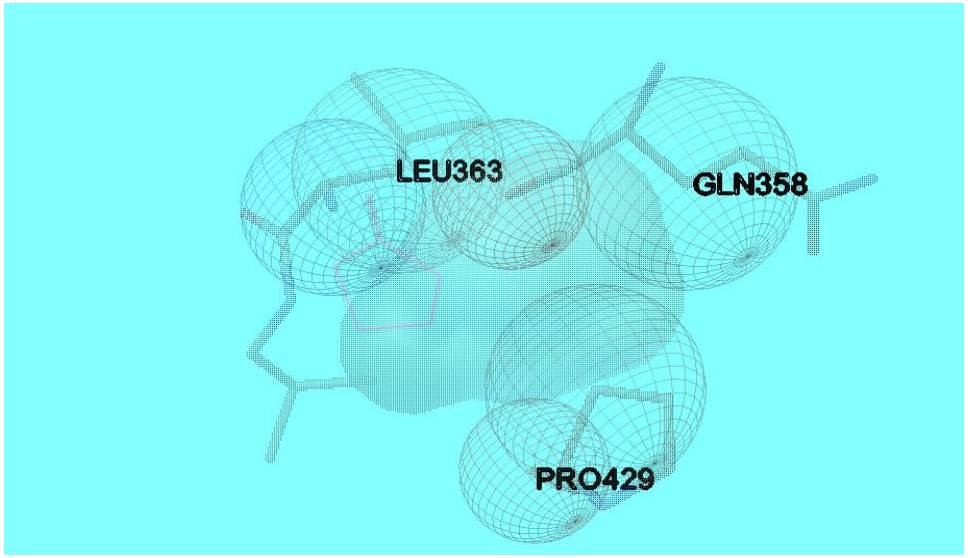


**Fig 7:Cyclopentanone against CYP2E1**

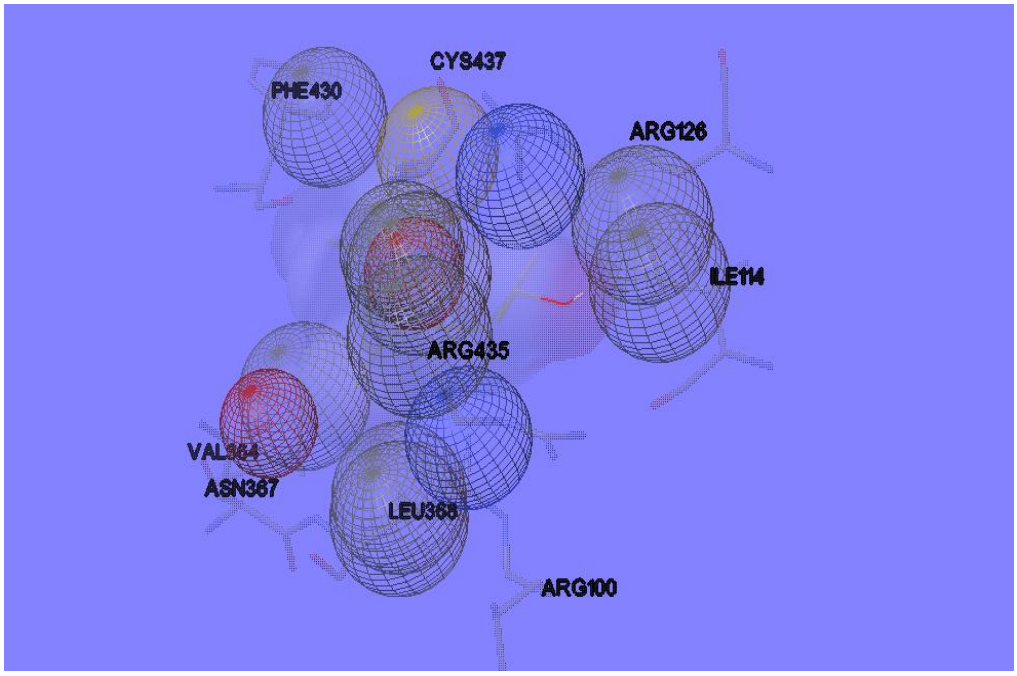


**Fig 8: Farnesol against CYP2E1**

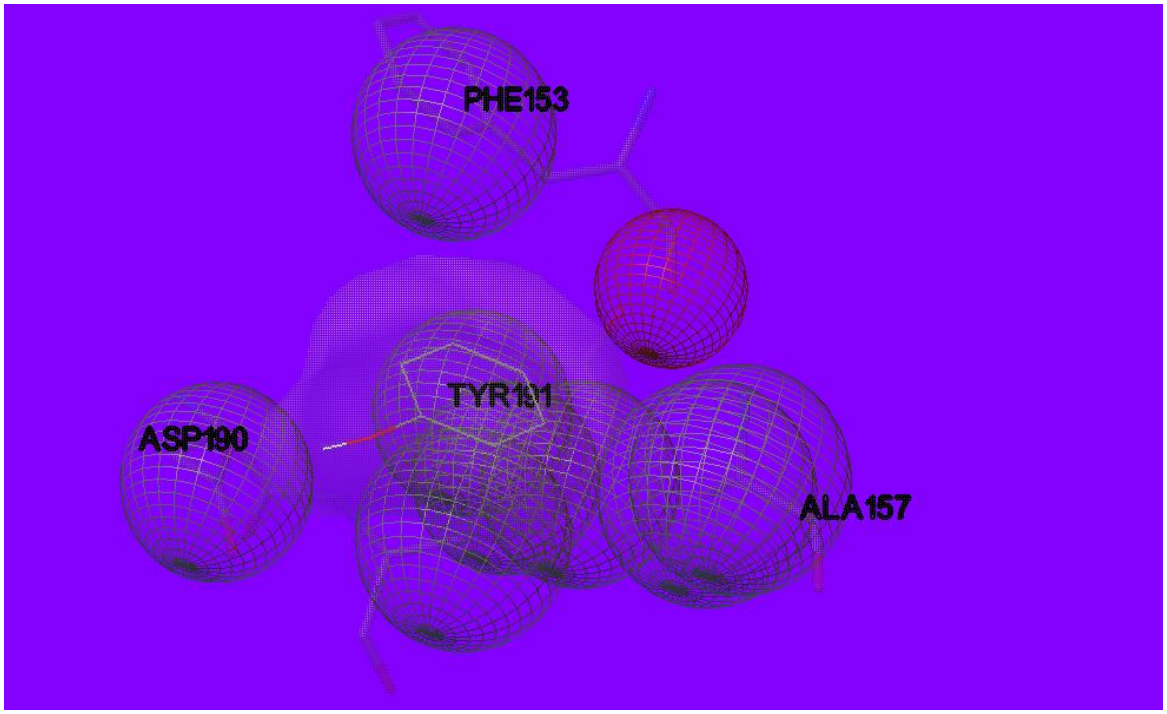


**Fig 9:Phenol against CYP2E1**

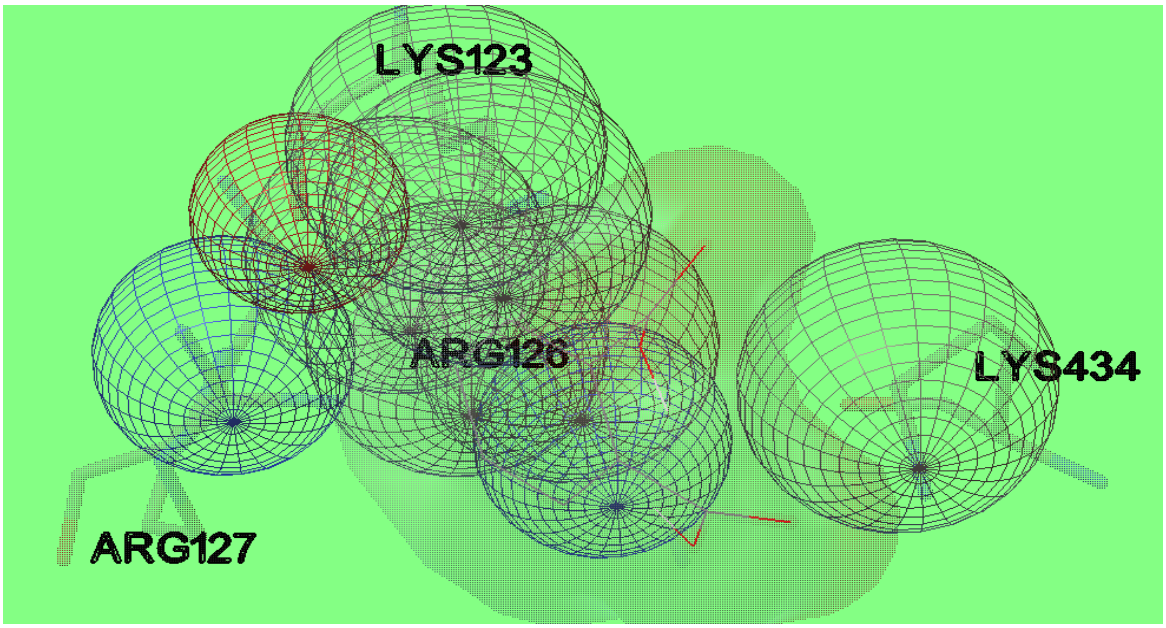


**Fig10: Phthalic acid against CYP2E1**

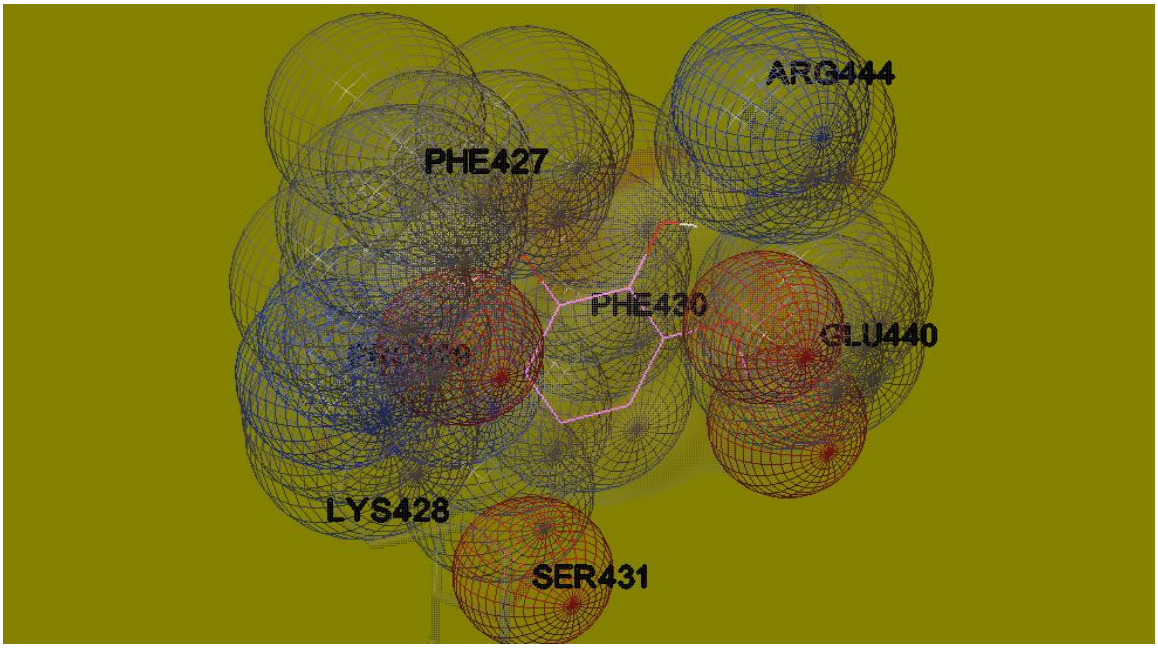


**Fig 11:Pyrogallol against CYP2E1**

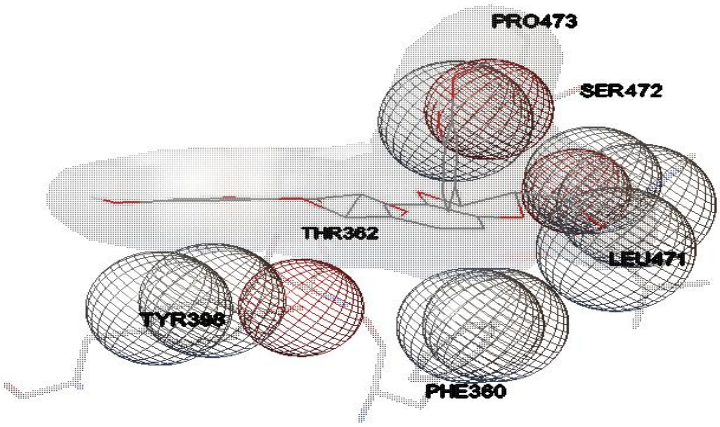


**Fig 12:Silymarin against CYP2E1**

**Table 10: PreADMET properties of selected phytoconstituents of flowers of L. *Caryota urens***

| Phytoconstituents | BBB | Buffer solubility mg/L | CaCo2 | CYP 2C19 inhibition | CYP2C9 inhibition | CYP2D6 inhibition | CYP2D6 substrate | CYP3A 4 inhibit ion | CYP3A4 substrate | HIA | MDCK | Plasma protein | Pure water solubility | Skin permeability | SKlogP Value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cyclopentanone** | 0.84591 | 17974.1 | 24.3716 | Inhibitor | inhibitor | Non | Non | inhibitor | weakly | 100 | 123.592 | 81.820699 | 13172.5 | -3.09696 | 0.843420 |
| **Phenol** | 1.66761 | 6123.63 | 21.6871 | 1nhibitor | inhibitor | Non | Non | inhibitor | non | 100 | 67.6023 | 89.710895 | 14380.3 | -2.38046 | 1.380310 |
| **(2E,6E)-Farnesol** | 13.6863 | 3.29364 | 31.9961 | Inhibitor | inhibitor | Non | Non | inhibitor | substrate | 100 | 85.6893 | 100.000000 | 27.7853 | -0.627479 | 5.167270 |
| **Phthalic acid** | 0.519934 | 53249.5 | 20.3475 | Inhibitor | inhibitor | Non | Non | non | non | 81.584502 | 29.0222 | 49.198454 | 4718.74 | -2.10851 | 1.572300 |
