
**“IN SILICO DOCKING BASED PHARMACOLOGICAL
SCREENING FOR THE HEPATOPROTECTIVE ACTIVITY
OF CUCUMIS MELO AND CUCURBITA PEPO IN
EXPERIMENTAL ANIMALS.”**

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**Accredited ‘A⁺’ Grade by NAAC (3rd Cycle)
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**For the award of the degree of
Doctor of Philosophy
In the Faculty of Pharmacy**

By

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ABBREVIATIONS

ADME	:	Absorption, Distribution, Metabolism, and Excretion
ALD	:	Alcoholic liver disease
ALP	:	Alkaline phosphatase
ALT	:	Alanine transaminase
AST	:	Aspartate aminotransferase
BBB	:	Blood brain barrier
BE	:	Binding Energy
<i>C. melo</i>	:	<i>Cucumis melo</i>
<i>C. pepo</i>	:	<i>Cucurbita pepo</i>
CAT	:	Catalase
Cus	:	Cucurbitacins
CYP-450	:	Cytochrome p450
DMSO	:	Dimethylsulfoxide
DTNB	:	5,5'-dithiobis-(2-nitrobenzoic acid
FDR	:	False discovery rate
FFAs	:	free fatty acids
FLD	:	Fatty liver disease
GSH	:	Glutathione
HBA	:	Hydrogen bond acceptor

HBD	:	Hydrogen bond donor
HCC	:	Hepatocellular carcinoma
HDL	:	High density cholesterol
HFD	:	High Fat Diet
IAEC	:	Institutional Animal Ethics Committee
INH	:	Isoniazid
KEGG	:	Kyoto Encyclopedia of Genes and Genomes
LC-MS	:	Liquid chromatography–mass spectrometry
LDL	:	Low density cholesterol
LPO	:	Lipid peroxidation
MD	:	Molecular dynamics
MTT	:	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
NAFLD	:	Non Alcoholic fatty liver disease
NASH	:	Non-alcoholic steatohepatitis
Pa	:	Probable activity;
Pi	:	Probable inactivity
ROS	:	Reactive oxygen species
SOD	:	Superoxide dismutase
STRING	:	Search Tool for the Retrieval of Interacting Genes/Proteins
TB	:	Tuberculosis

TC	:	Total cholesterol
TGs	:	Triglycerides
THM	:	Traditional herbal medicines
TTD	:	Therapeutic Target Database
VLDL	:	Very Low density cholesterol

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ABSTRACT

Background: *Cucumis melo* and *Cucurbita pepo* widely utilized vegetable fruit and traditionally claimed for hepatoprotective activity. However, their hepatoprotective molecular mechanisms are not yet elucidated. The current study utilized multiple *in silico* approaches to identify the mechanisms of action of bioactive phytochemicals of *C. melo* and *C. pepo* and potential targets of liver cirrhosis, validated through cytotoxic and hepatoprotective effect of both the herbs in HepG2 cell lines and experimental animals. Firstly, *in silico* studies viz., gene set enrichment and network analysis, molecular docking and dynamics were employed. Secondly, *C. melo* and *C. pepo* extract was subjected to LC-MS analysis and cytotoxicity was performed in HepG2 cell line by MTT assay. Thirdly, hepatoprotective activity of extracts and fractions was evaluated on isoniazid and HFD induced liver cirrhosis in rats & mice. Liver function biomarkers were estimated in blood serum.

Results: The result found that 18 phytochemicals from *C. pepo* were identified to modulated 4 main pathways (metabolic pathways, PPAR signalling pathway, insulin resistance, CYP450 and non-alcoholic fatty liver disease). and 16 phytochemicals from *C. melo* to modulate 6 main pathways (AMPK signaling pathway, Pathways in cancer, Non-alcoholic fatty liver disease and Hepatitis C pathways) involved in liver disease Among the predicted targets, NRIH3, HMGCR and FXR were identified as a hub gene within the network. Schottenol and Alpha-Spinasterol formed stable contacts with NRIH3, HMGCR. And Euphol phytochemical formed stable contact with FXR in MD simulation. >80% cell viability was seen at 100 µg/mL of the extracts and fractions of both the drugs. Isoniazid (50mg/kg/day) and HFD (10ml/kg) treatment elevated level of AST, ALT, ALP, Serum bilirubin and Total bilirubin, cholesterol and

antioxidants parameters. whereas, 500mg/kg/day extract and 50mg/kg/day of fraction reversed the Isoniazid and HFD mediated elevated liver biomarkers in rats and mice.

Conclusion: *In silico* combined with experimental study identified molecular mechanism action of *C. pepo* and *C. melo* activity for the management of liver cirrhosis.

Key words: *Cucurbita pepo*; *Cucumis melo*; Hepatoprotective; Network Pharmacology; Molecular docking; Molecular Dynamics; Isoniazid.

INTRODUCTION

Liver diseases causes approximately 2-million mortality annually, worldwide and constitutes 4% of global mortality (equivalent to 1 in every 25 deaths globally). About two thirds of all hepatic-related mortality is seen in males.¹ The majority of fatalities are mostly caused by consequences of cirrhosis and hepatocellular cancer, whereas a lesser number of mortality are attributed to acute hepatitis. The predominant etiologies of cirrhosis on a global scale include viral hepatitis, alcohol use and non-alcoholic fatty liver disease (NAFLD).²⁻³ Repetitive consumption of toxic substances is very likely to trigger damage to the liver, leading to the development of cirrhosis, liver cancer and unexpected failure of the liver.⁴

NAFLD is a most common form hepatitis that affects around 25% of the adult population worldwide. Nevertheless, the prevalence of NAFLD worldwide is uneven and varies based on geographical location, ethnicity, genetic variations and lifestyle variables.⁵ The global prevalence has been calculated to be 32.4% and has shown a rise in recent years.⁶ NAFLD may also occur without adiposity or physiological signs associated with metabolic syndrome. This disorder may occur in persons who have metabolic problems, including resistance to insulin or a higher likelihood of coronary artery disease. The North American population exhibits a prevalence ranging from 24% to 48%. The previous findings indicate that the prevalence in Europe ranges from 23% to 33%. A recent detailed and systematic evaluation found that the frequency of the disease in Asia is between 28% and 32.4%. NAFLD is now the second most common reason for liver transplantation in both the US (accounting for 21% of liver transplants in 2018) and Europe (accounting for 8.4% of liver transplants in 2016).¹ Due to the significant rise in metabolic risk factors worldwide, it is projected that the prevalence of NAFLD would more than double between 2016 and

2030. Metabolic risk factors in kids and teenagers are expected to pose a significant danger to global health in the next decades.⁷⁻⁸

Hepatotoxicity often arises from factors that include viral infections, excessive alcohol intake, NAFLD and drug-induced toxicity, including substances like Paracetamol, Thioacetamide, Isoniazid, antipsychotic medicines, and antidepressant pharmaceuticals.⁹ Also a chronic intake of a high-fat diet (HFD) leads to aberrant lipid buildup in the liver in the form of free fatty acids (FFAs) and triglycerides (TGs). This results in an increase in weight of the liver and triggers oxidative stress, which may lead to the development of steatohepatitis, cirrhosis and cancer.¹⁰ Cholesterol is a primary substance produced by liver cells. When there is an imbalance with regulating cholesterol levels, it affects the metabolism of lipoproteins, the intake of cholesterol from the diet, the production and elimination of cholesterol within the body. This leads to raised concentrations of total cholesterol (TC), low density lipoprotein (LDL) and triglycerides (TG) in the blood, as well as a reduction in high density lipoprotein. These changes are characteristic of dyslipidemia. This dyslipidemic disorder is responsible for causing hepatotoxicity, atherosclerosis, hypertension, stroke, and other related conditions.¹¹ The increased transport of FFAs from the fat cells to the liver leads to the accumulation of FFAs inside the liver, causing a growth of the intrahepatic pool of FFAs in the form of TGs. Excessive fat buildup in the liver causes hepatic lipo-toxicity, resulting in the production of pro-inflammatory cytokines by hepatic cells, the initiation of oxidative stress and the activation of hepatic stellate cells. These processes eventually result in liver inflammation.¹²⁻¹³

Currently, there's no officially approved pharmaceutical treatment for NAFLD. One important aspect is that the suggested approach for treating NAFLD involves

making changes to one's lifestyle, such as reducing calorie consumption and increasing physical exercise. Adopting lifestyle changes may lead to a reduction in body weight, which in turn can have a positive impact on hepatic pathologic syndrome and minimize the accumulation of fat in the liver.¹⁴⁻¹⁵ Traditional herbal medicines (THM) are a key and vital source of natural medicines and herbal products, notably for the production of hepatoprotective drugs.¹⁶ Despite the absence of conclusive evidence from extensive randomized controlled trials (RCTs) validating the therapeutic benefits of THM, a recent survey revealed that 20%–30% of patients in Indonesia use THM for the treatment of various ailments. Furthermore, the utilization of THM has witnessed a surge in certain Asian nations in recent times. Another investigation revealed 20 to 30% use of herbs for the treatment of chronic liver disease as complementary and alternative therapy.¹⁷⁻¹⁸

Cucumis melo, is a vegetable crop grown in India, China, Southeast Asia, Korea and Japan. In Japan, fruits are utilized to make pickles and juice and in India, for curry, chutney, etc. Seeds serve as an important source of oil for cooking. Diverse phytochemicals, including aromatic compounds, polyphenols and phytosterols are extracted from this plant which possess hepatoprotective, antioxidant, anticancer, anticarcinogenic, antidiabetic, antibacterial, and antimutagenic effects.¹⁹ Similarly, *Cucurbita pepo*, commonly referred to as pumpkin, is believed to contain a rich in antioxidants, polyunsaturated fatty acids (PUFA), fibers and possess hepatoprotective and antiatherogenic properties. Pumpkin is abundant in oleic acid, linoleic acid and tocopherols. Pumpkin oil has a significant impact in protecting against liver damage and the harmful effects of oxidative stress and possess diverse mechanism as anti-lipid peroxidation, increased detoxification, and protection towards glutathione reduction.^{20&21} Nevertheless, the specific bioactive

components molecular mechanism have not yet been fully understood. Therefore, this research used network pharmacology, molecular docking and dynamics approaches to trace the molecular mechanisms of phytochemicals for hepatoprotection, together with *in vivo* hepatoprotective experiments, to investigate the hepatoprotective effects of the phytochemicals enriched fraction of raw seeds from *C. melo* and *C. pepo* seeds on animal model.

AIMS AND OBJECTIVES

AIM

To assess the hepatoprotective effect of *Cucumis melo* and *Cucurbita pepo* by *in silico*, *in vitro* and *in vivo* methods

OBJECTIVES OF THE STUDY

1. To predict the molecular mechanism of hepatoprotective action of bioactive phytoconstituents from *C. pepo* and *C. melo* by *in silico*
2. To evaluate hydroalcoholic extract/fraction(s) of *C. pepo* and *C. melo* for hepatoprotective activity in the *in vitro* and *in vivo* models.

REVIEW OF LITERATURE

The liver plays a crucial function in human metabolism. The liver synthesizes and excretes bile, which is held in the gallbladder until it is required and is used for the breakdown and digestion of fatty acids. The liver produces prothrombin and fibrinogen, which are necessary components of blood coagulation, as well as heparin, a mucopolysaccharide sulfuric acid ester that stops blood clotting in the circulatory system. The body converts sugar into glycogen, which is stored until the muscles need energy. At that point, it is released into the bloodstream as glucose.²² The liver performs protein and cholesterol synthesis, as well as the conversion of carbs and proteins into lipids, which are then stored for future use. The liver produces blood proteins and many enzymes essential for digestion and several physiological processes.²³ The liver synthesizes urea by metabolizing proteins, which are derived from carbon dioxide and ammonia. The kidneys ultimately eliminate urea by excretion. Additionally, it retains essential trace minerals like iron and copper, along with Vitamins A, D and B12. The liver plays a crucial role in the detoxification process by converting and eliminating harmful chemicals and waste materials from the body.²⁴ The liver is responsible for managing five primary sources of bodily toxins and waste: toxins derived from food (including residual pesticides and preservatives), toxins from external sources (such as drugs, adulterants, and environmental pollutants), internally generated chemicals like hormones that are no longer required, nitrogen-containing waste resulting from protein recycling and waste produced during energy production.²² The liver metabolizes these poisons and wastes into less toxic compounds, which are subsequently excreted from the body.²⁴

LIVER INJURY

The liver is sensitive to a wide variety of metabolic and chemical changes²⁵ and liver disease process may be primarily linked to various common ailments, such as cancer and extrahepatic infection. Viral (Hepatitis A-E) or hepatotoxic substances, such as medicines, chemicals, and alcohol cause liver injury.²⁶

FIBROSIS

Fibrosis of liver consequences from constant harm to the hepatic-related accumulation of extracellular matrix molecules that is a trademark of several kinds of ongoing hepatic illnesses²⁷ (Figure 1). The fundamental driver of the hepatic fibrosis in developed nations incorporates ongoing hepatitis viral infection, liquor, drug misuse and nonalcoholic steatohepatitis (NASH). Because to the collapse of the hepatic parenchyma and subsequent replacement with collagen-rich tissue, hepatic fibrosis was formerly assumed to be an isolated and irreversible cycle, Cirrhosis, hepatic damage and hypertension are all symptoms of advanced hepatic fibrosis, which frequently necessitates hepatic transplantation.²⁸ Our comprehension of the cellular and molecular mechanisms underlying hepatic fibrosis has significantly progressed. Hepatic stellate cells, portal fibroblasts and bone marrow-derived myofibroblasts have all been recognized as key collagen-producing cells in the damaged liver; further, these cells are initiated by fibrogenic cytokines, for example, serum albumin, F- β 1, angiotensin II and leptin. Reversibility of progressive hepatic fibrosis in patients has been as of late archived, which has invigorated specialists to create antifibrotic drugs.^{27 & 29}

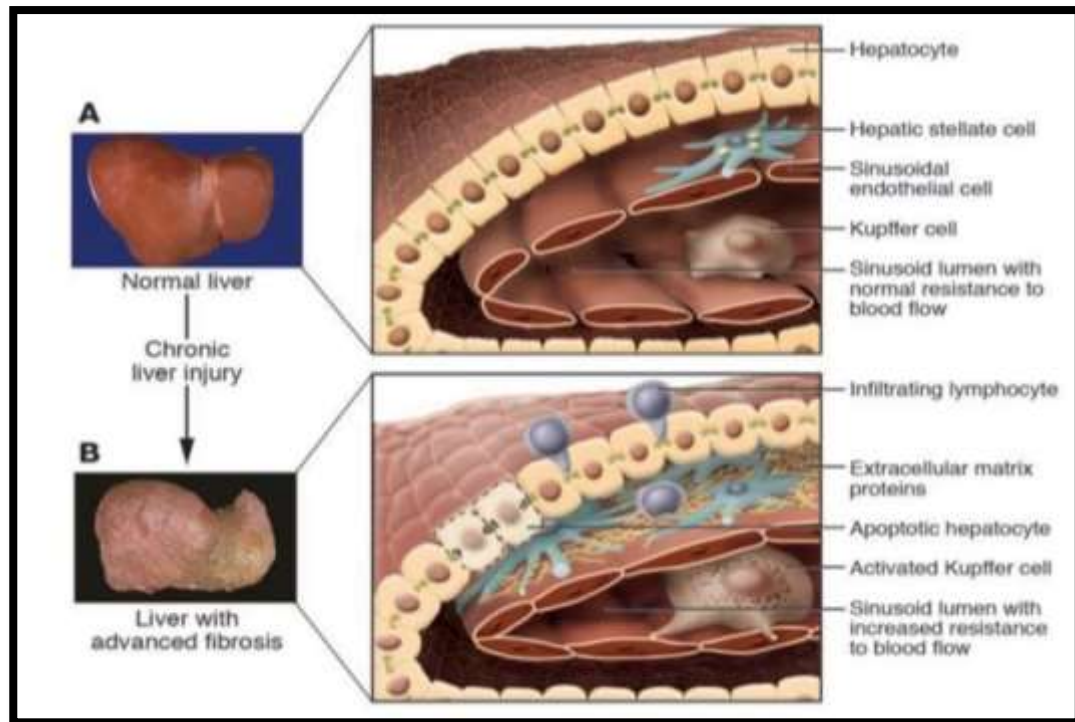


Figure 1: Changes in the hepatic architecture (A) associated with advanced hepatic fibrosis (B).

CIRRHOSIS

Cirrhosis is characterized by the formation of regenerative nodules surrounded by fibrous bands, resulting in increased blood pressure in the liver and advanced liver disease due to long-term liver damage.³⁰ Advancements in our understanding of the natural progression and cause of cirrhosis, together with the management of its complexities, resulted in improved treatment, quality of life, and prospects for individuals with cirrhosis. Cirrhosis represents the advanced stage of liver fibrosis characterized by the presence of hepato vascular distortion (Figure 2).²⁷ It obstructs the connection between the liver sinusoids and the surrounding hepatic parenchyma by redirecting the flow of blood from the gateway and blood artery directly into the liver surge. The liver sinusoids are lined with fenestrated endothelia, which rest on a layer of porous connective tissue that includes hepatic stellate cells

and a few mononuclear cells.³¹ It is crucial to note that bacterial infections are persistent in individuals with portal hypertension, particularly in those with decompensated cirrhosis.³² These patients are also at risk of developing encephalopathy. Therefore, it is imperative for individuals with cirrhosis to exercise caution and get comprehensive antibiotic treatment. The etiology of the cirrhosis-related systemic inflammatory syndrome involves enhanced bacterial migration in the digestive system, compromised immune function and excessive release of pro inflammatory cytokines.³³ The varied rates of cirrhosis progression across individuals with similar risk factors. Recently, there has been an increasing number of identified beneficial genetic variations that are likely to enhance the risk of fibrosis progression. Embroiled genes encode cytokines/chemokines and their receptors,³⁴ molecules associated to fibrogenesis or fibrolysis,³⁵ blood coagulation and several other molecules.³⁶ When any part of the body or organ sustains damage, the body forms a dense tissue known as scar tissue to protect the area. This process is referred to as scarring. Cirrhosis is a condition where scar tissue gradually replaces healthy liver tissue over a period of time. Therefore, the number of healthy liver cells is reduced. Similarly, the flow of blood through the liver is decelerated. Consequently, the liver is unable to perform its functions adequately.³⁷

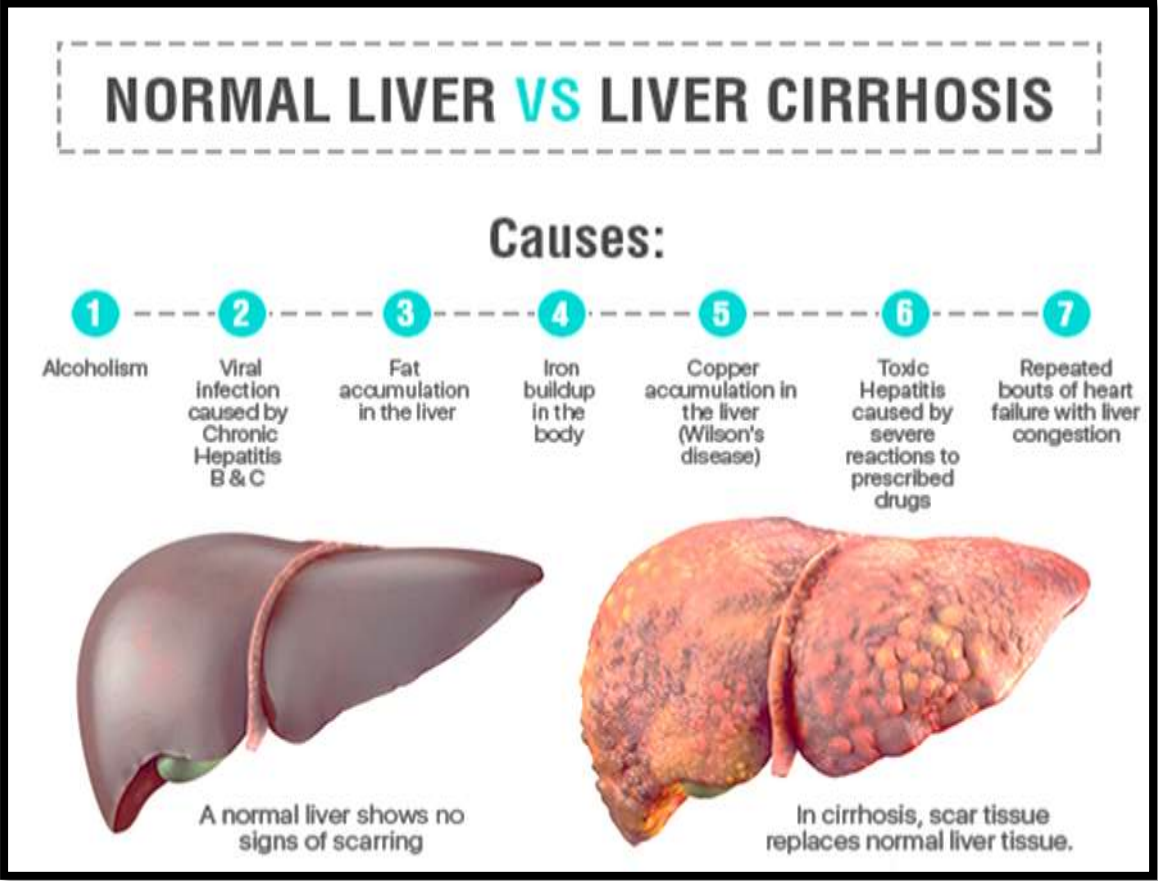


Figure 2: Healthy Liver versus Cirrhosis

NECROSIS

Necrosis, also known as cell death, is a process in which viable cells become nonviable, resulting in the breakdown of the cell components. Under biological conditions, individual debilitated cells are replenished by a well-regulated interaction.³⁸ However, cell death often occurs as a consequence of several types of tissue damage in obsessive conditions. Apoptosis or cell death is a common occurrence in people with severe and chronic liver disease. If the root cause of the disease remains untreated, it may lead to progressive fibrosis.³⁹ Figure 3 illustrates the phases of liver injury, whereas Table 1 outlines the specific patterns of necrosis seen in liver disease.