| **Pyrogallol 1,3-dimethyl ether** | 0.618765 | 84884.1 | 38.6698 | Inhibitor | inhibitor | non | Non | Inhibitor | non | 93.879152 | 367.26 | 95.701221 | 13217.4 | -1.95798 | 1.685280 |

Phytoconstituents exhibited optimum ADMET values. The leads were found to have good oral absorption, intestinal absorption, solubility and less interaction. Hence the AECU and EECU may show good oral bioavailability. Table 10.

# Discussion

# DISCUSSION

The liver performs many functions vital to the health of the organism. It transforms and excretes many drugs and toxins. These substances are frequently converted into inactive forms by reactions that occur in the hepatocytes. Certain enzymes in the endoplasmic reticulum catalyse the conjugation of many compounds. Transformations that occur in the liver render many drugs water-soluble and they are readily excreted by kidneys. The liver is the target organ for toxic drug-induced lesions. The physiological response to injury results in a variety of lesions such as necrosis, cholestasis, steatosis, inflammation and fibrosis. Ethanol induced hepatotoxicity serves as an excellent model to study the molecular, cellular and morphological changes in the liver.

Traditionally plants have been used in a folk medicine against different types of diseases [85]. Experimental research works on several plants has been carried out to evaluate their efficacy against chemically induced toxicity [82, 83, 84]. The therapeutic potential of plant and animal origin are being used since ancient times by simple process without isolation of pure compounds in the form of crude drugs. Based on the nature of its constituents the pharmacological actions of crude drugs were determined. World Health Organization (WHO) encourages, recommends and promotes traditional/herbal remedies in health care because these drugs are easily available at low cost and safe for the welfare of human health [86,87].

Carbohydrates, proteins and fats in spite of acting as a biosynthetic laboratory for chemical compounds, also acts as food for humans and they are used for a multitude of compounds including alkaloids, flavonoids and glycosides etc. which exert definite pharmacological activity. The plant materials by using suitable solvents are extracted to acquire the desired components and the resulting principle being employed as therapeutic agents to obtain the pharmacologic activities. Phytochemical (from the Greek word ‘phyto’, meaning plant) are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans [88].

The largest category of phytochemicals and the most widely distributed in plant kingdom are the phenolic compounds. The three most important groups of dietary phenolics are flavonoids, phenolic acids, and polyphenols. Phenolic compounds are large and complex group of chemical constituents present in plants.

In our study, the preliminary phytochemical screening of active constituents was carried out for aqueous and ethanol extracts of *Caryota urens* L. The presence of flavonoids, steroids, terpenoids, carbohydrates, tannins, phenolics and glycosides were revealed in aqueous extract of *Caryota urens L*. In ethanol extract of *Caryota urens* L. carbohydrates, glycosides, flavonoids, saponins, alkaloids, tannins and phenolics were revealed. Table 1.

MTT assay was carried out in Hep G2 cell lines to determine the protective effect on cell viability by the extract against paracetamol induced toxic effects. Tetrazolium salt (3-(4, 5 dimethylthiazole –2 yl)-2, 5 diphenyl tetrazolium bromide) is taken up into the cells and reduced in a mitochondria dependent reaction to yield a blue coloured formazan product. The product accumulates within the cell, due to the fact that it cannot pass through the plasma

membrane. On solubilisation of the cells, the product is liberated and can be readily detected and quantified by simple colorimeric method. The ability of cells to reduce MTT provides an indication of mitochondrial integrity and activity which in turn may be interpreted as a measure of viability. Determination of their ability to reduce MTT to the formazan derivative after exposure to test compounds compared to the control, enables the relative protection of extract. The results obtained from the study suggested that AECU showed increased in percentage of cell viability with increase in concentration (6.25-100 µg/ml) suggesting that the extract possess a protective effect against the toxicity on cell lines compared to EECU. Cells treated with increasing concentrations of extract showed decreased cellular damage confirming that the extract has a good protective effect against hepatotoxicity [70]. Table2.