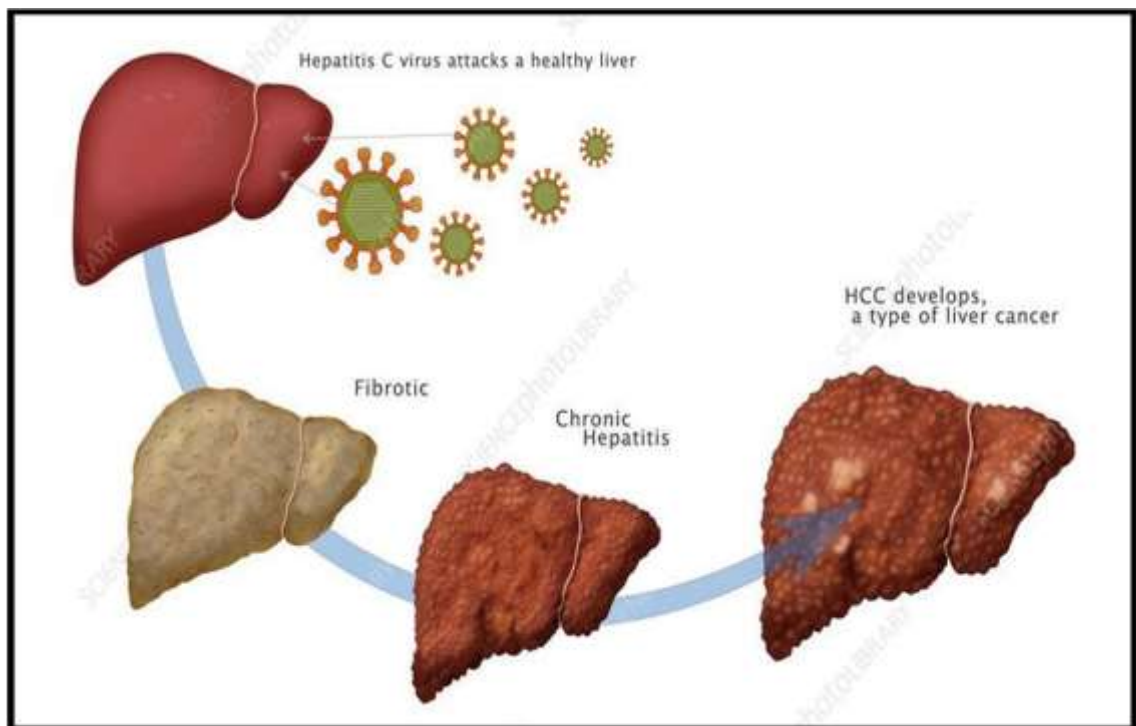


Figure 3: Different stages of liver damage

Table 1: Patterns of necrosis in liver disease.

Pattern of Necrosis	Description	Underlying Cause
Apoptosis (apoptotic body, acidophil body, Councilman body)	Necrosis of single cells	Hepatitis (viral, autoimmune, drug)
Focal/spotty necrosis	Necrosis involving small clusters of hepatocytes	Hepatitis (viral, autoimmune, drug)
Zonal necrosis (Centrilobular) Periportal (IH/piecemeal necrosis)	Necrosis around the central vein (acinar zone 3) Necrosis of periportal zone (acinar zone 1)	Ischemia, drugs, venous outflow impairment Hepatitis (viral, autoimmune, drug)
Confluent necrosis Localized (Multilobular) Necrosis Submassive/ massive necrosis	Necrosis of a part of the liver Subtotal/total hepatic necrosis (often with prominent ductular reaction)	Localized ischemia, transarterial embolization treatment Hepatitis (viral, autoimmune, drug), acute allograft failure, fulminant Wilson's disease

Liver injury due to Virus

Liver disease caused by viruses is still a prevalent and challenging issue. Hepatitis may be induced by several factors such as pharmaceuticals, viral agents, bacterial pathogens, and some types of fungi. The liver is often affected by hepatitis viruses, including hepatitis A-E.⁴⁰⁻⁴¹ Hepatitis caused by Hepatitis B and C are very dangerous illnesses that now lack a recognized and effective treatment. Hepatitis C will gradually emerge as a substantial contributor to death.⁴²

Drug induced liver injury

Substances may have an effect on the liver. Toxic compounds that induce liver damage include anti-tubercular medications (rifampicin, isoniazid), thioacetamide, antibiotics, chemotherapeutics, peroxidized oil, aflatoxins, carbon tetrachloride, chlorinated hydrocarbon acetaminophen and excessive alcohol use.⁴³ The vast majority of medications and chemicals mainly injure liver cells by causing lipid peroxidation and other types of oxidative damage.⁴⁴ Drugs alter the functioning of the liver by increasing the activity of microsomal enzymes, a process known as enzyme induction. Enzyme induction has an important function in determining the severity of liver damage.⁴⁵ Medicines are a major cause of severe liver damage. Drugs for treatment have been deemed accountable for cases of chronic hepatitis, fatty liver and cirrhosis, as well as many vascular and neoplastic liver diseases.⁴⁶⁻⁴⁷

Hepatotoxicity and drug metabolism

The liver has a physiological function in converting lipophilic substances into water-soluble derivatives, which can be eliminated more easily. The liver gets about 80% of its blood supply from the gastrointestinal system and has a significant ability to carry out both Phase I and II bio-transformations (Figure 4).⁴⁸ CYP-450 enzymes have a crucial function in metabolizing a wide variety of foreign substances, including medicinal drugs. These chemicals have the ability to accumulate in the liver via several mechanisms, such as active transport systems.⁴⁹ While drug metabolism primarily serves the purpose of detoxification, it may also function as a process of intoxication. Hence, foreign substances may undergo biotransformation to produce metabolites that possess inherent chemical reactivity towards cellular molecules. The tendency of a molecule to create these chemical and structural signals is now well characterized.⁵⁰

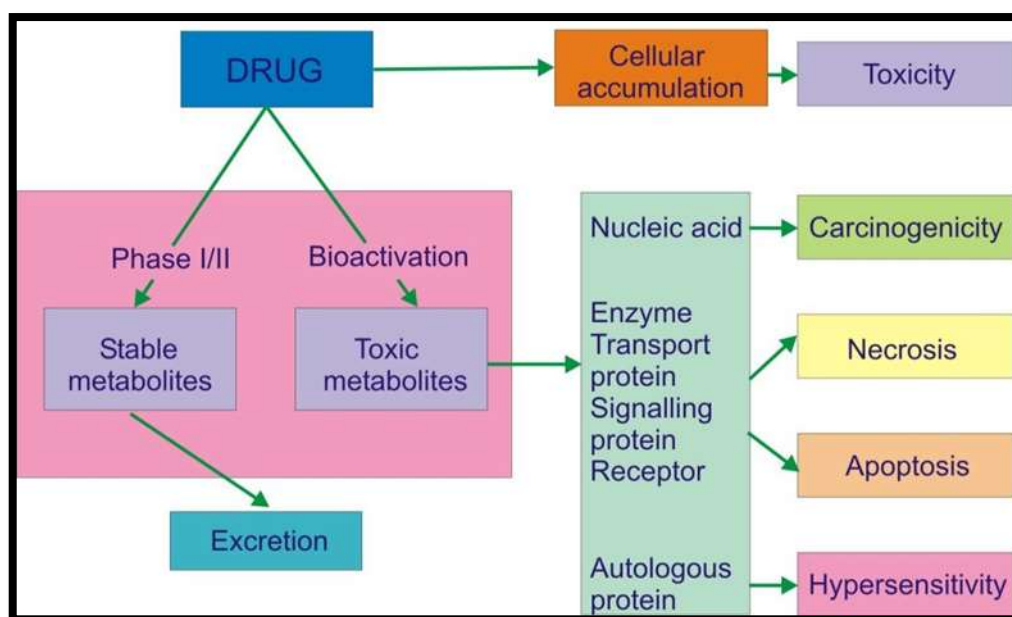


Figure 4: Representation of drug metabolism and hepatotoxicity

Fatty liver disorders, sometimes referred to as hepatic steatosis, is the process of fat buildup in hepatocytes or the liver. Fatty liver disorders are often categorized as alcoholic and non-alcoholic FLD (ALD and NAFLD) respectively.⁵¹ Both ALD and NAFLD have the potential to develop into steatohepatitis, fibrosis, cirrhosis and HCC (Figure 5). The significant risk factors for NAFLD are associated with lifestyle characteristics, namely an elevated consumption of a high-calorie diet rich in glucose, fructose, and saturated fat.⁵² A high-calorie diet activates the mechanism of hepatic de-novo lipogenesis, which causes inflammatory conditions in the liver and adipose tissue, as well as triggers IR in adipose tissue, liver and skeletal muscle.⁵³ Nonalcoholic fatty liver and steatohepatitis (NASH) are the two histologically distinct subtypes of NAFLD.⁵⁴⁻⁵⁵ NAFLD is defined by a diagnosis of a minimum of 5% liver steatosis, which is the buildup of fat in the tissues of the liver, without any signs of hepatocellular damage such as hepatocyte ballooning.⁵⁶ NASH is characterized by the existence of at least 5% accumulation of fat in the liver and inflammation accompanied by damage to liver cells, with or without the development of fibrosis.⁵⁷

NAFLD commonly results in an imbalance of pro-inflammatory cytokines, persistent inflammation caused by liver injury, accumulation of free radicals leading to oxidative stress in the liver, dysfunction of mitochondria and fatty oxidation and the buildup of extracellular matrix, specifically fibril forming collagens, known as fibrosis.⁵⁸ NAFLD may advance to cirrhosis or directly to HCC and it is a significant contributor to cryptogenic cirrhosis.⁵⁹

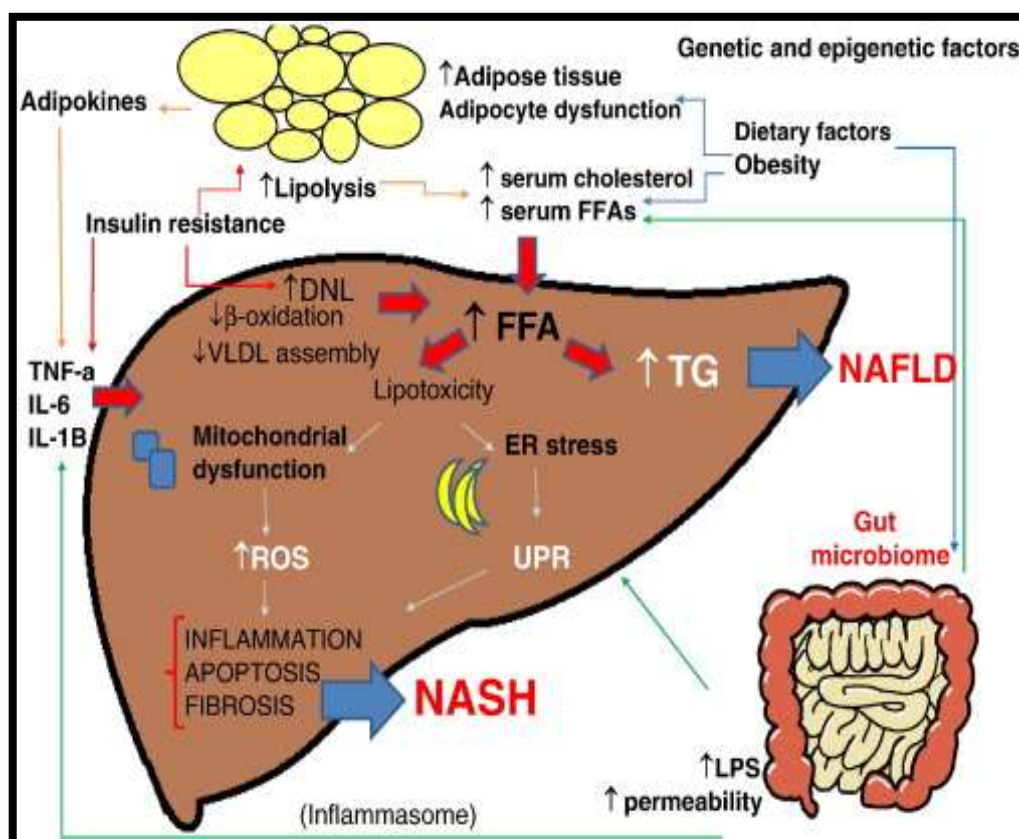


Figure 5: Pathogenesis of liver Cirrhosis including NASH and NAFLD

Drug induced animal models for screening novel pharmaceuticals.

INH induced hepatotoxicity as animal model

Isoniazid (INH) induced hepatotoxicity

Isoniazid (INH), also known as isonicotinylhydrazide or isonicotinic acid hydrazine (Figure 6), is a synthetic medication used as a first-line treatment for tuberculosis.

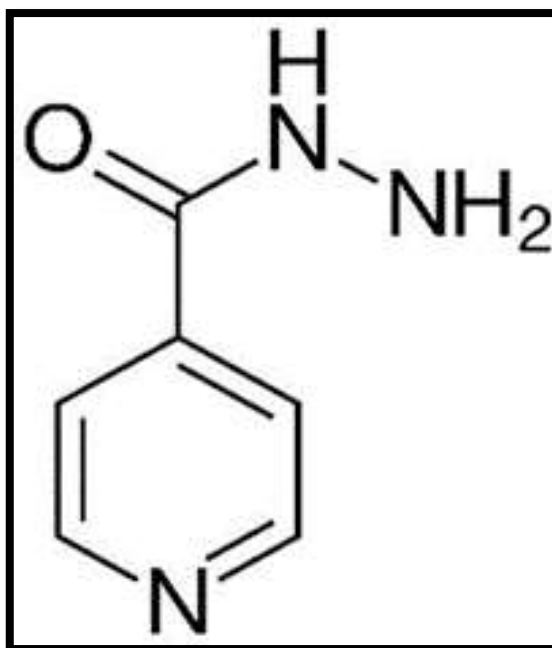


Figure 6: Structure of Isoniazid (INH)

Hepatotoxicity caused by isoniazid (INH) is an important health problem in developing nations such as India. Isoniazid-induced hepatotoxicity may manifest as a spectrum of liver damage, ranging from modest and temporary increases in aminotransferases to more severe cases of hepatitis.⁶⁰ The liver is responsible for the biotransformation of INH. INH undergoes metabolism via the enzyme N-acetyltransferase, resulting in the formation of acetyl isoniazid. Subsequently, acetyl isoniazid is further hydrolyzed to form acetyl hydrazine. The CYP2E1 enzyme metabolizes acetyl hydrazine into a highly reactive acetylating species.⁶¹ This species then covalently bonds to macromolecules found in the hepatic membrane, resulting in hepatocellular damage (Figure 7).⁶⁰ Three metabolites, including acetyl hydrazine (AcHz), hydrazine (Hz), and a metabolite formed from the bioactivation of INH itself, have been suggested as the potential causes of liver damage generated by INH.⁶² INH has shown an ability to affect cytochrome P450 enzymes, leading to its own harmful effects due to its role as an inducer of CYP2E1.⁶³

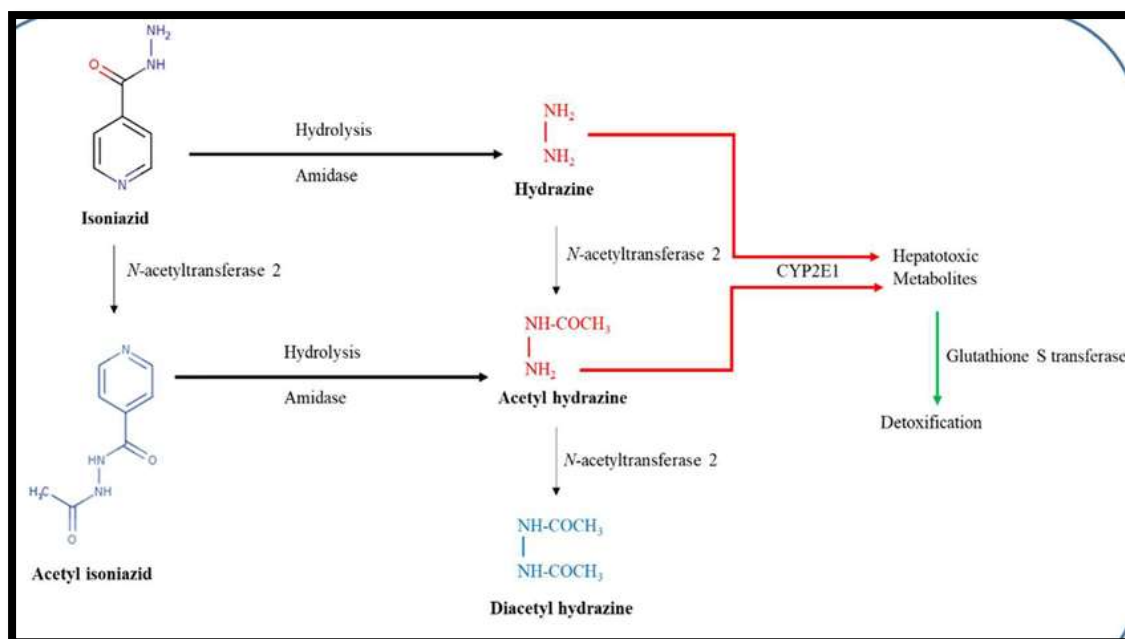


Figure 7: Biotransformation of isoniazid in liver

Severe liver damage caused by INH is characterized by hepatic injury, multi-lobular necrosis, and infiltration of mononuclear cells. Additionally, steatosis occurs early in the process. Isoniazid induces a minor kind of mitochondrial injury, leading to the production of damage-associated molecular pattern molecules.⁶⁴ This process triggers an immunological response that ultimately causes liver injury. The primary cause of liver injury from isoniazid is the buildup of harmful metabolic byproducts. Research suggests that variations in the genes N-acetyltransferase 2 and glutathione-S-transferase are potential risk factors for liver damage caused by isoniazid.⁶⁵ The toxicity of INH is associated with the release of AcHz and Hz, which have been shown to cause liver necrosis in animal experiments. Hydrazine, one of the byproducts of INH, can lead to hepatic steatosis, hepatocyte vacuolation and oxidative stress.⁶⁶ This oxidative stress is linked to a decrease in glutathione levels and a reduction in the activity of enzymatic antioxidants such as glutathione S-transferase, catalase and superoxide dismutase.⁶⁷

The administration of isoniazid (INH) caused oxidative stress in the liver of rats by reducing the activities of superoxide dismutase, catalase, and reduced glutathione, while considerably increasing lipid peroxides.⁶⁸ The administration of INH therapy in rats leads to hepatotoxicity by increasing the levels of serum γ glutamyl transpeptidase, CYP2E1 - alanine hydroxylase, urea, creatinine, and uric acid. This is then followed by a reduction in the levels of serum total protein and albumin, it was shown that HepG2 cell lines treated to anti-TB medications had elevated levels of cytotoxicity index.⁶⁹ Phase contrast microscopy study detected changes in cellular structure, characterized by cellular swelling and the formation of membrane blebs, suggesting the presence of necrosis in the afflicted cell lines. The examination of cells using confocal microscopy showed changes in the electrical potential across the mitochondrial membrane, while flow cytometry analysis demonstrated the occurrence of programmed cell death in the cell lines treated to anti-TB medicines.⁷⁰⁻⁷¹ Oxidative stress is the primary causative factor for the development of disease and it is also one of the contributing processes responsible for the liver damage generated by INH and RIF in rats. A substantial mismatch between the body's production of reactive oxygen species (ROS) and its capacity to use antioxidants to protect itself from them is known as oxidative stress. When mitochondria create energy, ROS hydroxyl radicals, superoxide and hydrogen peroxide are formed.⁶⁸ The CYP450 system is responsible for carrying out detoxifying activities. INH serves as a catalyst for the production of free radicals by stimulating the CYP450 system.⁶⁹ The occurrence of liver damage caused by INH is a result of the synthesis of reactive metabolites, namely HYZ, during the process of biotransformation. This leads to the production of ROS and carbonyl compounds.^{64, 72}

High fat diet induced hepatotoxicity as animal model

Mechanism action of high fat diet

Prolonged intake of a high fat diet leads to the development of chronic dyslipidemia and steatosis (Figure 8). While cholesterol is a necessary element of cells, an excessive amount of it may have significant consequences for the health of a human being. Hepatotoxicity occurs due to the accumulation of excessive lipoprotein and triglyceride (TG) in the liver, leading to liver damage.⁷³⁻⁷⁴

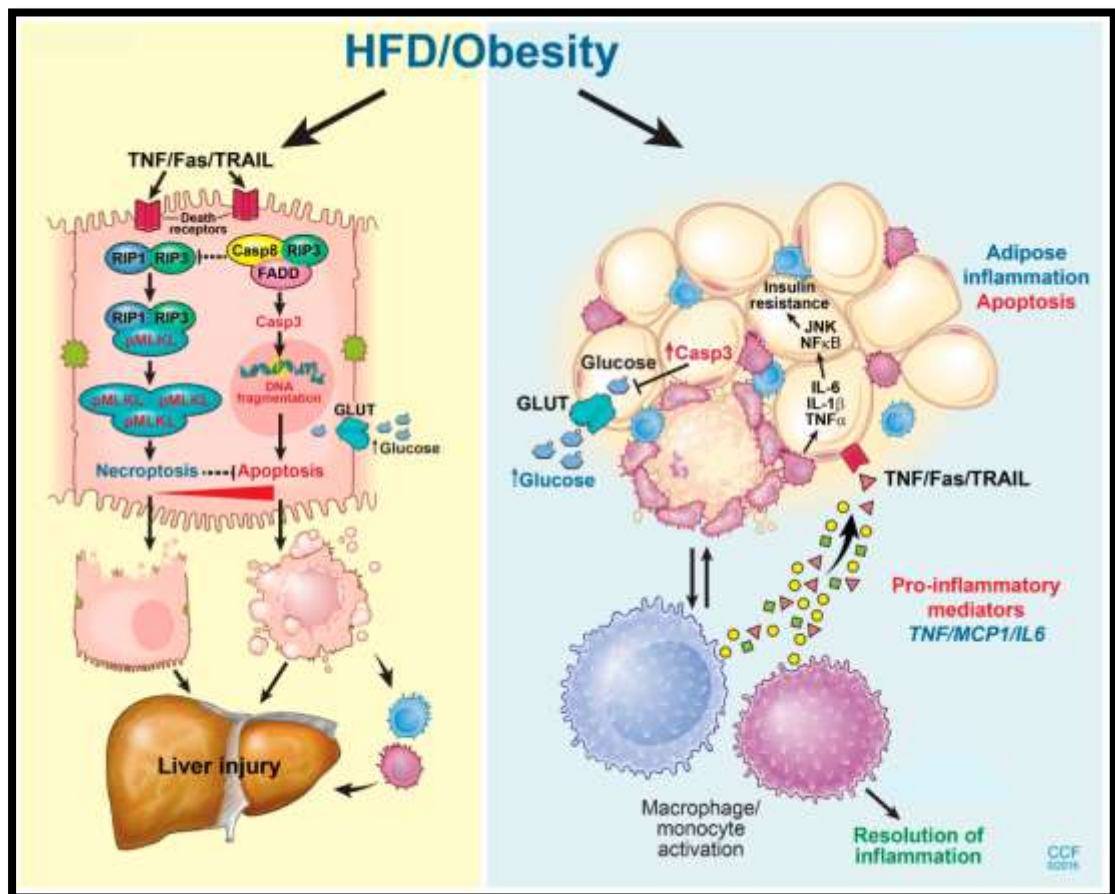


Figure 8: Hepatoma cells for hepatoprotective activity.