Alcoholic hepatitis is inflammation of the liver and can exist as either acute or chronic conditions. Symptoms can vary greatly, from asymptomatic to severe fever, nausea and abdominal pain. Acute hepatitis can often cause death and the chronic form often leads to cirrhosis. The disease is characterized by regenerative nodules of hepatic tissue completely surrounded by fibrous scar tissue. The scar tissue grows faster than liver cells can regenerate and the growing network of scar tissue inhibits blood flow [27]. Ethanol is known to have a profound effect on the metabolism of lipids and lipoproteins. Accumulation of lipids in the hepatocytes is the most striking initial manifestation of alcohol induced liver injury [54].

Ethanol is metabolised in the liver through alcohol dehydrogenase and microsomal ethanol oxidizing system (MEOS) pathway. Ethanol metabolised in to the highly toxic acetaldehyde is then oxidized to acetate by acetaldehyde oxidase giving rise to ROS via cytochrome P4502E1. Prolonged consumption of alcohol increases nitric oxide level which leads to formation of toxic oxidant peroxynitrite. Low capacity of antioxidant in this situation leads to damage of the cells of the hepatic cells and the cell organelles with the release of reactive aldehydes and ROS [38].

The animals treated with ethanol 40% v/v, 2ml/100g (Group II) showed significant ($P<0.01$) increased levels of AST, ALT, ALP and LDH and also significant ($P<0.01$) decrease in total protein levels were observed when compared with normal control group (Group I). Animal groups (III & IV) pre-treated with aqueous extracts of flowers of *Caryota urens* L. AECU at a dose of 100mg and 200mg/kg, orally for 42days reversed the serum level by decreasing the above-mentioned indices like AST, ALT, ALP, LDH and also it showed the increased levels of TP significantly ($P < 0.01$), whereas the animals pre-treated with the ethanolic extract of *Caryota urens* L. (EECU) at a same dose of 100mg and 200mg/kg, orally for 42days significantly ($P<0.01$) decreased and significantly ($P<0.05,0.01$) increased in EECU low and high dose parameters respectively. In addition, animals pre-treated with silymarin, a standard drug for liver disorders also significantly ($P<0.01$) decrease in serum AST, ALT, ALP and LDH levels and increased level in TP significantly ($P<0.01$) in the group II animals.

Ethanol administration in rats disrupted the membrane permeability of the plasma membrane causing leakage of the enzymes from the cells which lead to elevation in levels of serum enzymes AST, ALT, ALP, LDH and decrease in TP levels. Elevated serum enzymes are

indicative of cellular leakage and loss of functional integrity of the cell membrane in liver induced by ethanol administration was significantly($P<0.01$) normalized by AESA and EESA suggesting that hepatoprotective activity which might be due its effect against cellular leakage and loss of functional integrity of the cell membrane in hepatocytes. In our study, ethanol administration showed severe hepatotoxicity with heavy infiltration of inflammatory cells around portal tract and in the liver parenchymal cells. Group of animals pre-treated with AECU and EECU and silymarin for 42days protected the rat livers from ethanol induced hepatotoxicity.

In addition, ethanol contributed to increased oxidative stress and formation of the reactive oxygen species, which are also fatal to the cell and result in hepatocyte necrosis. Further, the oxidative stress causes a misbalance in antioxidant steady state due to generation of increased number of oxidants resulting in cellular damage as manifested by necrosis. Oxidative stress can be induced by toxins, the source for these toxins may be virus, bacteria or a xenobiotic compound and it causes accumulation of reactive oxygen/nitrogen species, by the activation of nitric oxide synthase. SOD, CAT, Gpx, GSSH and GSH are major antioxidant enzymes.

Reduced glutathione (GSH) is a powerful antioxidant that protects cells from oxidative injury by scavenging reactive oxygen/nitrogen species and a homeostatic decrease in the GSH pool can make cells more vulnerable to further damage by toxins. In addition to antioxidant action of GSH, the antioxidant enzymes SOD, GPx and catalase work together to counteract the oxidation of proteins, lipids and DNA, by removing ROS from the cell. SOD is a specific enzyme to reduce superoxide ion into hydrogen peroxide, which is further reduced to water by the action of catalase and glutathione peroxidase. Various response of these enzymes may be in indication to cellular needs in fight against increased levels of reactive oxygen species in induced oxidative stress states. In order to elucidate the protection mechanism of the flower extracts, ethanol induced rat liver was examined for lipid peroxide levels. Reactive oxygen species and particularly free radical induced lipid peroxidative tissue damage has been implicated in pathogenesis of various diseases.

The determination of malondialdehyde (MDA) level is one of the most commonly used methods for monitoring lipid peroxidation. MDA is a three-carbon low molecular weight aldehyde and spontaneous breakdown product of peroxides that can be produced from free radical attack on polyunsaturated fatty acids. Several methods are available for the quantification of MDA in biological samples. TBA is the most frequently used method since it is a very sensitive and reliable method [89]. Our results suggested that there was an increase in lipid peroxidation after ethanol administration and it was inhibited by the treatment with the extracts revealing that the plant extracts exhibit potent hepatoprotective activity.

Measurement of protein concentration is mainly used to calculate the level of purity of a specific protein [75]. Lowry method is commonly used in estimation of protein level. In case of diseased condition levels of protein are lowered due to the degradation of protein by free radical formation [90]. High level dose of ethanol cause depletion of total proteins indicating tissue damage which is also evidenced in our study. Treatment with the AECU and EECU significantly ($P<0.01$, 0.05) increased ethanol induced tissue damage respectively.

The role of GSH as a protective agent against liver injury has been extensively studied [92]. At higher dose of ethanol, the levels of GSH becomes depleted and reversed in groups of animals pre-treated with plant extracts. At higher dose of ethanol, the levels of GSSH becomes depleted and reversed in groups of animals pre-treated with plant extracts. GPx has a major role in degrading low levels of $H_2O_2$ in cells. Since GPx acts on hydroperoxides of unsaturated fatty acids, the enzyme plays an important role in protecting membrane lipids, and thus the cell membranes from oxidative disintegration. Catalase is present in almost all the mammalian cells localized in the peroxisomes or the microperoxisomes. It catalyses the decomposition of H2O2 to water and oxygen and thus protects the cell from oxidative damage by $H_2O_2$ and □OH. The first enzyme which involved in the anti-oxidant defence is superoxide dismutase (SOD), a metalloprotein found in both prokaryotic and eukaryotic cells. The oxygen radicals, generated by interaction of Fe++ and $H_2O_2$ are the species responsible for oxidation of epinephrine at pH 10.2 and was strongly inhibited by superoxide dismutase[91].

In our study, treatment with ethanol significantly ($P<0.01$) depleted hepatic GSH, GSSH, GPx, CAT and SOD enzymes levels. All the plant extracts increased the antioxidant enzyme levels significantly ($P<0.01$) and reduced the ethanol-induced oxidative injury, thus proving its antioxidant potential.

In the present study, *in silico* docking was carried out for the compounds present in flowers of *Caryota urens* L.in comparison with standard Silymarin against CYP2E1 enzyme.

CYP2E1 is a member of cytochrome P450 family, mixed function oxidase system which is involved in the metabolism of xenobiotics in the body. It is largely responsible for the breakdown of foreign compounds in mammals. CYP2E1 is a membrane protein expressed in high levels in the liver, where it composes nearly 50% of the total hepatic cytochrome P450 mRNA and 7% of the hepatic cytochrome P450 protein. The liver is therefore where most drugs undergo deactivation by CYP2E1, either directly or by facilitated excretion from the body [67,68,69]. CYP2E1 metabolizes mostly small, polar molecules. Oxidations by CYP2E1 are often of benefit to the body. Bio activation of certain carcinogens and toxins by CYP2E1, leads to onset of hepatotoxicity caused by certain classes of drugs. The study was carried out to investigate the inhibitory activity of the compounds on hepatotoxicity by molecular docking studies and to analyse the ADMET properties of the compounds such as Cyclopentanone, Phenol, (2E,6E)-Farnesol, Phthalic acid and Pyrogallol 1,3-dimethyl ether was used for docking on CYP2E1 enzyme to confirm the therapeutic effect of the flowers of this plant. In the field of molecular modelling, docking is a method which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex. Knowledge of the preferred orientation in turn may be used to predict the strength of association or binding affinity between two molecules using for example scoring functions. Docking is frequently used to predict the binding orientation of small molecule drug candidates to their protein targets in order to in turn predict the affinity and activity of the small molecule. Hence docking plays an important role in the rational design of drugs.

The bioactivity score and drug likeness properties of the selected phytoconstituents were studied. The calculated values of various parameters of the selected phytoconstituents of flowers of *Caryota urens* L. for drug likeness were calculated and tabulated.

Lipinski's rule is widely used to determine molecular properties that are important for drug's pharmacokinetic *in vivo*. According to Lipinski's rule of five (Ro5), a candidate molecule is more likely to be orally active if: a) the molecular weight is below 500, b) the calculated octanol/water partition coefficient (log P) is less than 5, c) there are not more than 5 hydrogen bond donors (OH and NH groups), d) there are not more than 10 hydrogen bond acceptors (notably N and O). It was found that among the 5 phytoconstituents used for docking studies, satisfied Lipinski parameters tabulated in table 6. It was found that all the 5 phytoconstituents used for docking studies satisfied Lipinski parameters and showed no violation of the rule.