In vitro biological tests play a crucial role in assessing the potential hepatoprotective properties of herbs. Research reveals that *in vitro* models of normal liver cells have proposed human hepatoma cell lines as an alternative to human

hepatocytes.⁷⁵ Hepatoma cells provide several potential advantages. Firstly, being an immortalized cell line, they can be easily obtained in substantial quantities. Secondly, they are simple to maintain due to their ability to be cryopreserved. Lastly, unlike primary cultures of human hepatocytes, their drug-metabolizing enzyme activities remain normal during culturing. Nevertheless, a clear drawback is that the processes involved in drug metabolism and toxicity could be abnormal in cells that are transformed. The HepG2 hepatoma cell line is extensively used in research on liver functionality, metabolism, and drug toxicity. HepG2 cells exhibit several biochemical and morphological features that are similar to those of normal hepatocytes. These cells are used in investigations to assess the hepatoprotective properties of medicinal herbs because to their similar several features of normal liver cells.⁷⁶

Herbal medicines and Conventional Therapies

Plant-derived bioactives are a plentiful reservoir of antioxidants utilized for the treatment of several illnesses. The natural world is rich in pharmaceutical substances, making it a fascinating and productive field of scientific research. The topic of health and well-being has been an essential focus for humanity throughout history. Through initial exploration of herbs and plants found in surroundings and subsequent treatment of diseases.⁷⁷ The utilization of plants, its extracts or isolated pure substances derived from natural sources for the purpose of treating ailments is a therapeutic approach that has stood over the course of time, despite the fact that most of the scientific understanding behind this kind of treatment is still in its early stages. Analysis of the chemical compounds found in plants and animal remedies used by ancient civilizations has often shown that trees had bioactive substances that were responsible for their medicinal efficacy.⁷⁸

Currently, synthetic pharmaceuticals outweigh medicines derived from natural sources in modern medicine, and the study of isolating and understanding the effects of natural compounds is significantly lagging behind the research on synthetic drugs. However, there are still several medications that are made from natural substances obtained from animals or plants.⁷⁹ Today, it is true that a significant number of pharmacological medication classes use a natural molecule as their prototype such as Morphine, digoxin, quinine, atropine, reserpine, physostigmine, pilocarpine, vinblastine, vincristine, artemisinin and taxol are among the therapeutic compounds derived from plants.⁸⁰ Currently, an extensive number of herbal extracts are being used in naturopathic treatments alongside the conventional medicines. While herbal treatments are widely used by many people and a small portion of plants have been thoroughly studied in terms of their pharmacological properties. It is crucial to assess the safety and efficacy of plant-based pharmaceuticals throughout their development due to the intrinsic biological complexity of plants.⁸¹⁻⁸²

Silymarin: An effective hepatoprotective agent

Milk thistle, or *Silybum marianum* (Family: Asteraceae/Compositae), is a plant that has been studied extensively for its potential to cure liver ailments. The dried seeds of the milk thistle plant contain the highest amounts of silymarin, which is subsequently extracted and used.⁸³ In the years 1968–1974, the active principle was initially isolated and structurally characterized. Four different flavonolignan isomers—silybin, isosilybin, silydianin, and silychristin—make up silymarin.⁸⁴⁻⁸⁵ Silymarin's protein synthesis facilitative properties are thought to be due to its structural similarities to steroid hormones. For example, silybin accounts for 60–70% of the isomers, whereas silychristin and silydianin each account for 10% and isosilybin for 5%.⁸⁶ The bioavailability of silybin is greatly improved by its combination with

phosphatidylcholine; this complex is known as silipide. Researchers from all over the world have shown that silymarin protects the liver from acetaminophen, diethyl nitrosamine, carbon tetrachloride, ethanol, D-galactosamine, Amanita phalloides toxin, and other toxins using partial hepatectomy models and toxic models in experimental animals.⁸⁷ After 7 days of treatment with 100 mg/kg (p.o), silymarin significantly increased liver weight in mice compared to animals treated with CCL₄. In addition, silymarin protected the livers of 87.5% of mice against the hepatotoxicity caused by paracetamol. While silymarin did protect against CCL₄-induced liver necrosis, the proportion of protection was low.⁸⁸

Details on *Cucumis melo*

Muskmelon (*Cucumis melo* L.), (Figure 9) belongs to the Cucurbitaceae family and it include a variety of essential nutrients including vitamin E, C, A and B6, as well as dietary fiber and carbohydrates.⁸⁹ In Ayurveda it is known as Madhuphala (nutrient-rich fruit). Muskmelon contain significant quantities of potassium, which may aid in the regulation of blood pressure, modulation of heartbeats, and perhaps prevent strokes. A research investigated the antioxidant and anti-inflammatory characteristics of a melon extract; showed significant superoxide dismutase activity. Muskmelon has the ability to lower overall body temperature, relieve lethargy, stimulate the appetite, and act as a diuretic. Muskmelon also serves as a source of folic acid. Therefore, it assists in reducing homocysteine levels, which are responsible for the development of atherosclerotic plaques in arteries. Having muskmelon juice is advantageous for those experiencing lack of appetite, undergoing weight loss, or suffering from UTI.⁹⁰ On looking to toxicity, *C. melo* is widely consumed and generally considered safe. High doses (5000 mg/kg) of *C. melo* extracts have shown no significant acute toxicity in animal models, with no mortality or severe adverse effects. Long-term

administration of *C. melo* extracts (100-500 mg/kg) did not result in significant adverse effects. There were no notable changes in body or organ weights, nor any histopathological alterations in major organs such as the liver and kidneys.⁹¹

Cucurbitacins (Cus) are a group of tetracyclic triterpenes that include at least five distinct compounds: Cucurbitacin B (CuB), Cucurbitacin D (CuD), Cucurbitacin E (CuE), Cucurbitacin I (CuI), and Cucurbitacin Q (CuQ). CuB, CuE and CuI have been extensively researched.⁹² They have an extensive distribution within the Cucurbitaceae family as well as other plant families and demonstrate a range of pharmacological and biological characteristics. Cucurbitacins (Cus) were first utilized as an anti-hepatitis medication in China during the late 1970s⁹³ and in the recent years, there has been an increasing interest in Cus; several studies have shown that Cus analogues possess a diverse array of biological functions, such as hepatoprotective, anti-cancer and anti-inflammatory effects. However, neither the precise biological targets of the Cus nor the complex molecular mechanisms regulating their biological function are understood.⁹⁴ Several studies have shown that the anti-proliferative effects of these substances are linked to the preventing of cell division and programmed cell death. This is accomplished by inhibiting the Jak-STAT3 signaling pathway. Additionally, other studies have suggested that these substances target cofilin, an important regulator of actin dynamics, leading to the disruption of actin assembly.⁹² CuB has been implicated in the suppression of Aurora phosphorylation in human multiple myeloma cells, which may result in cell death and G2-M phase arrest. Hence, Cus has significant pharmacological effects by modulating several cellular pathways, prompting us to investigate other possible targets of Cus.⁹⁵



Figure 9: Representing the pictorial image of *Cucumis melo*

Details on *Cucurbita pepo*

Cucurbita pepo (Pumpkin) (Figure 10) belongs to the family Cucurbitaceae and its health benefits include antioxidant, anti-inflammatory and anti-fatigue properties. Previous study has elucidated the effect of pumpkin on the CNS, particularly in relation to the intake of pumpkin seed oil (PSO).⁹⁶ It has been shown that PSO effectively alleviates symptoms associated with ischemic stroke after ischemia-reperfusion. Furthermore, it has been shown that extracts derived from pumpkin seeds have antidepressant properties in rats that are equivalent to those of imipramine, a medication utilized to treat depression. Pumpkin seeds are abundant in unsaturated FAs, antioxidants and fibers.⁹⁷ These components are recognized for their anti-atherogenic and hepatoprotective properties. Pumpkin is a plant that is often used as a functional food or medication. One of its prevalent use in many countries is for the treatment of diabetes. Pumpkin seed oil, which contains high levels of unsaturated fatty acids such as linoleic and oleic acid, as well as tocopherols, is very stable and has potential health benefits. It is recommended as a nutritious supplement to the human diet and may be used in many food preparation and industrial applications.⁹⁸ Pumpkins contain various biologically active ingredients, including carotenoids, sterols, proteins and peptides, polysaccharides, para-aminobenzoic acid

and fixed oils and the primary components included in pumpkin seed oil include important fatty acids such as omega-6 and omega-9, as well as phytosterols and antioxidants such as carotenoids, vitamin A and vitamin E; the Linoleic acid, a polyunsaturated fatty acid found in pumpkin seed oil, has the ability to enhance the flexibility of cell membranes, facilitating the process of osmosis and the exchange of gases both within and outside the cells. The composition of pumpkin seed oil consists of the following fatty acids: palmitic acid, stearic acid, oleic acid and linoleic acid.⁹⁹ Studies have reported no significant adverse effects or mortality up to 5000 mg/kg, indicating a low risk of acute toxicity. Also, sub-chronic and chronic toxicity (100 to 500 mg/kg) also shown no significant adverse effects on body weight, organ weights or histopathological changes.¹⁰⁰



Figure 10: Representing the pictorial image of *Cucurbita pepo*

Network pharmacology

Principle similar compounds tend to bind the same proteins is similar to master key can unlock multiple locks. In this aspect, one can observe that how one protein can regulate various proteins and these proteins interact with each other to regulate multiple pathways associated with disease pathogenesis. This concept can be explained via the network pharmacology, the term was coined by Hopkins in 2007 to

understand the action and interaction of drugs with multiple targets. It helps to list the drug-mediated molecular interaction within the cells and is an important tool to overview the hidden relationship of a drug(s) and the biological system. Further, it helps in drug repurpose for multiple therapeutic conditions and identify the new lead(s) via unbiased investigation depending on potential target spaces. The next generation medications might be intelligently designed by merging systems biology with NP breakthroughs. In addition to providing novel treatment alternatives, NP analysis seeks to enhance the effectiveness and safety of already prescribed drugs.¹⁰¹⁻¹⁰²

MATERIALS AND METHODS

The current study was designed into three form (1) *in silico* studies, (2) *in vitro* studies, and (3) *in vivo* studies (Figure 11).

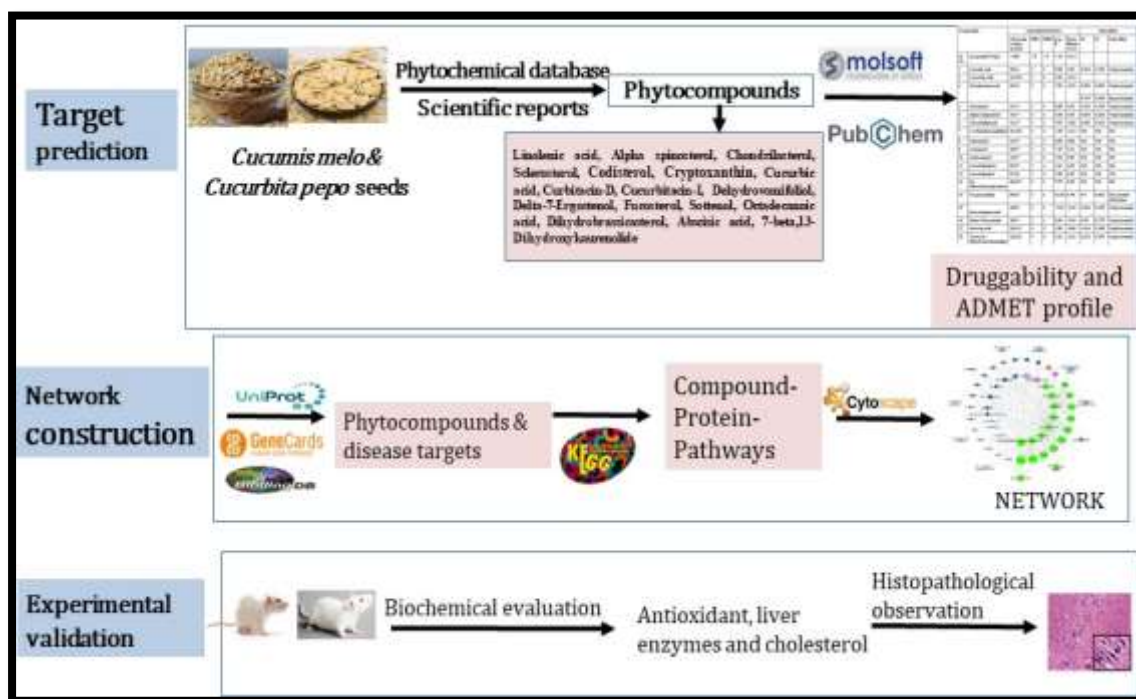


Figure 11: Brief workflow of the current study.

Study 1: *In silico* studies

Retrieval of phytochemicals from *C. pepo* and *C. melo*

Phytochemicals from *Cucurbita pepo* and *Cucumis melo* were obtained from phytochemical databases, and structural data were retrieved from PubChem and other open-source libraries. Potential protein targets for these phytochemicals in the context of liver cirrhosis were identified.¹⁰³ During dataset construction, duplicate phytochemicals and proteins associated with liver cirrhosis were removed. canonical SMILES for each phytochemical's were sourced from PubChem database.¹⁰⁴

Phytocompounds probable protein targets

Canonical SMILES of each compound were queried in Swiss Target Prediction to identify targets with a similarity greater than 0.1%. By referencing the Therapeutic Target Database (TTD), proteins associated with liver cirrhosis were identified among the existing targets for liver cirrhosis.¹⁰³

G-Profiler web server

G: Profiler server (<https://biit.cs.ut.ee/profiler>) was used to identify enriched biological categories within gene lists. GOST utilizes g: Convert to map gene/protein IDs across various namespaces, g: Orth to map orthologous genes across species, and g: SNPense to map human SNP identifiers to genes for the enrichment analysis of single or multiple gene lists.¹⁰⁵

Gene set pathway enrichment analysis

A set of genes obtained for INH, HFD, phytocompounds from *C. pepo* and *C. melo* were submitted to STRING database to identify the molecular pathways regulated by these set of genes. The pathways that are only associated with liver related diseases were traced via KEGG database.¹⁰⁶⁻¹⁰⁷

Network analysis

Cytoscape 3.7.2 was used to construct the network between INH, HFD, phytocompounds, their protein molecules and identified pathways.¹⁰⁸ To determine how many connections there were between targets, pathways, and phytocompounds, the edge count topological parameter was selected. During the analysis, the network was treated as direct to capture the direct connections between phytocompounds,

targets, and pathways. Node, edge sizes and shapes were adjusted during network construction.¹⁰⁹

Computational docking

Protein identification and preparation

The 3D structures of all phytocompounds were retrieved from the PubChem online database and minimized using the MMFF94 force field.¹¹⁰⁻¹¹¹ The RCSB database (<https://www.rcsb.org/>) identified the proteins and PDB IDs for NR1H3 (1UHL) and HMGCR (2R4F) for *C. pepo* compounds, and FXR (6HL1) for *C. melo* compounds. Protein preparation was performed using the Protein Preparation Wizard in Schrödinger's software (2021-2). This process included removing water molecules beyond 5 Å from hetero groups, creating zero bond orders to metals, assigning polar hydrogens, restoring methionine residues and performing restrained energy minimization using the OPLS3e force field.¹¹²

Ligand Preparation

The LigPrep panel within Schrödinger's 2021-2 software was utilized to generate ligand conformations at the target pH employing the Epik module. Chirality was preserved for the chosen ligands and a single stereoisomer was generated per ligand. Subsequently, energy minimization was conducted using the OPLS3e force field.¹¹³

Grid generation

Schrödinger's Glide module, which includes the receptor grid creation panel, was used to generate a grid at the binding location. The grid was produced in accordance with the location of the co-crystal ligand, which designated the binding site.¹¹⁴

Ligand docking

The Glide module from Schrodinger's was utilized for ligand docking, while Maestro 12.3 was used to display the binding interactions.¹¹⁵ Extra precision (XP) docking mode was used to determine the binding energy and ligand orientation within the binding pocket. To visualize the interaction between the ligand and the protein after molecular docking, the posture with the lowest BE was chosen.¹¹⁶

Molecular Dynamics using DESMOND

To assess the stability of complex formation, Desmond software was used to perform a 30-ns MD simulation on ligand-protein complexes. The SPC was used to solve a cubical box system with periodic boundary conditions of $10 \text{ \AA} \times 10 \text{ \AA} \times 10 \text{ \AA}$. Na⁺/Cl⁻ counterions were also added to the solution, further neutralising it. The SHAKE algorithm limited the geometry of heavy atom bond angles and lengths to.¹¹⁷ Long-range interactions were computed using the "particle mesh Ewald" approach. The cut-off for "Lennard-Jones interactions" was chosen at 10.0 Å. Furthermore, default parameters were used to minimize the system. The final 30 ns of the production run were then spent using a Nosé-Hoover chain thermostat with a 1.0 ps relaxation time and the Martyna-Tobias-Klein barostat method with a 2.0 ps relaxation time. The temperature and pressure were set to 300 K and 1.01325 bar, respectively, and a Coulombic short range cutoff radius of 9.0 Å. Protein radius of gyration (rGyr), complex root mean-square deviation (RMSD), and root mean-square fluctuation (RMSF) of residues were examined in order to estimate the structural stability. To deduce stable contacts between the ligand and protein during MD simulation, the percentage proportion of interactions between the ligand and protein was examined over a 30-ns simulation.¹¹⁸

Molecular Dynamics using GROMACS

MD simulations were performed using GROMACS 2021.3 (<http://www.gromacs.org/>) to evaluate FXR structural stabilities for 100ns. Antechamber with “bcc” charge model produced ligand partial charge and Amber ff99SB-ildn force field. Amber Tool’s xleap module (<https://ambermd.org/AmberTools.ph>) was used to build ligand complexes with solvated rectangular box boundaries (10.0 Å) from the protein edge in all directions using the (TIP3P) model.¹¹⁹ The simulated system was neutralized with enough (NA+/CL-) counter ions. Using steepest descent and conjugate gradient energy reduction, near-global state least energy conformations were produced. After 1ns of equilibration by (NVT) and (NPT) ensemble, the system maintained constant atom count, volume, temperature, and pressure. We maintained (300 K) and 1 bar in this study. H-bonds were constrained using the ‘LINear Constraint Solver (LINCS) method and Coulomb electrostatic interactions using PME. At the end simulation run produced trajectories which was further examined using built-in Gromac tools, including "gmx rmsd," "gmx rmsf," "gmx hbond," "gmx gyrate," and "gmx sasa."¹²⁰

In vitro studies

Collection and Authentication of *C. pepo* and *C. melo*

The ripe fruits of *C. pepo* and *C. melo* were purchased locally in the June month of 2020 from local area of Belagavi region, Karnataka then plant seeds were authenticated by Central Research Facility B.M.K. Ayurveda Mahavidyalaya, Belagavi, with voucher specimen number CRF/Auth./2020/1 and CRF/Auth./2020/2.

Preparation of plant extract and fraction of *C. pepo*

Procedure for *C. pepo*

The *C. pepo* fruits were peeled, the seeds were carefully hand-removed from the pulp, and cleaned, rinsed, and damaged seeds were separated. The remaining seeds were then dried in an oven at 40°C for 24 hours. Dried seeds were then grinded in electrical grinder. 70% v/v ethanol used as solvent for maceration of dried seeds and 5 % chloroform (preservative) for 72 hrs. Extract was filtered, and the raw material remained in the maceration was subjected to soxhlation (70% v/v ethanol). Filtrates of maceration and soxhlation were concentrated at 40°C under reduced pressure using a rotary evaporator which yielded total extract. Finally, the extract was subjected to fractionation. Fractionation was performed as explained by Paul Cos et al. (2006).¹²¹ The hydroalcoholic extract was dissolved in water and dichloromethane mixture and dichloromethane fraction was further mixed with methanol (90%) to obtain the sterols fraction (Figure 12).¹²²

Yield of the extract (%) = (weight of extract / weight of dried seeds) × 100.

Percentage yield of extract/ 100gm of powder was found to be 14% and the percentage yield of fraction using 14 g of hydroalcoholic extract of *C. pepo* was found to be 4%.

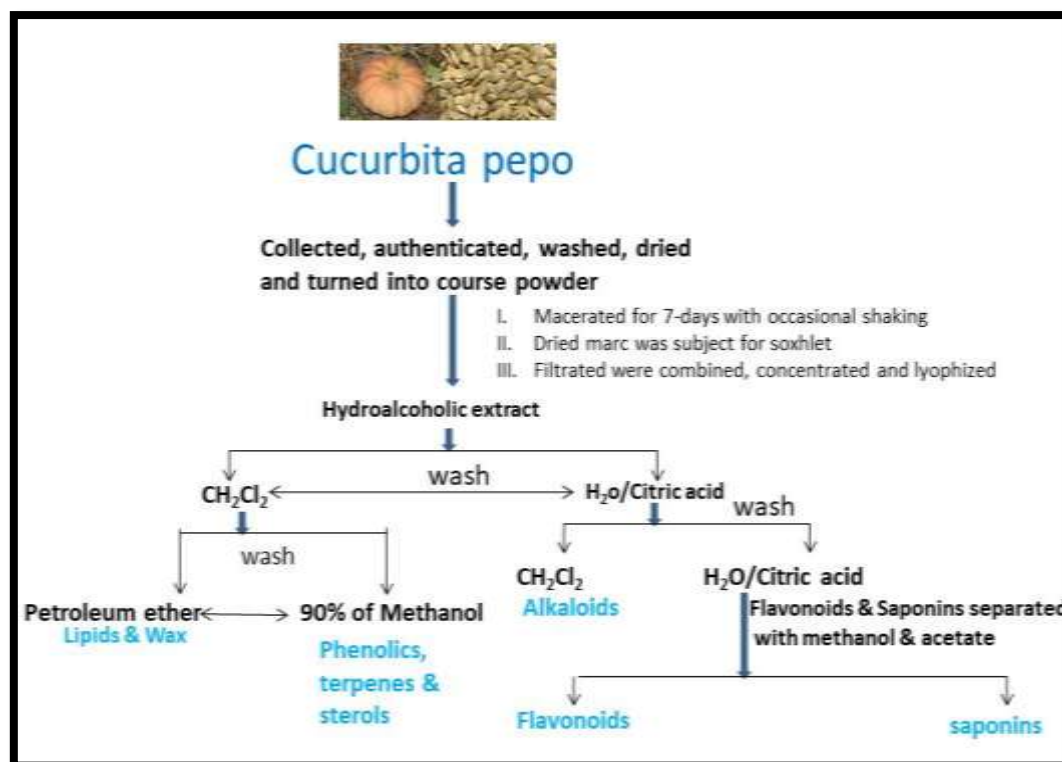


Figure 12: Hydroalcoholic extraction and crude fractionation of *C. pepo*

Procedure for *C. melo*

Proper precautions were taken to ensure that any potential active metabolites are present during the extraction procedure. The *C. melo* seeds were pulverised after being broken up into smaller pieces and shade-dried at room temperature. In order to extract the coarse powder from 100 g of *C. melo* seeds were subjected to Soxhlet extractions (95% v/v ethanol). Using a rotary evaporator, extract was concentrated under reduced pressure. Further, separate fractionation from the entire extract was done to separate polar from less-polar elements (Figure 13). This enables more accurate differentiation of fractions.¹²¹

Hydroalcoholic extract yield of *C. melo* seeds per 100g of powder is 16%. The yield of the fraction employing 16 g of *C. melo* hydroalcoholic extract is 6%.