Molinspiration software was also used to predict the bioactivity scores of each derivative. The predicted bioactivity scores of screened compounds for GPCR ligand, ion channel modulator, kinase inhibitor, nuclear receptor ligand, protease inhibitor and enzyme inhibition were studied and tabulated in table 7. As a general rule, larger is the bioactivity score, higher is the probability that investigated compound will be active. Therefore, a molecule having bioactivity score more than 0.00 is most likely to possess considerable biological activities, while values -0.50 to 0.00 are expected to be moderately active and if score is less than -0.50 it is presumed to be inactive. The drug likeliness scores as calculated through molinspiration reveals that the selected compounds satisfy maximum parameters.

The bioactivity score of the selected phytoconstituents is given in Table 7. The GPCR ligand activity phytoconstituents of flowers of *Caryota urens* L. were within the range -3.58to -0.13 indicate their moderate bioactivity score. The ion channel modulatory effects were found to be moderate within a range -3.42 to 0.22. Predicted kinase inhibitory values were found to be in the range between -3.79to -0.60, indicating their moderate bioactivity score. Biological nuclear receptor ligand activity was found to be in range between -3.47 to 0.20. Protease inhibitory (-3.56 to -0.43) and enzyme inhibitory (-3.29 to -0.42) actions predicted was found to be moderate.

The docking results of the selected phytoconstituents against CYP2E1 enzymes are tabulated in Table 8. All phytoconstituents (2E,6E)-Farnesol shows excellent properties was found to possess excellent binding energy(ΔG) (-6.07), inhibition constant (35.8μM) and intermolecular energy (8.45 kcal/ mol). Based on the docking analysis, the flowers of *Caryota urens*. L have potential for hepatoprotective activity by inhibiting CYP2E1. All the selected constituents had similar binding energy when compared to the standard silymarin (-6.29). This proves that constituents consist of potential activity against the selected enzyme CYP2E1 when compared to the standard silymarin.

The binding interactions of the selected phytoconstituents and silymarin is given in table 9. It was found that the phytoconstituents of flowers of *Caryota urens* L. has the similar binding site as that of standard silymarin.

ADMET studies were carried out using PreADMET software for the compounds selected for synthesis and the results were tabulated in Table 10. Numerous in vitro methods have been used in the drug selection process for assessing the intestinal absorption of drug candidates. Among them, Caco2-cell model and MDCK (Madin-Darby canine kidney) cell model has been recommended as a reliable in vitro model for the prediction of oral drug absorption. In absorption, this module provides prediction models for in vitro Caco2-cell and MDCK cell assay. Additionally, in silico HIA (human intestinal absorption) model and skin permeability model can predict and identify potential drug for oral delivery and transdermal delivery. In distribution, BBB (blood brain barrier) penetration can give information of therapeutic drug in the central nervous system (CNS), plasma protein binding model in its disposition and efficacy.

Chemicals which are strongly bound will have more than 90% of protein binding and Chemicals weakly bound will have less than 90% of protein binding. BBB value more than 2.0 shows high absorption to CNS and 2.0 ~ 0.1 shows middle absorption to CNS and less than 0.1shows Low absorption to CNS. For prediction of HIA in PreADMET, chemical structures at pH 7.4 are applied, because HIA is measured by in vivo test. HIA values between 0 ~ 20 % shows poorly absorbed compounds and values between 20 ~ 70 % shows moderately absorbed compounds. Values between 70 ~ 100 % shows well absorbed compounds. For prediction of Caco-2 cell permeability in PreADMET, chemical structures at pH 7.4 are applied, because Caco-2 cell permeability and MDCK cell permeability are measured at about pH 7.4. Caco-2 cells are derived from human colon adenocarcinoma and possess multiple drug transport pathways through the intestinal epithelium. Values less than 4 showed Low permeability and values between 4 ~ 70 shows middle permeability. Caco-2 value between more than 70 shows high permeability.

Phytoconstituents exhibited optimum ADMET values. The leads were found to have good oral absorption, intestinal absorption, solubility and less interaction. Hence the AECU and EECU may show good oral bioavailability. Table 10.

# Summary & Conclusion

# SUMMARY AND CONCLUSION

Liver is one of the most important organs in the human body. It plays a supreme role in the metabolism of xenobiotics, detoxification and excretion of many endogenous and exogenous compounds. However, liver is one of the most frequently injured organs in the body. A number of hepatotoxins such as viruses, bacteria, chemicals, medicines and alcohol target the liver and cause liver injury. The magnitude of liver hepatotoxins is generally measured by the levels of serum enzyme biomarkers and antioxidants. These antioxidants are rich in natural sources of drugs, especially plants. Natural remedies from traditional plants and their derivatives are still used all over the world in one from another as they are effective and safe alternate treatments for hepatotoxicity. In this aspect, plants that were chosen for the study are the flowers of *Caryota urens* L.

The study investigated the *in silico, in vitro* and *in vivo* hepatoprotective activities of flowers *Caryota urens* L. Phytochemical screening of various plant extracts viz, aqueous and ethanol was carried out. *In vitro* hepatoprotective activity was based on the protection of liver cells from ethanol cytotoxicity in MTT assay. The aqueous extract of *Caryota urens* L showed good activity than ethanolic extract. *In vivo* hepatoprotective activity of various extracts of the plant at two different doses (lower and higher) was determined. AECU and EECU at doses of 100mg/kg and 200mg/kg possessed significant increasing ($P<0.01$) hepatoprotective activity.

The results of the present study revealed that the AECU and EECU can protect the liver in a dose dependent manner from damaging effects of ethanol by considerably decreasing the serum marker enzymes. The decreased serum levels of these enzymes were further accompanied by the improvement of liver histology in AESA and EESA at higher doses (200mg/kg) which remarkably exhibited the hepatoprotective effect of flowers Caryota urens L. The presence of active constituents (flavonoids, tannins, triterpenoids and phenolics) in these plants might be responsible for the hepatoprotective activity. Therefore, AECU and EECU at high dose (200mg/kg) proposed to protect the liver against ethanol-induced oxidative damage in rats. The *in vivo* hepatoprotective properties of the plants can consequently propose a liver protection to the population whoever consumes it and prevent liver damage.

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# Annexures

भारत सरकार
GOVERNMENT OF INDIA
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भारतीय वनस्पति सर्वेक्षण
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सं. भा.व.स./द.क्षे.के./No.: BSI/SRC/5/23/2019/Tech. /141

दिनांक/Date: 15 July 2019

## पौधे प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen brought by you for authentication is identified as *Caryota urens* L. - ARECACEAE. The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.

15/07/2019

डॉ सी मुरुगन/Dr. C. Murugan
वैज्ञानिक 'ई' एवं कार्यालय अध्यक्ष /
**Scientist 'E' & Head of Office**
वैज्ञानिक 'ई' एवं कार्यालय अध्यक्ष
SCIENTIST 'E' & HEAD OF OFFICE
भारतीय वनस्पति सर्वेक्षण
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SOUTHERN REGIONAL CENTRE
कोयम्बत्तूर / COIMBATORE - 641 003

सेवा में / To

Ms. Dhanya. R
II M. Pharm. (3rd Semester) Student
Department of Pharmacology
College of Pharmacy
Sri Ramakrishna Institute of Paramedical Sciences
Coimbatore – 641 044

# INSTITUTIONAL ANIMAL ETHICS COMMITTEE

**(CPSCEA Registration # 1559/PO/Re/S/11/CPCSEA)**

## College of Pharmacy

Sri Ramakrishna Institute of Paramedical Sciences

**(Educational Service of M/s SNR Sons Charitable Trust)**

Coimbatore – 641 044.

## IAEC PROTOCOL APPROVAL CERTIFICATE

**Date: 14/09/2019**

**Approval #:1559/PO/Re/S/11/CPCSEA**

**IAEC PROTOCOL#: COPSRIPMS/IAEC/PG/Pharmacology/002/2019-2020**

**IAEC PROTOCOL TITLE:** *In silico, in vitro, in vivo* hepatoprotective activity of extracts of *Caryota urens* flowers against ethanol-induced liver toxicity in rats

Dear Dr. V. Subhadradevi,

This is to certify that above mentioned animal study protocol has been approved in IAEC meeting held on 14/09/2019 with following conditions:

PI : Ms. R. Dhanya

Duration of Study : months/years (From 14/09/2019 to13/09/2020)

Animal Sanctioned : 42 Male *Wistar* Rats

Species : Rats

Strain : *Wistar*

Sex/Age : Male / 12 Weeks

Total No. : 42

It is requested to get prior approval of IAEC in case of any deviation/changes in submitted protocol. Please maintain the Form D & provide the photocopy to IAEC along with project report at defined interval.

**Member Secretary**
**IAEC, COP, SRIPMS**

**Chairman**
**IAEC, COP, SRIPMS**

14/9/19
**Main nominee**
**CPCSEA**
(G. ARUMARASIVAKUMAR)