Paul Cos et al.'s method for fractionation was used.¹²¹

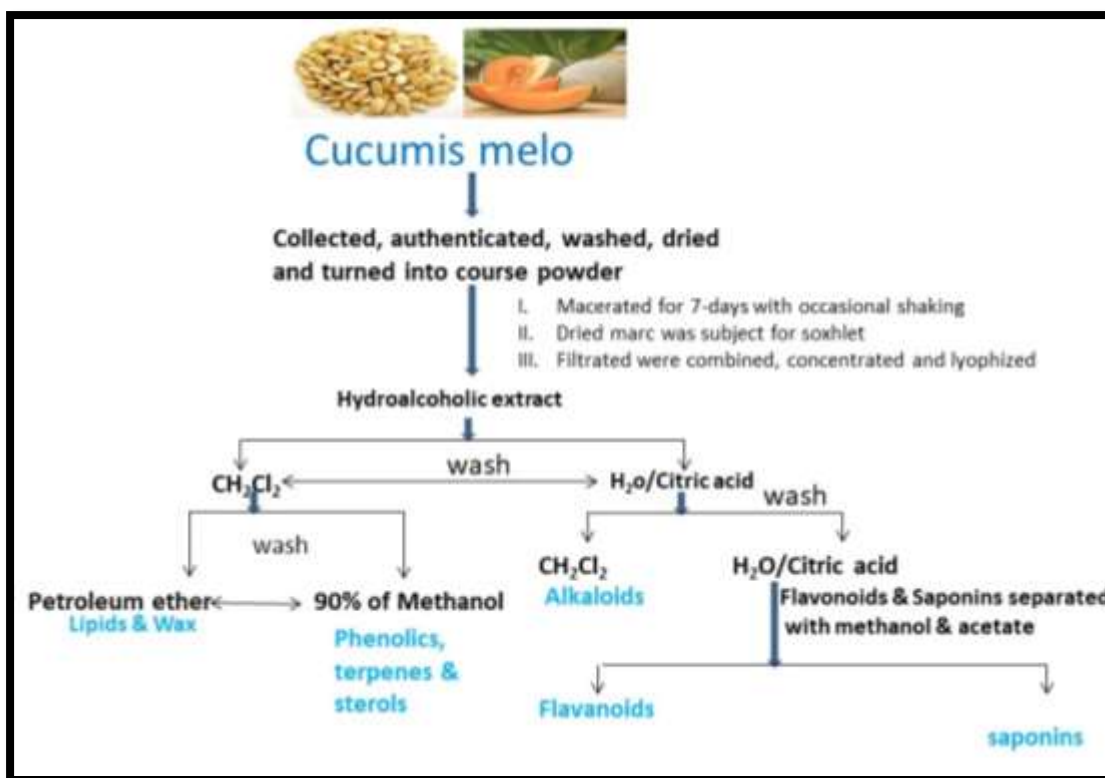


Figure 13: Hydroalcoholic extraction and crude fractionation of *Cucumis melo*

LC-MS profile of the hydroalcoholic extract of *C. melo* and *C. pepo*

When using the LC-MS 2010A (Shimadzu Japan) to run the sample, the following requirements were upheld. Methanol and water were employed in a 90:10 v/v ratio as the mobile phase, with a flow rate of 200min^{-1} . The C18 column served as the stationary phase. The sample was injected ($10\mu\text{L}$) after being dissolved in the mobile phase. The chemicals in the sample were identified using the electrospray ionisation peaks.¹²²⁻¹²³

Estimation of cytotoxic by MTT assay

From NCCS, Pune, India, HepG2 cell line was acquired. "HepG2 cells at a density of 1×10^4 cells were seeded in 96-well plates and the test substances in varying concentrations, standard medicine (Silymarin), and FFA were added to 96-well culture plates, which were then incubated at 37°C and $5\% \text{CO}_2$ for 24h;¹²⁴ After that,

the hepatoprotective parameters were estimated using the supernatant and the plate with cells were incubated for 4 hours at 37°C in the dark with 20µl of MTT solution (5 mg/ml in phosphate buffer saline) and after this to dissolve the formazan crystals, 100µl of DMSO was added to each well and absorbance at a 570 nm was recorded and the percentage of cell viability was determined using the formula,¹²⁵⁻¹²⁶

$$\% \text{ Cell viability} = (\text{Mean OD control} - \text{Mean OD of test} / \text{Mean OD of control}) \times 100$$

Assessment of hepatoprotective activity of *C. pepo* and *C. melo*

Induction of hepatotoxicity by INH

Experiment was performed in accordance with standard guidelines of bioethics at KLE College of Pharmacy, Belagavi, approved by Institutional Animal Ethics Committee (IAEC), Reg.no 221/Po/Re/S/2000/CPCSEA in its meeting date 21/01/2022. Rats weighing 160 to 180 g were housed in standard laboratory conditions of temperature (25±2 °C), 12 h light and dark places and with food and water ad libitum. Isoniazid (50mg/kg p.o.) was administered daily for 28 days in drug induced liver toxicity model, to all animals except group 1. Silymarin (50 mg/kg, p.o) was used as standard. The animals were segregated in to seven groups of six each as follows.¹²⁶ **Group –I:** received food and water. **Group-II:** (control) received Isoniazid (50mg/kg p.o.) up to 28 days. **Group III:** received Silymarin 50 mg/kg¹²⁷ orally and Isoniazid (50mg/kg) p.o. up to 28 days. **Group IV:** received 500mg/kg of *C. pepo* extract and Isoniazid (50mg/kg). **Group V:** received 50mg/kg of *C. pepo* steroid fraction and Isoniazid (50mg/kg). **Group VI:** received 500mg/kg of *C. melo* extract and Isoniazid (50mg/kg). **Group VII:** received 50mg/kg of *C. melo* steroid fraction and Isoniazid (50mg/kg). At the end of the study, ketamine: 300 mg/kg (i.p.) and xylazine 30 mg/kg was injected to euthanize the animals and immediately blood was collected from each rat via the retro-orbital plexus for biochemical analysis as shown in ref.¹²⁷

Induction of hepatotoxicity by HFD

Experiment was performed in accordance with standard guidelines of bioethics at KLE College of Pharmacy, Belagavi, approved by Institutional Animal Ethics Committee (IAEC), Reg.no 221/Po/Re/S/2000/CPCSEA in its meeting date 21/01/2022. Male albino mice weighing 25-30 g were utilized for the experiment and temperature of room was maintained at $22 \pm 3^{\circ}\text{C}$ with 12-h light-dark cycles with free access of food and drinking water. HFD (10ml/kg p.o.) was administered daily for 28 days to all group animals except group-I. The percentage of fat is conformed according to the RM value.¹²⁸ Silymarin (50 mg/kg, p.o.) was used as standard. The animals were segregated into seven groups of six animals in each group as follows. **Group –I:** received food and water. **Group-II:** (control) received HFD (10ml/kg) p.o. up to 28 days.¹²⁹ **Group III:** received Silymarin 50mg/kg orally HFD (10ml/kg) p.o. up to 28 days. **Group IV:** received 500mg/kg of *C. melo* extract and HFD (10ml/kg). **Group V:** received 50mg/kg of *C. melo* steroid fraction and HFD (10ml/kg). **Group VI:** received 500mg/kg of *C. pepo* extract and HFD (10ml/kg). **Group VII:** received 50mg/kg of *C. pepo* steroid fraction and HFD (10ml/kg). At the end of the 4 weeks, animals were euthanized by overdose of ketamine: 300 mg/kg (i.p.) and xylazine 30 mg/kg (i.p.) as per standard guidelines. Blood was withdrawn via “retro-orbital plexus puncture” route for biochemical investigation.¹³⁰

Measurements and methods

Body weight

The body weight of each mice was recorded every 3 consecutive and the percentage change in body weight was calculated.¹³¹

Euthanasia and organ collection

The animals were euthanasized using ketamine: 300 mg/kg (i.p.) and xylazine 30 mg/kg (i.p.) while withdrawing the blood and isolating the organs respectively. Subsequently, the samples were washed with cooled phosphate buffer solution at a pH of 7.4. The liver was sectioned into 2 halves; one part liver was immersed in 10 % v/v formalin for histopathological examination. The 0.5 g of the second half of the liver was homogenized in chilled PBS (100mg/mL), centrifuged and the supernatant was deep-frozen for further use.¹³²

Serum biochemical parameters

Blood was collected from the medial canthus of the eye on the last day of the experiment. Serum lipid profiles viz., TC, LDL, HDL, VLDL and the liver markers ALT, AST, ALP, total and serum bilirubin, utilizing Erba kit.¹³³⁻¹³⁴

Estimation of anti-oxidant biomarkers

The enzymatic & non-enzymatic anti-oxidant profiles of *C. melo* and *C. pepo* were quantified in liver homogenate (10% w/v, 0.5 mL) which are detailed below.

The **glutathione (GSH) level** was determined by incubating (30 min) the liver homogenate (250 µL) with DTNB (50µL) and sodium-phosphate buffer (2.5mL) and the Abs was recorded at 412 nm.

Lipid peroxidation was assessed by incubating (95°C, 1h) liver homogenate (200 µL) within the mixture of thiobarbituric acid (1.5mL, 0.8% w/v), CH₃COOH (20% v/v), and SLS (200 µL, 8.1 w/v); cooled and centrifuged (10min, 4000rpm) to record the organic layer absorbance at 532nm; presented as TBARS nmol/mg of protein.¹³⁵

SOD determination: SOD's ability to inhibit adrenaline's auto-oxidation to adrenochrome in the presence of an alkaline pH. Added 0.1mM of epinephrine in

carbonate buffer (pH 10.2) to 25µl of homogenate sample. Using an ELISA plate reader, the amount of adrenochrome formed in the combination above was quantified at 295 nm. Additionally, the total protein value was utilized to calculate the SOD level, which was then represented in U/mg of protein.

Estimating Catalase Level: 50µl of tissue homogenate, 1.95ml of PBS 7.0pH, and 1ml of 0.7mM H₂O₂ solution were mixed. At 240 nm, noted the absorbance.¹³⁶

Liver histopathology

Tissue samples were sectioned and were fixed with formalin (10% v/v); stained with hematoxylin-eosin to assess the effect of *C. melo* and *C. pepo* over hepatic Kupffer cell hyperplasia, venous congestion, portal triditis, ballooning degeneration, apoptosis, sinusoidal congestion, spotty necrosis, cholestasis and inflammation at 10X and 40X magnification.¹³⁷

Statistics

Statistical analysis of all data

Gene set enrichment analysis was performed with a false discovery rate (FDR) cut-off of 0.05, and the network was analysed using the "Edge count" topological parameter. Docking scores were expressed in kcal/mol, and the parameters describing structural stability were represented by C- α RMSF, backbone, and complex RMSD. MTT assay data were represented in % cell viability and CC₅₀ value. The *in vivo* experimental data were presented in Mean $\pm$ SEM wherever applicable. One-way and two-way analysis of variance (ANOVA) followed by Tuckey's multiple comparison test multiple comparison test was applied to evaluate the quantitative data wherever applicable. Both quantitative and categorical data were evaluated using GraphPad Prism 8.0 version.

RESULTS

***In silico* pharmacology**

In silico* pharmacology of *C. pepo

Mining of phytoconstituents and their target prediction

135 phytoconstituents from *C. pepo* were mined from literature and open source phytochemical databases. These 135 phytocompounds were predicted to target 408 protein molecules in Swiss Target Prediction server with $p > 0.1$.

Gene enrichment and network analysis

The gene set enrichment analysis of 408 targets revealed, 26 targets that are potential to modulate 11 molecular pathways associated with liver cirrhosis. Out of 135 compounds, 18 compounds were found to target these 26 targets. These phytoconstituents majorly are of steroids. Among 11 pathways (Figure 14) targeted by phytocompounds, PPAR signaling pathway was the hub pathway within the network with gene count of 10 (PPARG, FABP1, FABP3, FABP4, PPARG, RXRG, RXRB, PPARG, RXRA and NR1H3) and lowest FDR of $1.40E-14$ and Insulin resistance as second modulated pathway with lowest FDR value. Metabolic and Adipocytokine signaling pathway, Th17 cell differentiation, (NAFLD), Bile secretion, Linoleic acid metabolism, AMPK signaling pathway, Arachidonic acid metabolism, and Drug-metabolism cytochrome P450 are directly linked in the pathogenesis of liver disease against INH induced hepatotoxicity (Table 2).

Docking studies:

The binding energy and mode of interaction of each phytocompounds against NR1H3 and HMGCR receptor and are summarized in Table 3 and Table 4. Within the

network, NR1H3 and HMGCR were identified as hub genes and were considered for further docking analysis. Among the 18 compounds targeting NR1H3, Schottenol formed lowest BE of -14.2kcal/mol by forming highest number of bonds with NR1H3 i.e. 24 (His421, Gln424, Val425, Leu428, Leu439, Phe234, Phe257, Thr238, Leu260, Ala261, Ser264, Ile339, Phe326, Phe335, Leu331, Arg305, Thr302, Glh301, Met298, Ile295, Trp443, Thr314, Ile313 and Tyr321) (refer Figure 15). Likewise, Among the 17 compounds targeting HMGCR, Alpha-Spinasterol formed lowest BE of -12.4kcal/mol by forming 10 bonds with HMGCR (Lys691, Asp690, Met655, Met657, Asn658, Ser661, Lys662, Glu665, Arg590 and Val683) (refer Figure 16).

Molecular dynamics

Stability of Schottenol with NR1H3

The RMSD of both backbone and complex was found to be stable throughout 30 ns MD simulation (Figure 17). The average RMSD of backbone was found to be ~ 2.0 Å and the complex was ~ 2.2 Å. The residues involving the ligand interaction i.e. Phe329, Phe245, Leu274, Phe271, Ala275, Trp457, Met312 showed least fluctuation compared to the N- and C- terminal residues. The compound “Schottenol” was found to interact with Phe329, Phe245, Leu274, Phe271, and Ala275, Trp457, and Met312 of NR1H3 throughout 30ns MD simulation.

Druggability (ADME) and side effects of compounds

Except (+)-Dehydrovomifoliol, Clerosterol, Codisterol, Isofucosterol, Cucurbitacin-D, Cucurbitacin-I, 22-Dihydrobrassicasterol the potential adverse consequences of the remaining phytosterols were projected (Table 5). The likelihood of phytosterol side effects is mostly nephrotoxicity with Pa 0.5, which indicates least probability to induce the effect. The ADME profile of each phytosterols such as GI

absorption, BBB permeation, P-gp substrate, CYP1A2 inhibitor, CYP2C19inhibitor, CYP2C9inhibitor, CYP2D6inhibitor, CYP3A4 inhibitor and LogKp (skin permeation) by Swiss ADME are mentioned in (Table 6).

Table 2: Pathways modulated by *Cucurbita pepo* compounds.

KEGG ID	Pathway name	Gene count	FDR	Genes set within the pathway
hsa03320	PPAR signaling pathway	10	1.40E-14	PPARG, FABP1, FABP3, FABP4, PPARG, RXRG, RXRB, PPARG, RXRA, NR1H3
hsa04931	Insulin resistance	5	4.68E-05	NR1H2, PTPRF, PPARG, IL6, NR1H3
hsa01100	Metabolic pathways	11	0.00013	CYP26B1, CYP51A1, CYP24A1, CYP26A1, CYP2C9, HMGCR, CYP11B1, CYP11B2, CYP17A1, CYP2C19, CYP19A1
hsa04920	Adipocytokine signaling pathway	4	0.00013	RXRG, RXRB, PPARG, RXRA
hsa04659	Th17 cell differentiation	4	0.00037	RXRG, RXRB, IL6, RXRA
hsa04932	Non-alcoholic fatty liver disease (NAFLD)	4	0.0014	PPARG, IL6, RXRA, NR1H3
hsa04976	Bile secretion	3	0.0021	HMGCR, RXRA, NR1H4
hsa00591	Linoleic acid metabolism	2	0.0067	CYP2C9, CYP2C19
hsa04152	AMPK signaling pathway	3	0.0067	PPARG, HMGCR, HNF4A
hsa00590	Arachidonic acid metabolism	2	0.0219	CYP2C9, CYP2C19
hsa00982	Drug-metabolism cytochrome P450	2	0.0233	CYP2C9, CYP2C19

Table 3: Binding energy and mode of interaction of each phytochemicals of *C. pepo* against NR1H3

Ligand	Binding energy (kcal/mol)	NHB	Hydrogen bond residue
Linolenic-Acid	-4.8	6	Val400, Asn377, Leu422, Leu425, Val426, Arg429
Cucurbit acid	-6.7	8	Leu453, Leu449, Phe271, Thr316, Phe329, Met312, Ala275, Trp457
Octadecanoic acid	-7.5	6	Trp457, His322, Tyr320, Arg361, Phe329, Arg319
Schottenol	-14.2	24	Hie421, Gln424, Val425, Leu428, Leu439, Phe234, Phe257, Thr238, Leu260, Ala261, Ser264, Ile339, Phe326, Phe335, Leu331, Arg305, Thr302, Glh301, Met298, Ile295, Trp443, Thr314, Ile313, Tyr321
Alpha-Spinasterol	-7.6	6	Gln280, Val279, Leu452, Ile456, Ile301, Leu304
Chondrillasterol	-8.9	6	Asn239, Phe329, Leu345, His435, Val439, Trp457
Dehydrovomifoliol	-5.8	1	Lys305
Clerosterol	-7.5	3	Leu452, Ile301, Val283
Codisterol	-7.5	4	Leu452, Val279, Ile456, Ile301
Isofucosterol	-7.5	4	Gln280, Val279, Leu452, Ile301

Cucurbitacin-D	-7.4	6	Glu323, His322, Tyr320, Arg361, Leu365, Arg319
Cucurbitacin-I	-7	3	Asp367, Arg318, Arg361
Dihydrobrassicasterol	-7.4	4	Gln280, Ile301, Leu301, Val279
Cryptoxanthin	-7.8	3	Leu330, Arg319, Arg316
Eicosadienoic acid	-6.4	3	His322, Arg316, Trp457
Delta-7-Ergosterol	-7.5	5	Gln280, Leu452, Val279, Ile456, Ile301
Absciscic acid	-6.8	5	Leu452, Val279, Arg361, Phe329, Met312,
7-beta,13-Dihydroxykaurenolide	-5.8	6	Val279, Leu452, Met312, Phe243, Arg316, Trp457

Table 4: Binding energy and mode of interaction of each phytochemicals of *C. pepo* against HMGCR

Ligand	Binding energy (Kcal/mol)	NHB	Hydrogen bond residue
Linolenic-Acid	-9.4	8	Leu345, His435, Val439, Trp457, Val279, Ile456, Ile301, Arg318
Alpha-Spinasterol	-12.4	10	Lys691, Asp690, Met655, Met657, Asn658, Ser661, Lys662, Glu665, Arg590, Val683
Chondrillasterol	-11.2	7	Pro813, Tyr517, Ile456, Ile301, His322, Tyr320, Arg361
Clerosterol	-9.9	6	Leu452, Ile301, Trp457, Val279, Ile456, Gln280, Leu452,
Codisterol	-10.4	6	Val279, Ile456, Ile301, His322, Tyr320, Arg318
Cryptoxanthin	-9.6	4	Leu811, Cys527, Gln280, Leu452,
Cucurbic acid	-10.5	5	Ile301, His322, Tyr320, Arg318
Cucurbitacin-D	-12.4	6	Cys527, Val530, Tyr519, Tyr533, Cys527, Val530
Cucurbitacin-I	-10.5	7	Cys527, Gln280, Leu452, His322, Tyr320, Arg361, Phe329
Dehydrovomifoliol	-8.9	8	Phe271, Thr316, Phe329, Leu449, Phe329, Phe271, Tyr320, Arg318
Delta-7-Ergosterol	-9.4	9	Val279, Leu452, Ile301, Val279, Ile456, Gln280, Leu452, Tyr320, Arg318

Fucosterol	-14.4	6	Arg361, Phe329, His322, Tyr320, Arg318.
Schottenol	-12.6	8	His322, Tyr320, Arg361, Phe329, His322, Tyr320, Arg318, Val530.
Octadecanoic acid	-11.5	6	Phe329, Leu449, Phe329, Phe271, Tyr320, Arg318
Dihydrobrassicasterol	-13.8	3	Phe329, Arg318, Gln280
Absciscic acid	-11.9	3	Arg319, Gln280, Phe243
7-beta,13-Dihydroxykaurenolide	-5.2	4	Met312, Arg316, Trp457, Gln280, Leu449

Table 5: Druggability and rule of five Drug likeness score of phytocompounds

Compounds		Lipinski Rule of Five					Side effects		
		Molecular weight (g/mol)	HBA	HBD	Log P	Drug likeness score	Pa	Pi	Side effect
S. No	Acceptable Values	< 500	< 5	< 5	< 10	0 to 2			
1	Linoleic acid	280.4	2	1	5.88	0.09	0.304	0.152	Nephrotoxicity
2	Cucurbic acid	212.28	3	2	1.82	-0.14	Nil	Nil	Nil
3	Octadecanoic acid	284.5	2	1	7.65	-0.54	0.500	0.050	Nephrotoxicity
							0.474	0.209	Hepatotoxicity
4	Schottenol	414.7	1	1	8.58	0.18	0.322	0.135	Nephrotoxicity
5	Alpha-Spinasterol	412.7	3	1	2.68	0.29	0.254	0.212	Nephrotoxicity
6	Chondrillasterol	412.7	1	1	7.87	-0.05	0.254	0.212	Nephrotoxicity
7	(+)-Dehydrovomifoliol	222.28	1	3	1.38	-1.14	Nil	Nil	Nil
8	Clerosterol	412.7	1	1	8.58	0.68	Nil	Nil	Nil

9	Codisterol	398.7	1	1	7.70	0.43	Nil	Nil	Nil
10	Isofucosterol	412.7	1	1	7.82	0.85	Nil	Nil	Nil
11	Cucurbitacin-D	516.7	7	4	2.61	0.39	Nil	Nil	Nil
12	Cucurbitacin-I	514.6	7	4	2.86	0.26	Nil	Nil	Nil
13	22-Dihydrobrassicasterol	400.37	1	1	7.87	0.59	Nil	Nil	Nil
14	Cryptoxanthin	552.9	1	1	12.83	0.78	0.7	0.012	Myocardial infraction
15	Eicosadienoic acid	308.5	2	1	-7.61	-0.30	0.304	0.152	Nephrotoxicity
16	Delta-7-Ergosterol	400.7	1	1	8.00	-0.03	0.28	0.179	Nephrotoxicity
17	Abscisic acid	264.32	4	2	2.56	-0.86	0.244	0.159	Nephrotoxicity
18	7 beta,13-Dihydroxykaurenolide	332.20	4	2	2.12	-0.34	0.312	0.294	Nephrotoxicity

Pa: Probable activity; Pi: Probable inactivity

Table 6: The ADME profile of each phytochemicals of *C. pepo* by Swiss ADME.

Compounds	GI absorption	BBB permeant	P-gp substrate	CYP 1A2 inhibitor	CYP2 C19 inhibitor	CYP 2C9 inhibitor	CYP 2D6 inhibitor	CYP 3A4 inhibitor	LogKp (skin permeation)
Linoleic acid	High	Yes	No	Yes	No	Yes	No	No	-3.05 cm/s
Cucurbitic acid	High	Yes	No	No	No	No	No	No	-6.26 cm/s
Octadecanoic acid	High	No	No	Yes	No	No	No	No	-2.19 cm/s
Schottenol	Low	No	No	No	No	No	No	No	-2.38 cm/s
Alpha-Spinasterol	Low	No	No	No	No	No	No	No	-2.92 cm/s
Chondrillasterol	Low	No	No	No	No	No	No	No	-2.92 cm/s
(+)-Dehydrovomifolol	High	Yes	No	No	No	No	No	No	-7.30 cm/s
Clerosterol	Low	No	No	No	No	Yes	No	No	-2.24 cm/s
Codisterol	Low	No	No	No	No	Yes	No	No	-2.53 cm/s
Isofucosterol	Low	No	No	No	No	No	No	No	-2.53 cm/s
Cucurbitacin-D	High	No	Yes	No	No	No	No	Yes	-7.99 cm/s
Cucurbitacin-I	Low	No	Yes	No	No	No	No	Yes	-7.54 cm/s
22-Dihydrobrassicasterol	Low	No	No	No	No	No	No	No	-2.50 cm/s
Cryptoxanthin	Low	No	Yes	No	No	No	No	No	-0.98 cm/s
Eicosadienoic acid	High	No	No	Yes	No	Yes	No	No	-2.47 cm/s
Delta-7-Ergosterol	Low	No	No	No	No	Yes	No	No	-3.44 cm/
Abscisic acid	Low	No	No	No	No	No	No	No	-2.24 cm/s
7 beta,13-Dihydroxykaurenone	High	No	No	Yes	No	No	No	No	-2.45cm/s



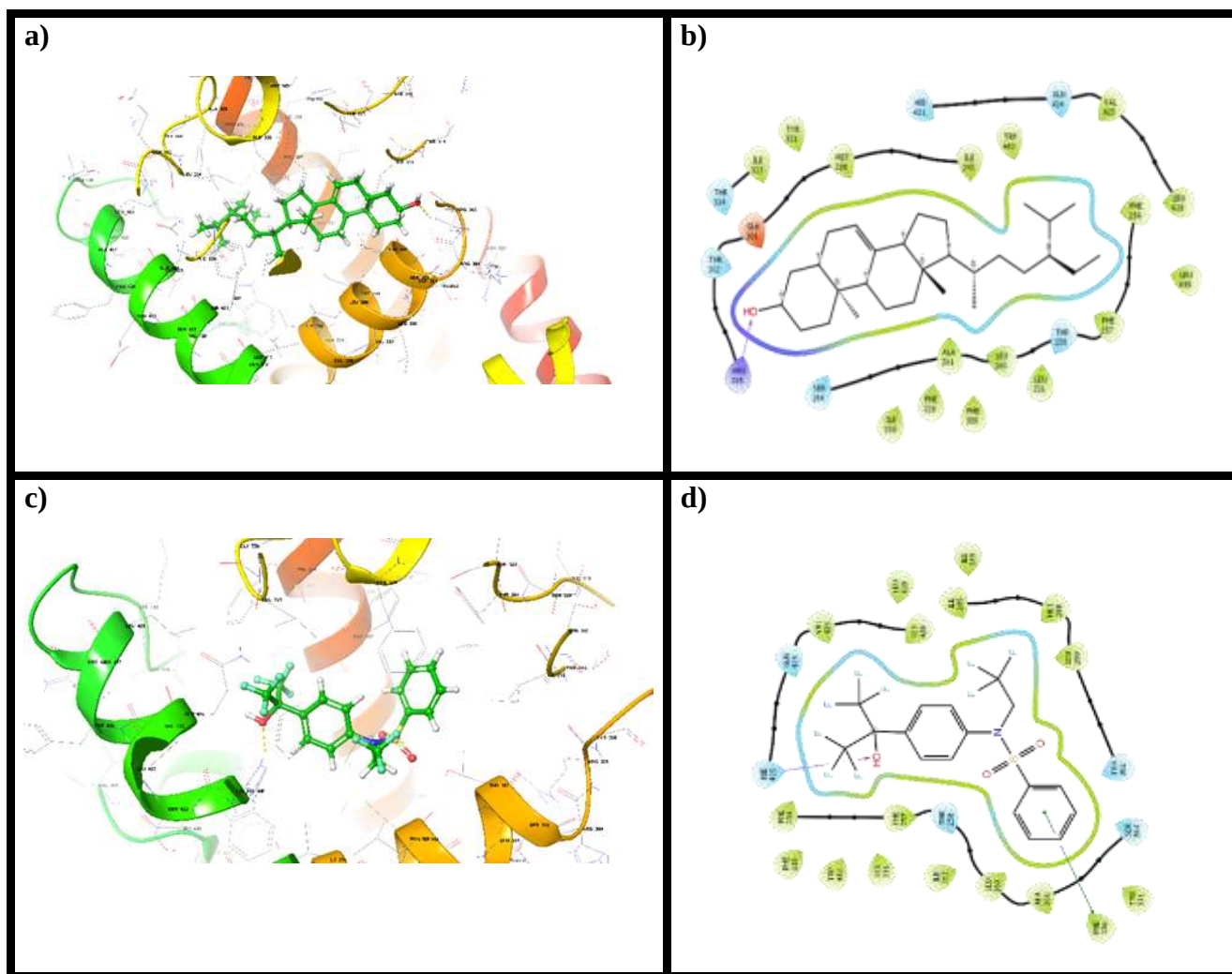


Figure 15: (a) 3D and (b) 2D binding orientation of Schottenol ligand in NR1H3 binding pocket. (c) 3D and (d) 2D binding orientation of cocrystal ligand in NR1H3 binding pocket.

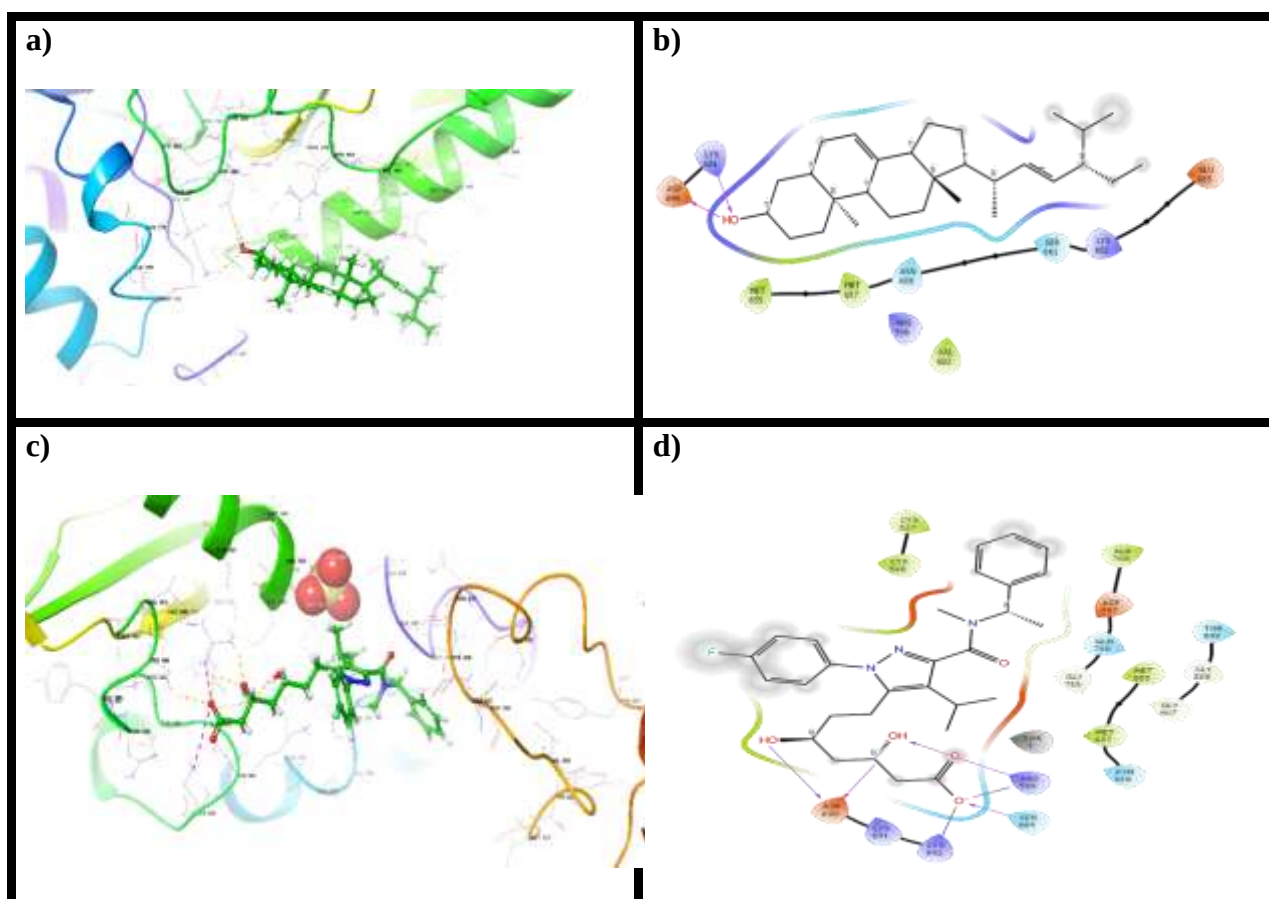


Figure 16: (a) 3D and (b) 2D binding orientation of Alpha-Spinasterol ligand in HMGR binding pocket. (c) 3D and (d) 2D binding orientation of cocrystal ligand in HMGR binding pocket.

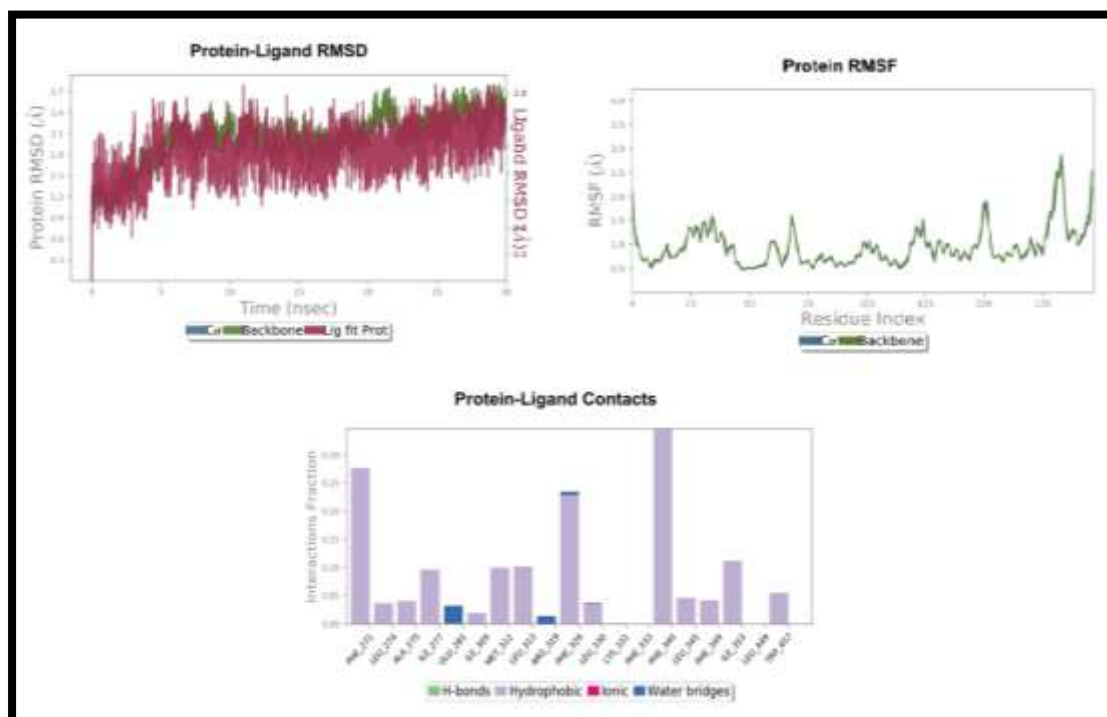


Figure 17: Stability of Schottenol with NR1H3.

In silico* pharmacology of *C. melo

Mining of phytoconstituents and their target prediction

160 phytoconstituents from *C. melo* were mined from literature and open-source phytochemical databases. These 160 phytocompounds were predicted to target 105 protein molecules in Swiss Target Prediction server with $p > 0.1$.

Gene enrichment and network analysis

The gene set enrichment analysis revealed, out of 105 targets, 20 targets potentially to modulate 6 molecular pathways directly associated with liver cirrhosis. Out of 160 compounds, 16 compounds were found to target these 20 targets. These phytoconstituents majorly are of steroids. Among 6 pathways (Figure 18) targeted by phytocompounds, PPAR signaling pathway was the hub pathway within the network with gene count of 8 (PPARG, FABP1, FABP3, FABP4, PPARG, RXRG, RXRB, PPARG, RXRA, NR1H3) and lowest FDR of $1.95E-13$ and Insulin resistance as second modulated pathway with lowest FDR value. AMPK signaling pathway, Pathways in cancer, Non-alcoholic fatty liver disease (NAFLD) and Hepatitis C pathways were directly linked in the pathogenesis of liver disease (Table 7).

Docking studies

The binding energy and mode of interaction of each phytocompounds against FXR receptor are summarized in Table 8. Within the network, FXR was identified as hub genes and were considered for further docking analysis. Among the 16 compounds targeting FXR, Euphol formed lowest BE of -10.033kcal/mol by forming highest number of bonds with FXR i.e. 21 (Trp454, Trp459, Ile352, Phe366, His447, Met365, Met450, Tyr369, Leu287, Met290, Ala291, Hie294, Phe336, Ile335, Ser332,

Arg331, Leu348, Phe329, Met328, Tyr361, Ile362) (refer Figure 19). Whereas, Silymarin scored BE of -10.973kcal/mol with forming 26 bonds (Hie294, Met265, Ala291, Met290, Leu348, Leu287, Tyr369, Trp454, Trp469, Phe366, Leu451, Met365, Met450, Ile357, Ile362, His447, Tyr361, Met328, Phe329, Ile352, Arg331, Ser332, Ile273, Ile335, Phe336, Thr270) (refer Figure 20).

Molecular Dynamics

Structural stability of the Silymarin with Farnesoid X receptor (FXR).

The complex “Silymarin- Farnesoid X receptor” showed a stable dynamic during the 100 ns of MD simulation run (Figure 21). After ~20 ns equilibration period, the average backbone root mean square deviation (RMSD) and complex was ~ 2.2 Å and ~ 2.8 Å respectively. The c-alpha atoms at N- terminal residues showed a highest fluctuation at (~ 4.82 Å), also due to loop formation on the binding site if the protein showed the highest fluctuation because of the enhanced flexibility was observed at ~ 5.3 Å with the residues between *Lys339* to *Gly356*. Similarly, a moderate peak were also observed at (~ 2.1 Å), (~ 2.6 Å), and (~ 3.06 Å) with the interacting residues *Thr270* to *Leu285*, *Gly435*, *Arg436* and *Glu467* respectively. There is a gradual increase in Rg value was seen and a stable complex formation was seen after ~ 15 ns. After ~ 15 ns, the complex Rg value was found to be ~ 1.85 Å. Similarly, the initial and final surface area occupied by the Silymarin complex with *FXR* were found to be 133.24 nm² and 128.7 nm² respectively. Additionally, the Silymarin- *FXR* complex formed 5 hydrogen bonds, of which 2 were consistent throughout the simulation run.

Structural stability of the Euphol with Farnesoid X receptor (FXR)

The complex Euphol- Farnesoid X receptor all-atom explicit MD simulation trajectory revealed stable dynamics during the 100 ns run. After ~ 10 ns equilibration period, the average RMSD for backbone and complex was observed to be ~ 1.92 Å and ~ 2.86 Å respectively. The c-alpha atoms at N- terminal showed a maximum fluctuation at (~ 5.89 Å) followed by (~ 3.8 Å) and (~ 3.24 Å) with residues *His242*, *Met243*, *Lys338* to *His344*, and *Lys420* to *Leu437* respectively. All the peaks formed a flexible loop located at the binding site, further due to the flexible nature towards the binding pocket and most having a contact with the complex formation (Figure 22). The gradual increase in Rg value was seen, and a stable complex formation was seen after the ~ 15 ns. After ~ 15 ns, the Euphol- FXR complex the Rg value was found to be ~ 1.832 Å. Additionally, it was observed that the initial and final SASA occupied by the complex were 129.94 nm^2 and 127.38 nm^2 respectively, which represents the higher flexibility of the binding pocket in the presence of Euphol. Euphol formed 3 hydrogen bonds, of which one was seen consistent over 100 ns run.

Druggability (ADME) and side effects of compounds

Out of 16 compounds, Sitosterol scored the highest DLS score of 0.78. Except for 6 compounds (Taraxerol, Lupeol, beta-Amyrin, Multiflorenol, 24-Methylenecycloartanol and Cycloartenol) all other compounds exhibited positive DLS score ranging from 0.10 to 0.78. Out of 16 compounds, only two compounds Avenasterol and Sitosterol showed Nephrotoxicity with Pa value 0.246 and 0.258 respectively. All the compounds were found to show low GI absorption, no BBB permeability, no inhibition for CYP1A2, CYP2C19, CYP2D6, CYP2C9 (except Stigmasterol and Codisterol), and CYP3A4. Table 9, Table 10, and Table 11 shows the ADMET profile of compounds.

Table 7: Liver cirrhosis pathway and gene enrichment analysis

Pathway	Description	Gene count	False Discovery Rate	Genes
hsa03320	PPAR signaling pathway	8	1.95E-13	FABP4, PPARG, FABP1, FABP5, PPARD, FABP3, PPARA, FXR
hsa04931	Insulin resistance	4	0.00012	NR1H2, PPARA, IL6, FXR
hsa04152	AMPK signaling pathway	3	0.0039	IGF1R, PPARG, HMGCR, FXR
hsa05200	Pathways in cancer	6	0.00014	IGF1R, PPARG, PPARD, AR, IL6ST, IL6
hsa04932	Non-alcoholic fatty liver disease (NAFLD)	3	0.0058	PPARA, IL6, FXR
hsa05160	Hepatitis C	2	0.042	PPARA, FXR

(Fatty acid binding protein adipocyte) FABP4; Fatty acid-binding protein, liver (FABP1); Fatty acid binding protein muscle (FABP3); Fatty acid binding protein skin (FABP5); Peroxisome proliferator-activated receptor alpha (PPARA); Peroxisome proliferator-activated receptor delta (PPARD); Peroxisome proliferator-activated receptor gamma (PPARG); Farnesoid X receptor (FXR); Androgen receptor (AR); Interleukin-6 (IL6); Insulin like growth factor1 receptor (IGF1R); Interleukin 6 cytokine family signal transduced (IL6ST); Nuclear Receptor Subfamily 1 Group H Member 2(NR1H2; Nuclear Receptor Subfamily 1 Group H Member 3(NR1H3); HMG-CoA reductase (HMGCR).

Table 8: Binding energy and mode of interaction of each phytochemicals of *C. melo* against FXR target.

Compound name	Binding energy (Kcal/mol)	NHB	Hydrogen bond residue
Silymarin	-10.973	26	His294, Met265, Ala291, Met290, Leu348, Leu287, Tyr369, Trp454, Trp469, Phe366, Leu451, Met365, Met450, Ile357, Ile362, His447, Tyr361, Met328, Phe329, Ile352, Arg331, Ser332, Ile273, Ile335, Phe336, Thr270
Euphol	-10.033	21	Trp454, Trp459, Ile352, Phe366, His447, Met365, Met450, Tyr369, Leu287, Met290, Ala291, His294, Phe336, Ile335, Ser332, Arg331, Leu348, Phe329, Met328, Tyr361, Ile362
Tirucallol	-9.024	14	Trp457, Ser377, Leu422, Leu425, Val426, Arg429, Met365, Met450, Ile357, Ile362, Phe329, Met312, Phe241, Thr318,
Alpha-amyrin	-9.011	12	Leu453, Leu449, Phe271, Thr316, Phe329, Met312, Asn275, Trp457, Leu422, Tyr321, Met332, Phe259.
Taraxerol	-8.019	7	Trp457, His322, Tyr320, Arg361, Phe329, Arg319, Leu422,
Avenasterol	-7.089	6	Val400, Asn377, Leu422, Leu425, Val426, Arg429
Stigmasterol	-7.045	8	Leu453, Leu449, Phe271, Thr316, Phe329, Met312, Ala275, Trp457

Sitosterol	-7.01	6	Trp457, Is322, Tyr320, Arg361, Phe329, Arg319, Et312, Ala275, Trp457
Lupeol	-6.089		Val400, Asn377, Leu422, Leu425, Val426, Arg429
beta-Amyrin	-6.045	8	Leu453, Leu449, Phe271, Thr316, Phe329, Met312, Ala275, Trp457
beta-Sitosterol	-6.034	6	Trp457, His322, Tyr320, Arg361, Phe329, Arg319
Multiflorenol	-6.012	6	Val400, Asn377, Leu422, Leu425, Val426, Arg429
Codisterol	-6.009	10	Leu453, Leu449, Phe271, Thr316, Phe329, Met312, Ala275, Trp457, Arg361, Phe329,
22-dihydrobrassicasterol	-5.081	9	Trp457, His322, Tyr320, Arg361, Phe329, Arg319, Met312, Ala275, Trp457
24-Methylenecycloartanol	-5.074	6	Val400, Asn377, Leu422, Leu425, Val426, Arg429
Campesterol	-5.074	8	Leu453, Leu449, Phe271, Thr316, Phe329, Met312, Ala275, Trp457
Cycloartenol	-5.045	6	Trp457, His322, Tyr320, Arg361, Phe329, Arg319

NHB: Number of hydrogen bonds

Table 9: Phytocompounds of *C. melo* which were retrieved from PubChem possess Drug likeness score.

S. No	Compounds name	Lipinski Rules				
		Molecular wt. (g/mol)	HBA	HBD	Log P	Drug likeness score
		Acceptable Values				
		< 500	< 5	< 5	< 10	0 to 2
1.	Euphol	426.7	1	1	8.74	0.55
2.	Tirucallol	426.7			8.74	0.55
3.	Alpha-amyrin	426.7	1	1	7.77	0.10
4.	Taraxerol	426.7	1	1	8.11	-0.90
5.	Avenasterol	412.7	2	1	7.95	0.25
6.	Stigmasterol	412.7	1	1	7.74	0.62
7.	Sitosterol	414.7	1	1	8.45	0.78
8.	Lupeol	426.39	1	1	8.35	-0.22
9.	beta-Amyrin	426.7	1	1	7.95	-0.22
10.	beta-Sitosterol	414.39	1	1	8.45	0.78
11.	Multiflorenol	426.39	1	1	7.95	-0.27
12.	Codisterol	398.7	1	1	7.70	0.43
13.	22- dihydrobrassica sterol	400.7	1	1	7.87	0.59
14.	24- Methylenecyclo artanol	440.7	1	1	8.49	-0.48
15.	Campesterol	400.37	1	1	7.87	0.59
16.	Cycloartenol	426.7	1	1	8.56	-0.27

Table 10: Toxicity screening of phytocompounds from *Cucumis melo*

S. No.	Cucumis melo	Pa	Pi	Side Effect
1.	Euphol	-	-	-
2.	Tirucallol	-	-	-
3.	Alpha-amyrin	-	-	-
4.	Avenasterol	0.246	0.228	Nephrotoxicity
5.	Sitosterol	0.258	0.205	Nephrotoxicity
6.	Taraxerol	-	-	-
7.	beta Amyrin	-	-	-
8.	beta-Sitosterol	-	-	-
9.	Multiflorenol	-	-	-
10.	Codisterol	-	-	-
11.	22-dihydrobrassicasterol	-	-	-
12.	24-Methylenecycloartanol	-	-	-
13.	Campesterol	-	-	-
14.	Cycloartenol	-	-	-
15.	beta-Sitosterol	-	-	-
16.	Multiflorenol			

Pa: Probable activity to cause toxicity;

Pi: Probable inactivity to cause nontoxicity

Table 11: ADME profile of each phytochemicals from *Cucumis melo*

Compounds	GI Absorption	BBB permeability	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	LogKp (skin permeation)
Euphol	Low	No	No	No	No	No	No	-2.58
Tirucallol	Low	No	No	No	No	No	No	-2.58
Alpha-amyrin	Low	No	No	No	No	No	No	-2.51
Taraxerol	Low	No	No	No	No	No	No	-2.3
Avenasterol	Low	No	No	No	No	No	No	-2.72
Stigmasterol	Low	No	No	No	Yes	No	No	-2.74
Sitosterol	Low	No	No	No	No	No	No	-2.2
Lupeol	Low	No	No	No	No	No	No	-1.9
beta-Amyrin	Low	No	No	No	No	No	No	-2.41
beta-Sitosterol	Low	No	No	No	No	No	No	-2.2
Multiflorenol	Low	No	No	No	No	No	No	-2.41
Codisterol	Low	No	No	No	Yes	No	No	-2.53
22-dihydrobrassicasterol	Low	No	No	No	No	No	No	-2.5
24-Methylenecycloartanol	Low	No	No	No	No	No	No	-1.67
Campesterol	Low	No	No	No	No	No	No	-2.5
Cycloartenol	Low	No	No	No	No	No	No	-1.96

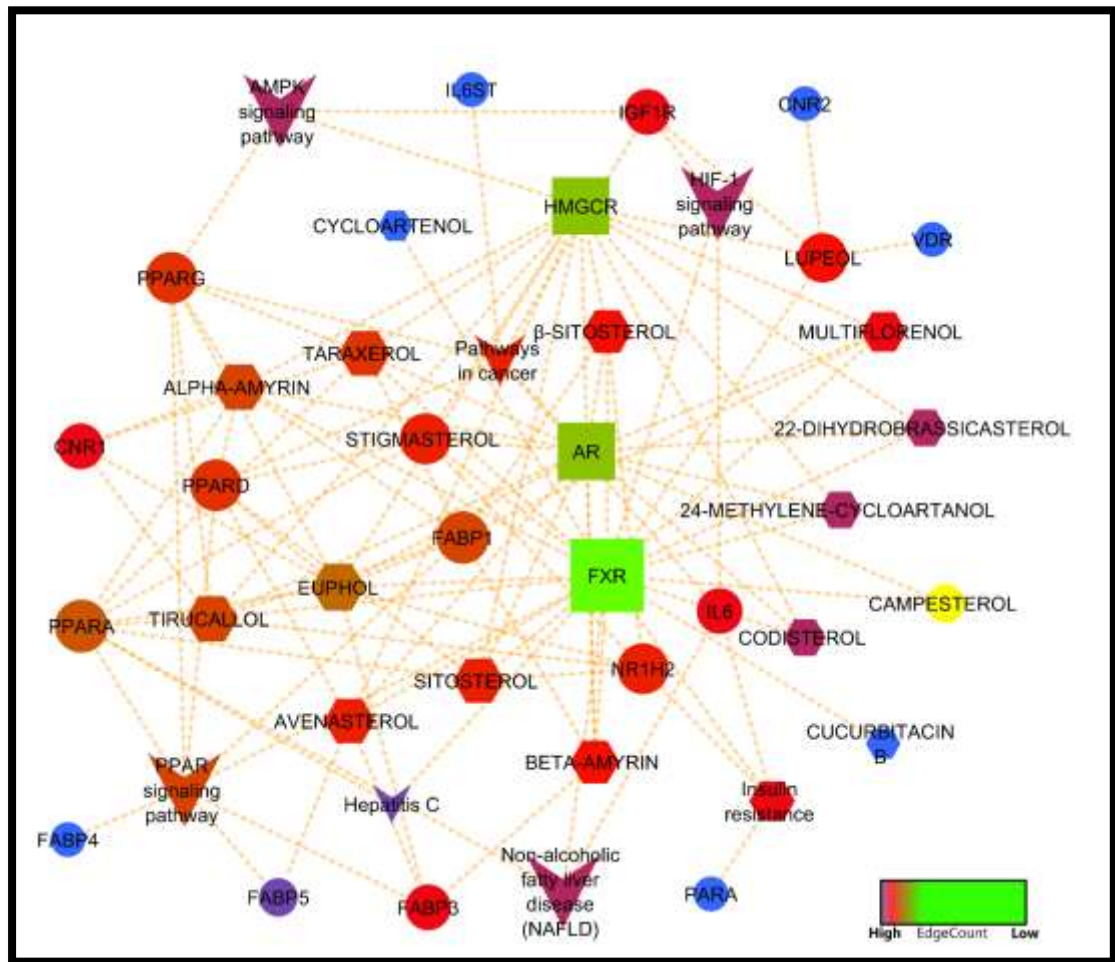


Figure 18: Network analysis of phytocompounds from *C. melo*, their targets, and modulated pathways of liver cirrhosis. Large size nodes and edges represent highly significant compounds, proteins, and pathways, whereas small size nodes and edges represent less modulated compounds, proteins and pathways.

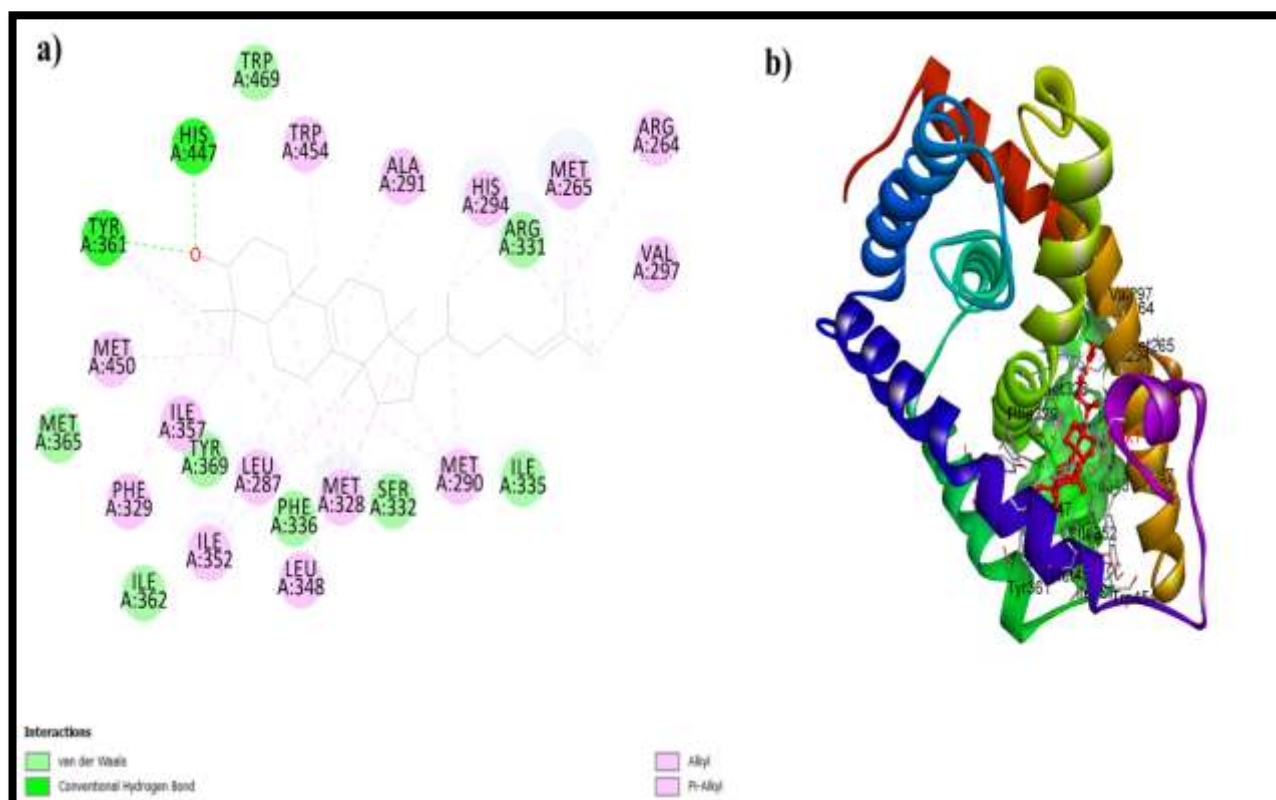


Figure 19: Shows (a) the 2D interaction of the binding mode of Euphol with FXR, (b) 3D binding mode of Euphol with FXR.

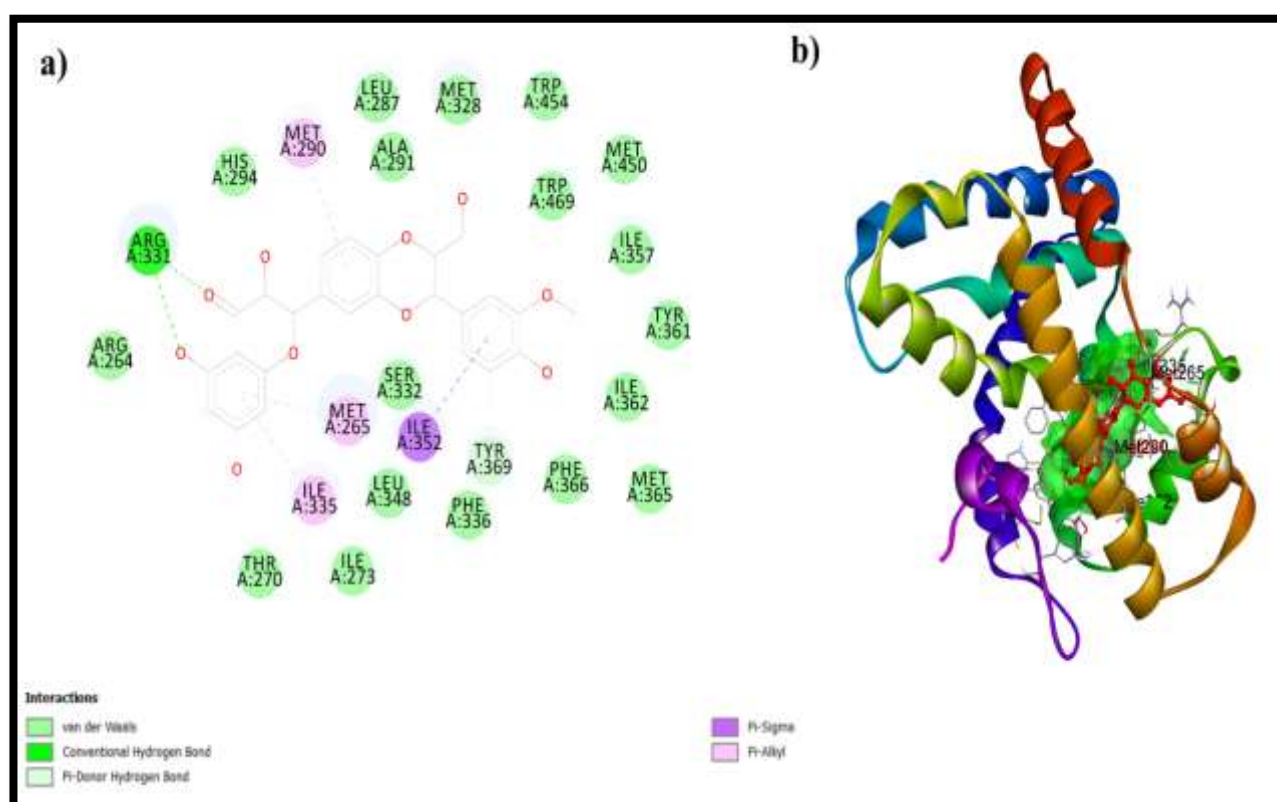


Figure 20: Shows (a) the 2D interaction of the binding mode of Silymarin with FXR, (b) 3D binding mode of Silymarin with FXR.

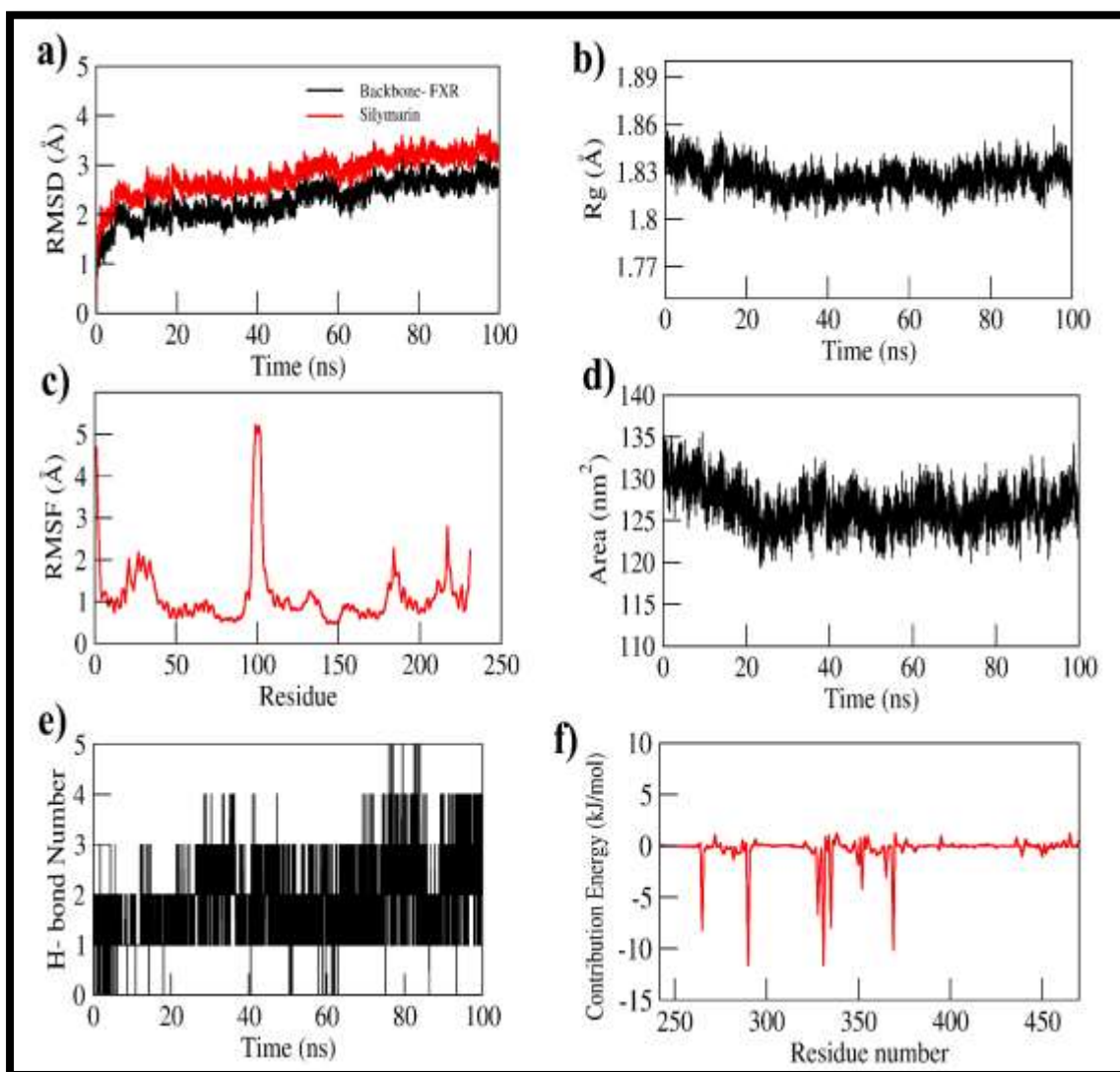


Figure 21: Parameters describing the stability of Silymarin with FXR complex.

(a) RMSD plot for FXR backbone and Silymarin complex, (b) Radius of gyration, (c) C-alpha RMSF, (d) Protein SASA, (e) number of hydrogen bond interactions formed during 100ns MD simulation and (f) per residue energy contribution.

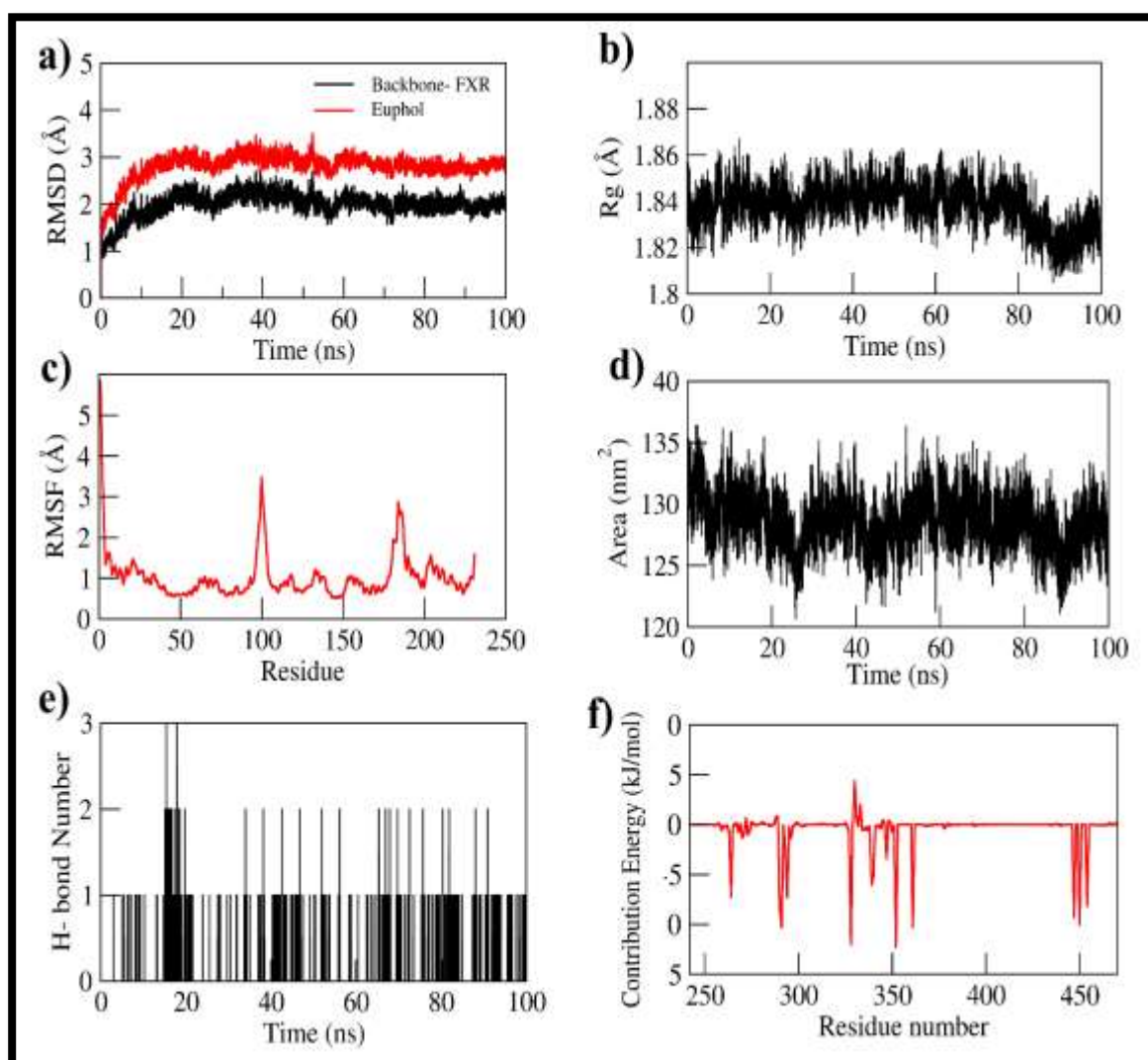


Figure 22: Parameters describing the stability of Euphol with FXR complex. (a) RMSD plot for FXR backbone and Euphol complex, (b) Radius of gyration, (c) C-alpha RMSF, (d) Protein SASA, (e) number of hydrogen bond interactions formed during 100ns MD simulation and (f) per residue energy contribution.

Isoniazid modulated molecular pathways (network analysis)

A total of 182 pathways were found to modulate by INH of which TNF and PPAR signalling pathways scored for highest gene count. Among the modulated genes, GSK3B and PPARG were found to be the hub genes within the network (Figure 23).

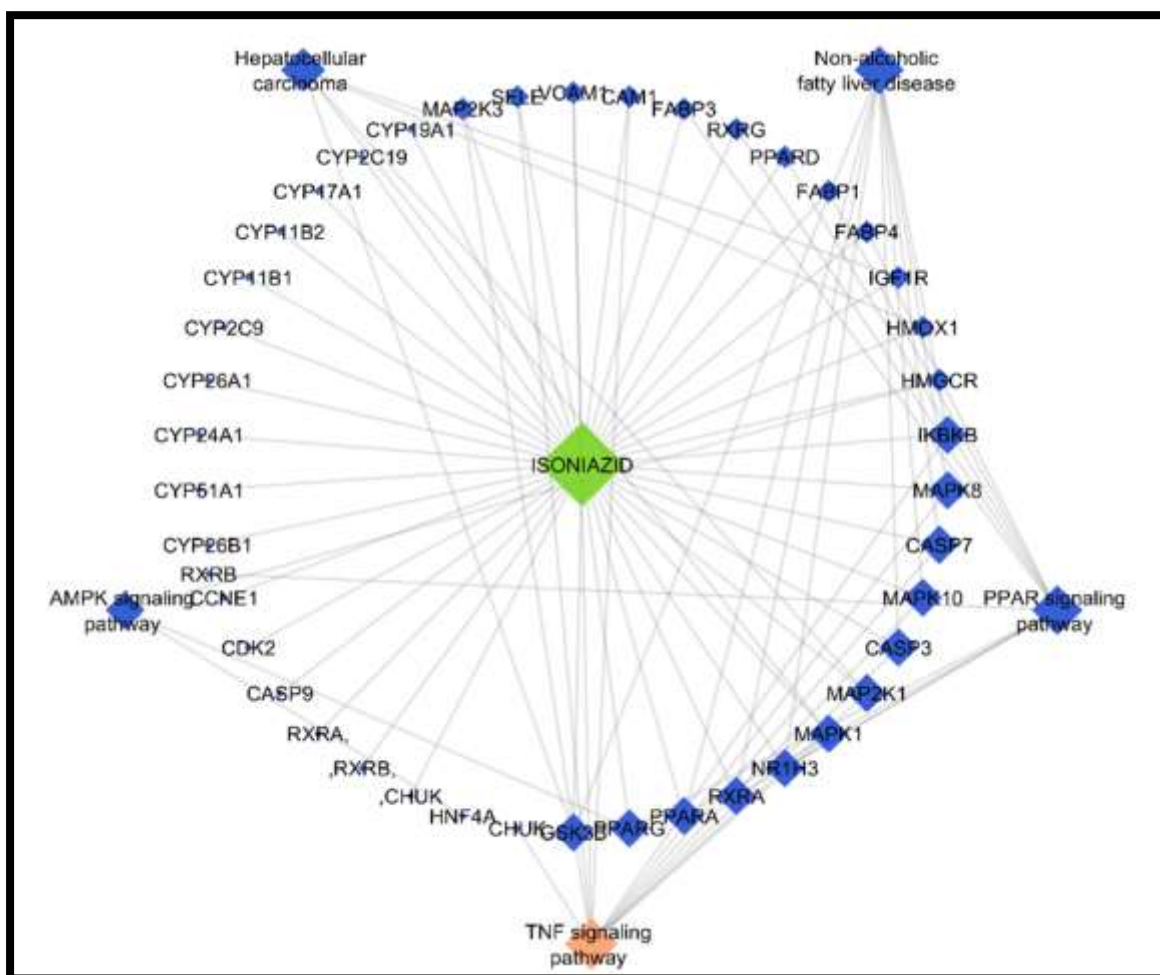


Figure 23: Network representation of Genes and Pathways modulated in INH induced hepatotoxicity





In vitro* studies*Extraction and preliminary phytochemical experiments**

1. The crude material of *C. pepo* showed LOD (Loss on Drying) 14% w/w, Total Ash 3%w/w, Acid Insoluble Ash Value 0.5% w/w, Water Soluble Ash Value 2% w/w, Alcohol Soluble Extract 10% w/w, and Water Soluble Extract 8% w/w. Whereas the crude material of *C. melo* showed LOD (Loss on Drying) 12% w/w, Total Ash-4%w/w, Acid insoluble Ash Value-0.5% w/w, Water Soluble Ash Value 3% w/w, Alcohol Soluble Extract-12% w/w, and Water Soluble Extract 10% w/w.
2. Both *C. pepo* and *C. melo* showed the presence of alkaloids, glycosides, saponins, steroids, tannins, flavonoids and proteins. (Table 12)

Table 12: Preliminary Phytochemical screening of *C. pepo* and *C. melo*

S. No.	Phytoconstituents	Test	Result
1	Alkaloid	Mayer's test	+ve
		Wagner's test	+ve
		Dragendroff's test	+ve
		Hager's test	+ve
2	Glycosides	Molisch test	+ve
		Fehling's test	+ve
		Barfoed's test	+ve
3	Saponins	Foam test	+ve
4	Steroids	Salkowski test	+ve
5	Tannins	FeCl ₃	+ve
6	Flavonoids	FeCl ₃	-ve
		NaOH solution test	+ve
		Lead acetate test	+ve
		Mineral acid test	-ve
7	Proteins	Biurette test	-ve

+ve: Present, -ve: absent

LC-MS analysis

The following compounds were found to be present in the prepared extract

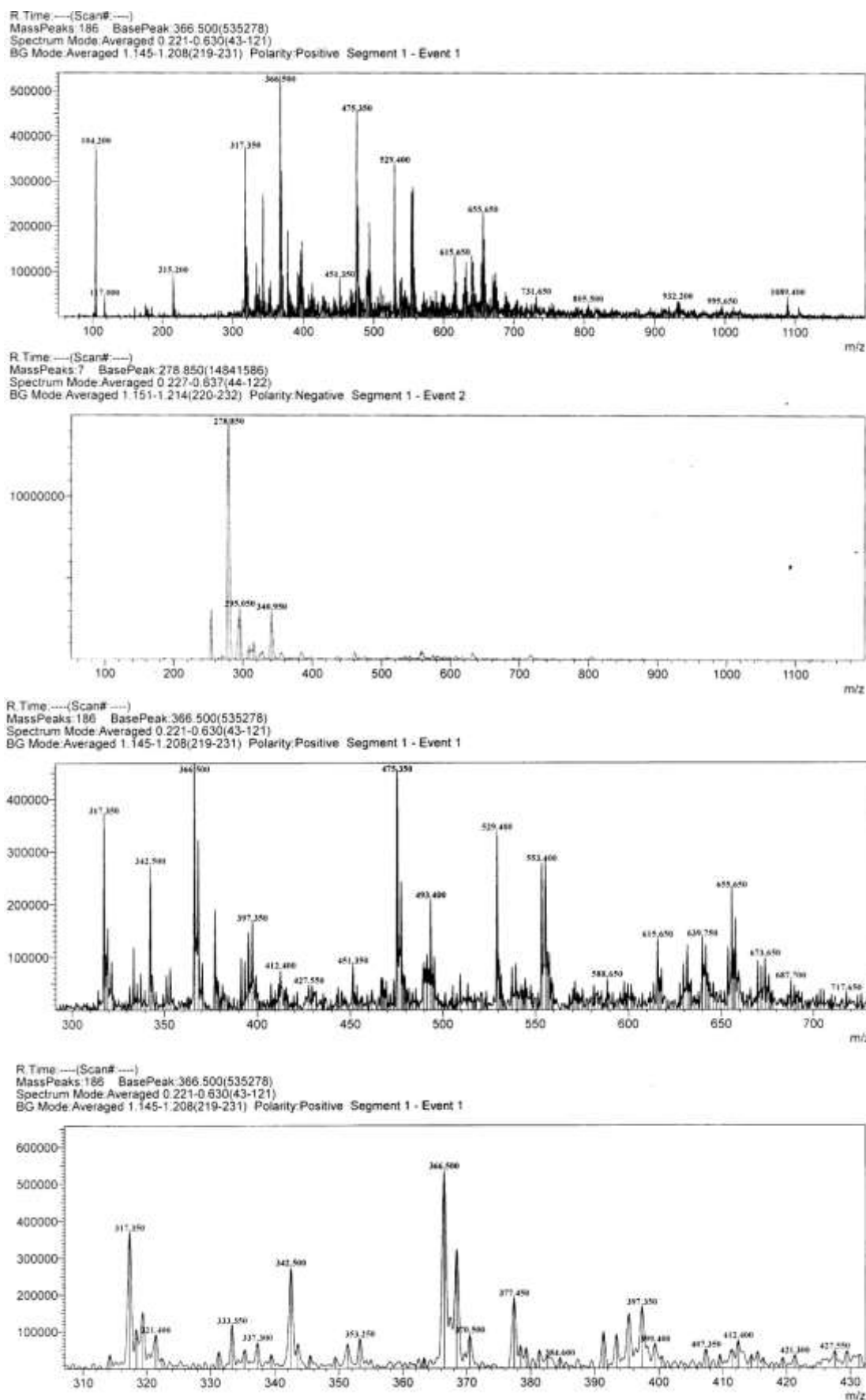
C. pepo (Figure 26 and Table 13) and *C. melo* (Figure 27).

Table 13: LC-MS identified molecules from *C. pepo*

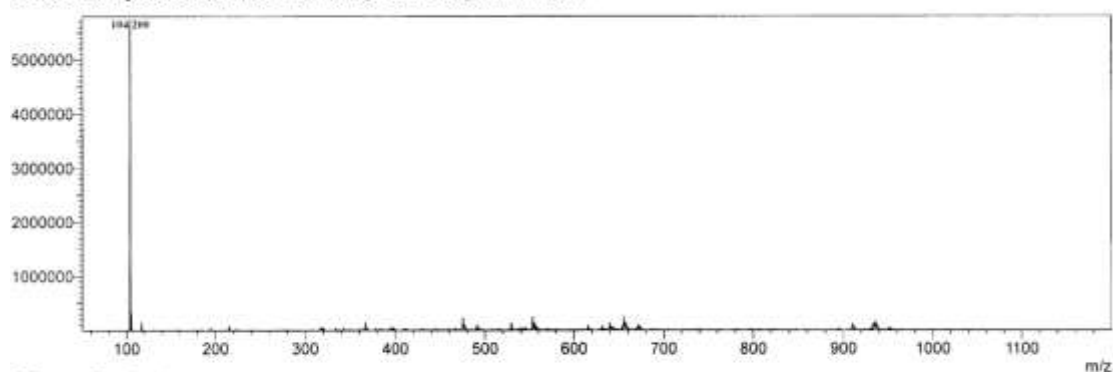
S. No	Compound	Molecular formula	Mol. Weight (g/mol)	Mol. Weight by m/z (g/mol)
<i>C. pepo</i>				
1.	Alpha-Linolenic-Acid	<u>C₁₈H₃₀O₂</u>	278.4	278.90
2.	Cucurbic acid	<u>C₁₂H₂₀O₃</u>	212.28	-
3.	Octadecanoic acid	C ₁₈ H ₃₆ O ₂	284.5	-
4.	Schottenol	<u>C₂₉H₅₀O</u>	414.7	-
5.	Alpha-Spinasterol	<u>C₂₉H₄₈O</u>	412.7	412.40
6.	Chondrillasterol	<u>C₂₉H₄₈O</u>	412.7	412.40
7.	(+)-Dehydrovomifoliol	<u>C₁₃H₁₈O₃</u>	222.28	-
8.	Clerosterol	<u>C₂₉H₄₈O</u>	412.7	412.40
9.	Codisterol	<u>C₂₈H₄₆O</u>	398.7	397.35
10.	Isofucosterol	C ₂₉ H ₄₈ O	412.7	412.40
11.	Cucurbitacin-D	<u>C₃₀H₄₄O₇</u>	516.7	-
12.	Cucurbitacin-I	<u>C₃₀H₄₂O₇</u>	514.6	-
13.	Dihydrobrassicasterol	<u>C₂₈H₄₈O</u>	400.7	399.4
14.	Cryptoxanthin	<u>C₄₀H₅₆O</u>	552.9	553.40
15.	Eicosadienoic-Acid	<u>C₂₀H₃₆O₂</u>	308.5	-
16.	Delta-7-Ergosterol	<u>C₂₈H₄₈O</u>	400.7	-
17.	Absciscic acid	<u>C₁₅H₂₀O₄</u>	264.32	-
18.	7-beta,13-Dihydroxykaurenolide	<u>C₂₀H₂₈O₄</u>	332.4	-

Table 14: LC-MS identified molecules from *C. melo*

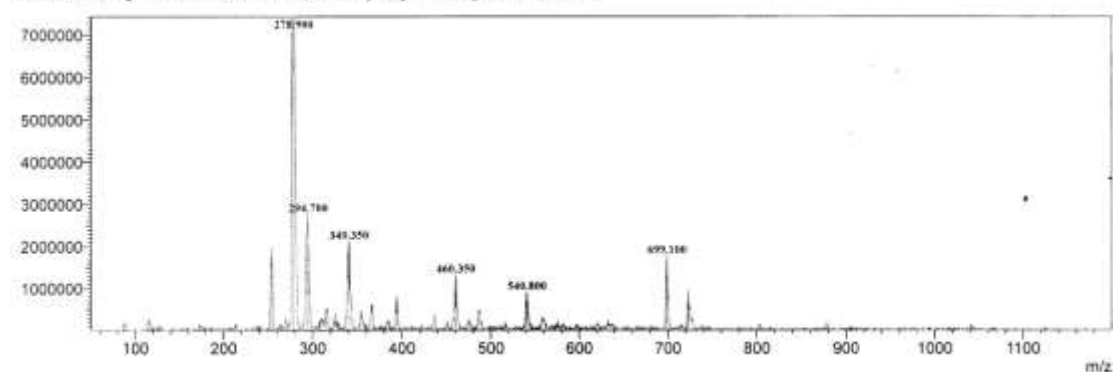
1.	Euphol	$\underline{C_{30}H_{50}O}$	426.7	460.25
2.	Tirucallol	$\underline{C_{30}H_{50}O}$	426.7	-
3.	Alpha-amyrin	$\underline{C_{30}H_{50}O}$	426.7	-
4.	Taraxerol	$\underline{C_{30}H_{50}O}$	426.7	-
5.	Avenasterol	$\underline{C_{29}H_{48}O}$	412.7	410.25
6.	Stigmasterol	$\underline{C_{29}H_{48}O}$	412.7	-
7.	Sitosterol	$\underline{C_{29}H_{50}O}$	414.7	-
8.	Lupeol	$\underline{C_{30}H_{50}O}$	426.7	-
9.	beta-Amyrin	$\underline{C_{30}H_{50}O}$	426.7	-
10.	beta-Sitosterol	$\underline{C_{29}H_{50}O}$	414.7	-
11.	Multiflorenol	$\underline{C_{30}H_{50}O}$	426.7	-
12.	Codisterol	$\underline{C_{28}H_{46}O}$	398.7	340.35
13.	22-dihydrobrassicasterol	$\underline{C_{28}H_{48}O}$	400.7	-
14.	24-Methylenecycloartanol	$\underline{C_{31}H_{52}O}$	440.7	-
15.	Campesterol	$\underline{C_{28}H_{48}O}$	400.7	-
16.	Cycloartenol	$\underline{C_{30}H_{50}O}$	426.7	-

Figure 26: LC-MS profile of *C. pepo*

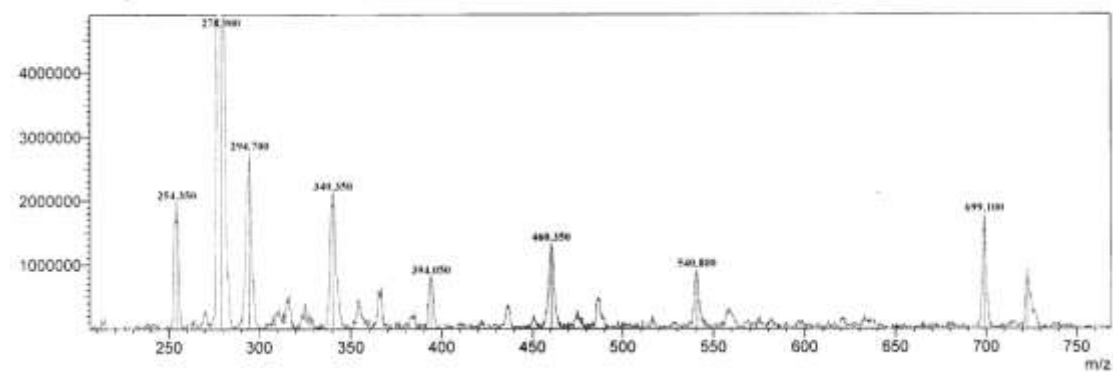
R Time:----(Scan#----)
MassPeaks:2 BasePeak:104.200(5739165)
Spectrum Mode:Averaged 0.200-0.546(39-105)
BG Mode:Averaged 0.882-0.914(169-175) Polarity:Positive Segment 1 - Event 1



R Time:----(Scan#----)
MassPeaks:9 BasePeak:278.900(14716492)
Spectrum Mode:Averaged 0.206-0.553(40-106)
BG Mode:Averaged 0.889-0.920(170-176) Polarity:Negative Segment 1 - Event 2



R Time:----(Scan#----)
MassPeaks:9 BasePeak:278.900(14716492)
Spectrum Mode:Averaged 0.206-0.553(40-106)
BG Mode:Averaged 0.889-0.920(170-176) Polarity:Negative Segment 1 - Event 2



R Time:----(Scan#----)
MassPeaks:9 BasePeak:278.900(14716492)
Spectrum Mode:Averaged 0.206-0.553(40-106)
BG Mode:Averaged 0.889-0.920(170-176) Polarity:Negative Segment 1 - Event 2

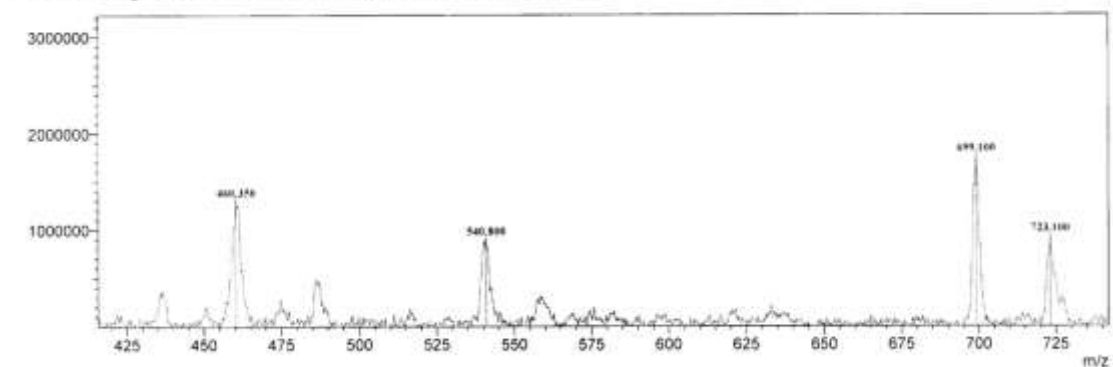


Figure 27: LC-MS profile of *C. melo*

DPPH free radical scavenging assay of *C. pepo* and *C. melo*

The IC₅₀ of ascorbic acid, *C. pepo* (Figure 28) and *C. melo* (Figure 29) for DPPH scavenging was found to be 49.89 µg/mL, 153 µg/mL and, 142.80 µg/mL and respectively.

Table 15: DPPH assay of *C. pepo*

Sample Absorbance				
Control	Ascorbic acid	C.pepo	$A=(A-B)/A*100$	$A=(A-C)/A*100$
A	B	C	39.26380368	35.58282209
0.163	0.099	0.105	57.05521472	48.46625767
0.163	0.07	0.084	63.19018405	57.66871166
0.163	0.06	0.069	75.4601227	69.93865031
0.163	0.04	0.049	87.73006135	80.98159509
0.163	0.02	0.031	$A=(A-B)/A*100$	$A=(A-C)/A*100$

% of Inibhition		
Concentration	%Ascorbic acid	% C.pepo
50	39.26	35.58
100	57.06	48.47
200	63.19	57.67
400	75.46	69.94
800	87.73	80.98

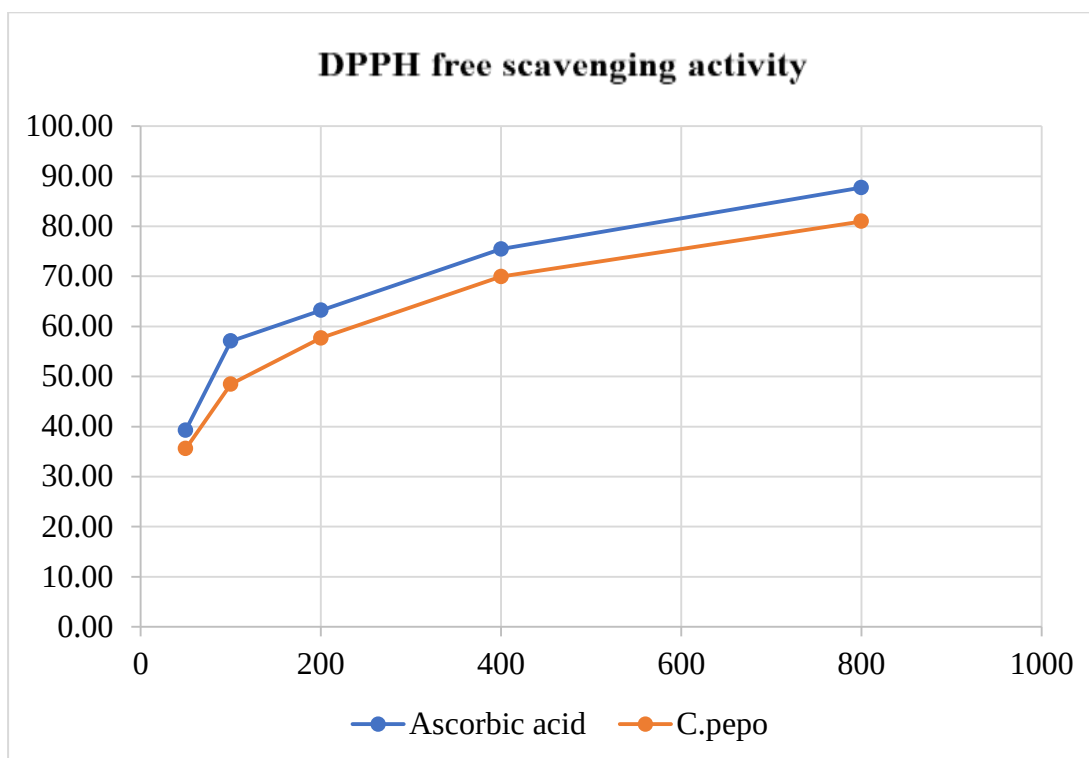


Figure 28: Linear regression curve and IC_{50} for *C. pepo* and Ascorbic acid in DPPH assay

			IC50 value
Ascorbic acid	$R^2 = 0.8686$	$y = 0.0529x + 55.496$	47.8919
C.pepo	$R^2 = 0.8688$	$y = 0.0543x + 41.692$	153.002

Table 16: DPPH assay of *C. melo*

Sample Absorbance				
Control Abs	Ascorbic acid	<i>C.melo</i>	% Ascorbic acid	% <i>C. melo</i>
A	B	C	$A=(A-B)/A*100$	$A=(A-C)/A*100$
0.163	0.099	0.106	39.26380368	34.96932515
0.163	0.07	0.086	57.05521472	47.2392638
0.163	0.06	0.065	63.19018405	60.12269939
0.163	0.04	0.044	75.4601227	73.00613497
0.163	0.02	0.032	87.73006135	80.36809816
0.163	0.04	0.034	75.4601227	46294.5538

% of Inibhition		
Concentrations	% Ascorbic acid	% <i>C.melo</i>
50	39.26	34.97
100	57.06	47.24
200	63.19	60.12
400	75.46	73.01
800	87.73	80.37

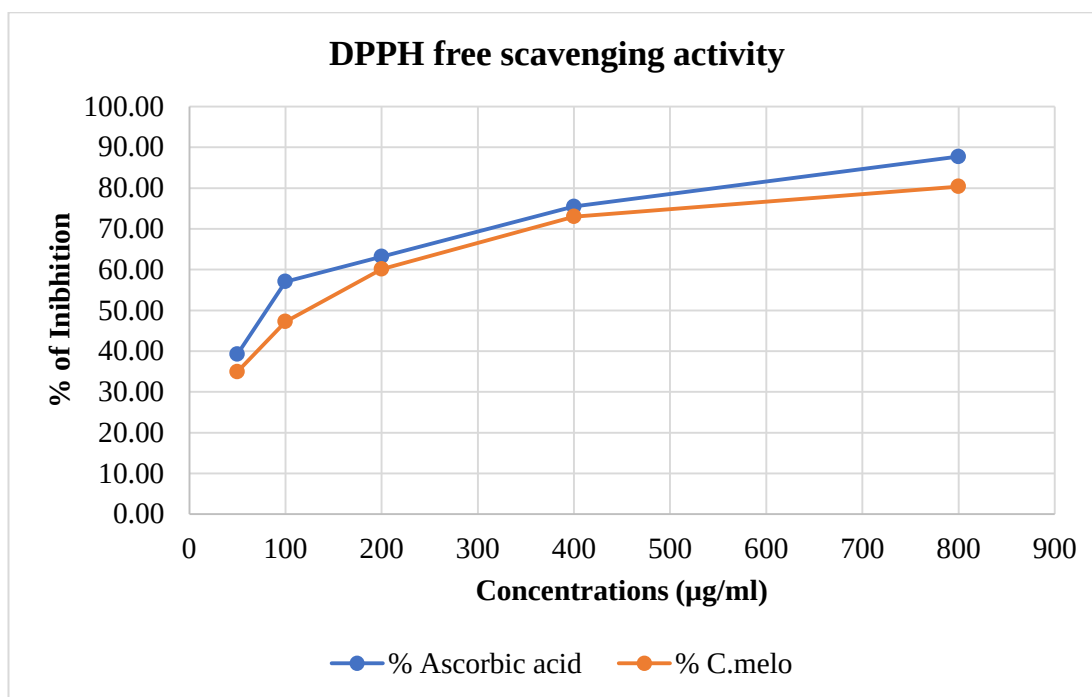


Figure 29: Linear regression curve and IC₅₀ for *C. melo* and Ascorbic acid in DPPH assay

			IC ₅₀ value
Ascorbic acid	$R^2 = 0.8458$	$y = 0.0555x + 47.342$	47.89189189
<i>C.melo</i>	$R^2 = 0.8108$	$y = 0.0546 + 42.203$	142.8021978

Study 2: *In vitro* studies

Cytotoxic effect of *C. melo* and *C. pepo* in INH and HFD treated HepG2 cell line

Cytotoxic effect of *C. melo* and *C. pepo* extract, fraction and standard drug on HepG2 cells was performed by using different concentration ranging 10-100 µg/mL (Table 13, Figure 30 and Figure 31). All the test drugs and standard drug showed changes in the viability of HepG2 cells, among them *C. melo* fraction (100 µg/mL) and *C. pepo* fraction (100 µg/mL) showed significant increase in viability on HepG2 cell lines.

Table 17: *In vitro* cytotoxic activity (MTT assay) of *C. melo* and *C. pepo* by INH and HFD model

Compound	Concentration	% of Cell Viability	% of Cell Viability
HepG2	-	99.89	99.89
INH	50 mM	54.11	--
HFD	1mM	--	59.40
Silymarin	100µg/ml	82.39	83.99
	50µg/ml	78.74	83.17
	25µg/ml	76.87	82.44
	12.5µg/ml	75.09	80.44
<i>C. melo</i> fraction	100µg/ml	76.07	81.98
	50µg/ml	75.09	79.81
	25µg/ml	73.59	78.36
	12.5µg/ml	72.89	74.49
	100µg/ml	74.62	80.89

<i>C. melo</i> extract	50µg/ml	73.68	78.49
	25µg/ml	72.09	75.36
	12.5µg/ml	69.87	71.49
<i>C. pepo</i> fraction	100µg/ml	75.37	80.59
	50µg/ml	73.68	76.28
	25µg/ml	72.03	74.38
	12.5µg/ml	70.88	72.12
<i>C. pepo</i> extract	100µg/ml	73.12	78.89
	50µg/ml	71.53	75.98
	25µg/ml	70.08	73.78
	12.5µg/ml	68.82	70.39

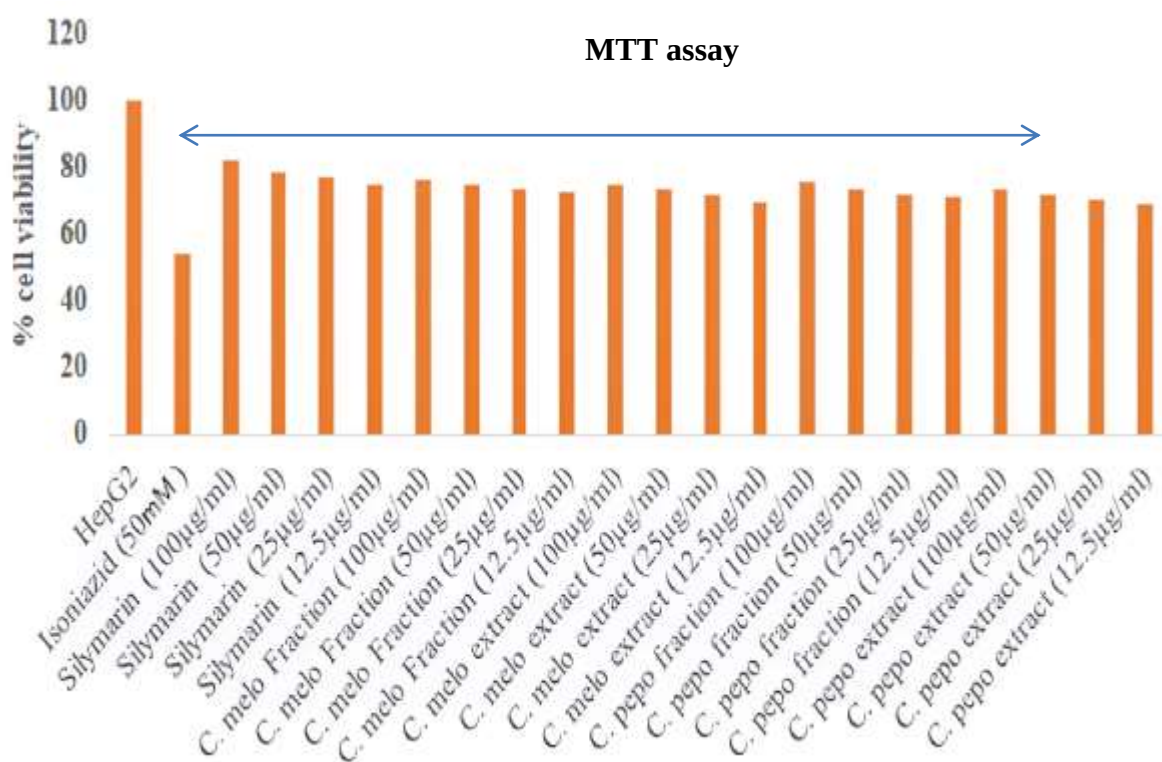


Figure 30: Cytotoxic effect of *C. pepo* and *C. melo* in HepG2 cell line

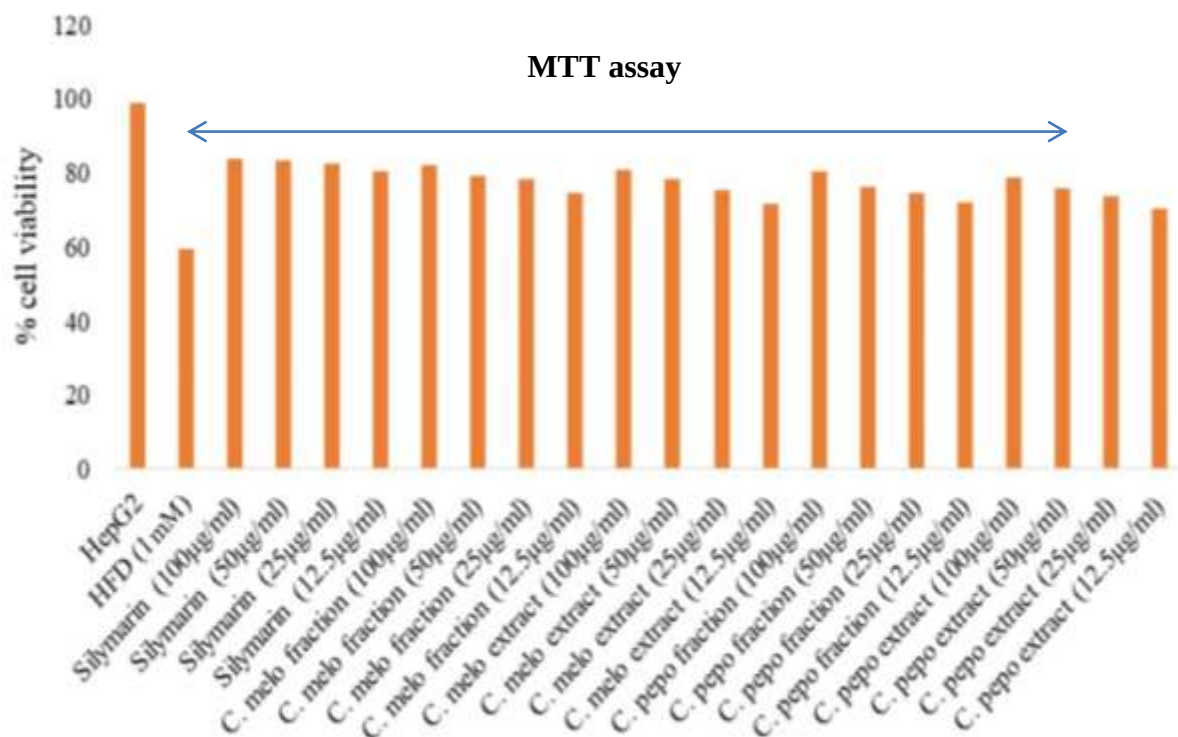


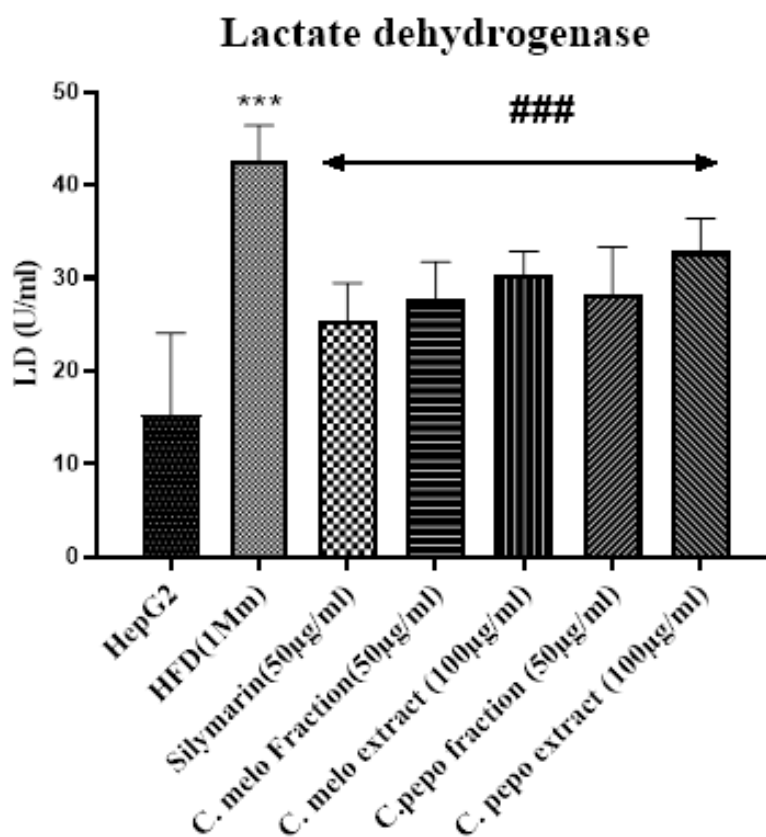
Figure 31: Cytotoxic effect of *C. melo* and *C. pepo* in HepG2 cell line.

Effect of the *C. melo* and *C. pepo* extract and fraction on palmitate-oleate (PO-BSA) induced steatosis in HepG2 cells

LDH, AST and ALT in the HepG2 cell supernatant was estimated to check the hepatoprotective effect (Figure 32 and Table 18). Pre-treatment with *C. melo* and *C. pepo* extracts and fractions showed decrease in the LDH, AST, and ALP level. Among them *C. melo* fraction and *C. pepo* fraction showed significant decrease ($p > 0.01$; 27.67, 30.67, 37.67 IU/L) ($p > 0.01$; 28.34, 32.67, 39.62 IU/L) in LDH, AST, and ALP level as compared to HFD treated group ($p > 0.01$; 42.67, 42.34, 52.68)

Table 18: *In vitro* hepatoprotective activity of *C. melo* and *C. pepo* using HepG2 cell lines.

Group Name	% LDH(IU/L)	ALT (IU/L)	AST (IUL)
HepG2	15.33±8.74	17.33±1.15	28.67±1.53
HFD (1Mm)	42.67±3.79***	42.34±1.53***	52.68±6.02***
Silymarin (50µg/ml) + HFD	25.38±4.09 ^{###}	28.58±1.29 ^{###}	32.14±4.08 ^{###}
<i>C. melo</i> fraction (50µg/ml) + HFD	27.67±4.05 ^{###}	30.67±1.52 ^{###}	37.67±5.03 ^{###}
<i>C. melo</i> extract (100µg/ml) + HFD	30.34±2.52 ^{##}	34.34±4.17 ^{##}	40.34±1.52 ^{##}
<i>C. pepo</i> fraction (50µg/ml) + HFD	28.34±5.03 ^{###}	32.67±5.51 ^{##}	39.62±3.09 ^{###}
<i>C. pepo</i> extract (100µg/ml) + HFD	32.86±3.58 ^{###}	35.18±3.17 ^{##}	42.12±1.45 ^{##}



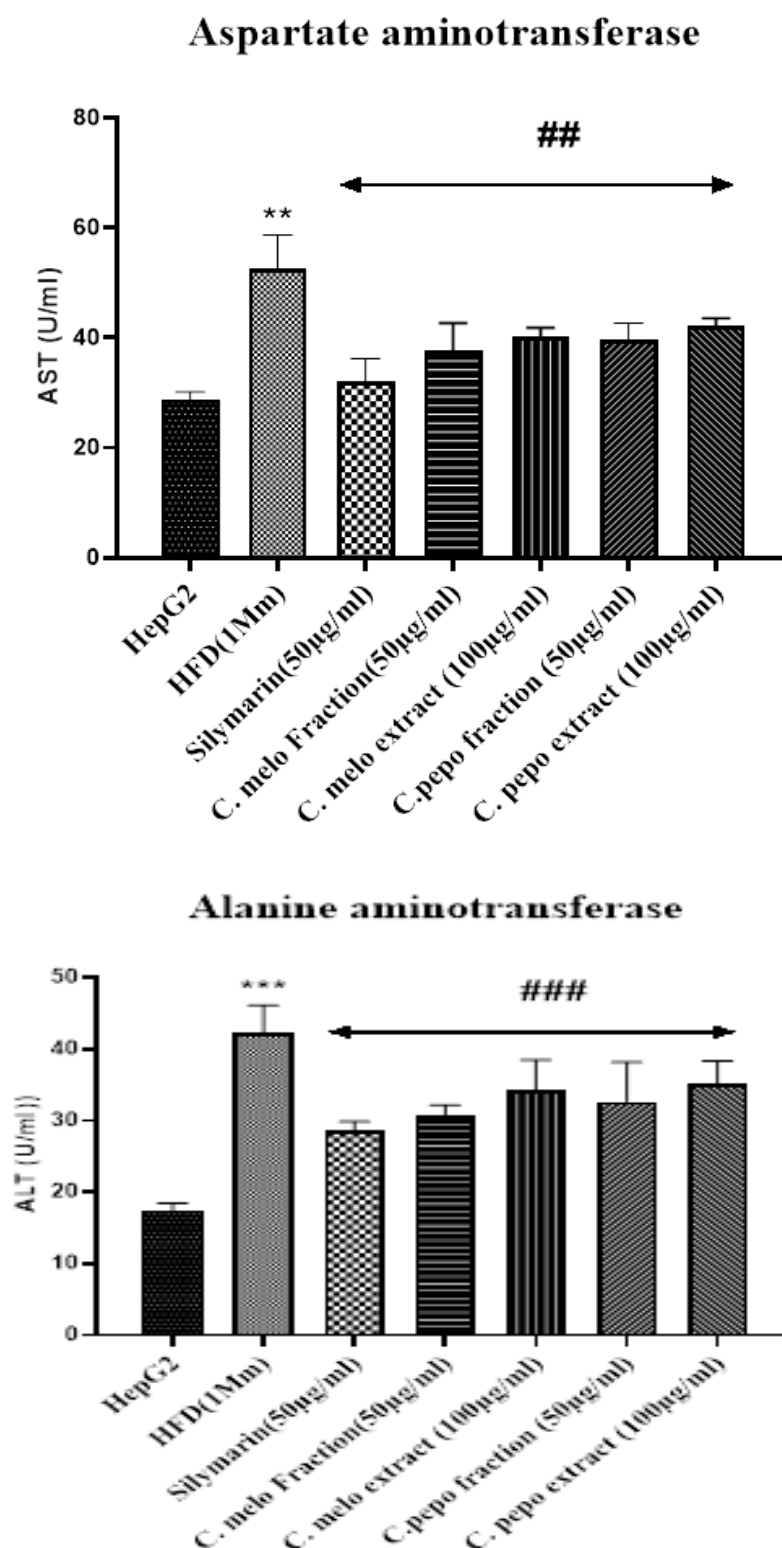


Figure 32: Effect of extract, fraction, and Silymarin on LDH, AST, and ALT in HepG2 cell line. Tukey's multiple comparison test was applied of one-way ANOVA on the data, which were presented as Mean $\pm$ SEM (n=6) using GraphPad prism 8.0.2. *** $p < 0.01$ compared with normal HepG2 group, ## $p < 0.01$ compared with HFD group.

Study 3: In vivo study**Effect of *C. melo* and *C. pepo* on INH induced hepatotoxicity****Effect on body weight and liver weight**

A significant increase ($p < 0.01$; 4.66g) in liver weight was observed in INH group compared to normal (2.99g) which was significantly reversed in *C. melo* extract ($p < 0.05$; 3.46g), *C. melo* fraction ($p < 0.05$; 3.48g), *C. pepo* fraction ($p < 0.05$; 3.42g), and Silymarin ($p < 0.05$; 3.43g) treated groups (Figure 34, Table 15). However, initially, no significant change on body weight was observed in all the treated groups (BW ~190 to 195g), however, after the treatment there is a observable increase in the body weight was seen in INH treated group (non-significant), and treatment with *C. melo* and *C. pepo* fraction also showed observable decrease in the body weight and *C. pepo* extract; 500mg/kg showed significant decrease ($p < 0.05$) in body weight compared to INH group. (Figure 33, Table 19).

Table 19: Effect of *C. melo* and *C. pepo* on body weight and liver weight

Groups	Body weight		Liver weight
	Initial	After	
I (Normal)	190.33 $\pm$ 4.48	221.5 $\pm$ 2.43	2.99 $\pm$ 0.04
II (INH control; 50 mg/kg, p.o)	192.83 $\pm$ 6.06	230.83 $\pm$ 4.39 ^{ns}	4.66 $\pm$ 0.14**
III (Standard; 100 mg/kg, p.o)	190 $\pm$ 7.97	209 $\pm$ 2.78 [#]	3.43 $\pm$ 0.13 ^{##}
IV (<i>C. melo</i> fraction; 50 mg/kg, p.o.)	191.67 $\pm$ 2.63	224 $\pm$ 6.27 ^{ns}	3.48 $\pm$ 0.09 ^{##}
V (<i>C. melo</i> Extract; 500mg/kg p.o.)	192.33 $\pm$ 6.93	221.67 $\pm$ 2.5 ^{ns}	3.46 $\pm$ 0.13 ^{##}
VI (<i>C. pepo</i> fraction; 50 mg/kg, p.o.)	191.83 $\pm$ 6.15	217.33 $\pm$ 7.11 ^{ns}	3.42 $\pm$ 0.12 ^{##}
VII (<i>C. pepo</i> extract; 500mg/kg p.o.)	192 $\pm$ 5.81	209.5 $\pm$ 6.41 [#]	3.24 $\pm$ 0.06 ^{##}

Data are presented as Mean $\pm$ SEM (n=6), one-way ANOVA followed by Tukey's t test. **p < 0.01 compared with normal group. #p < 0.05 compared with INH; "ns" represents non-significant.

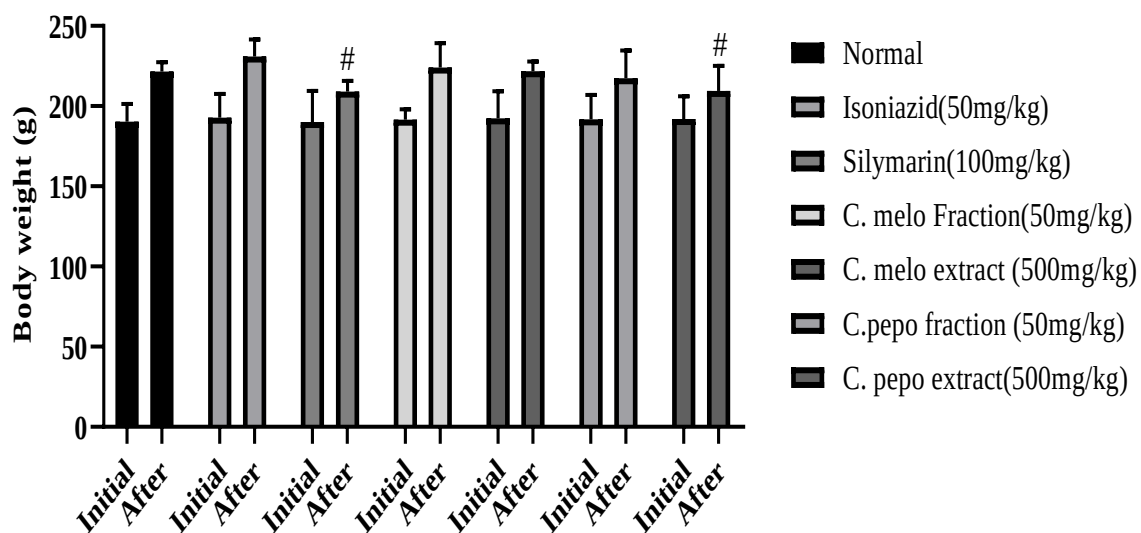


Figure 33: Effect of *C. melo* and *C. pepo* of body weight

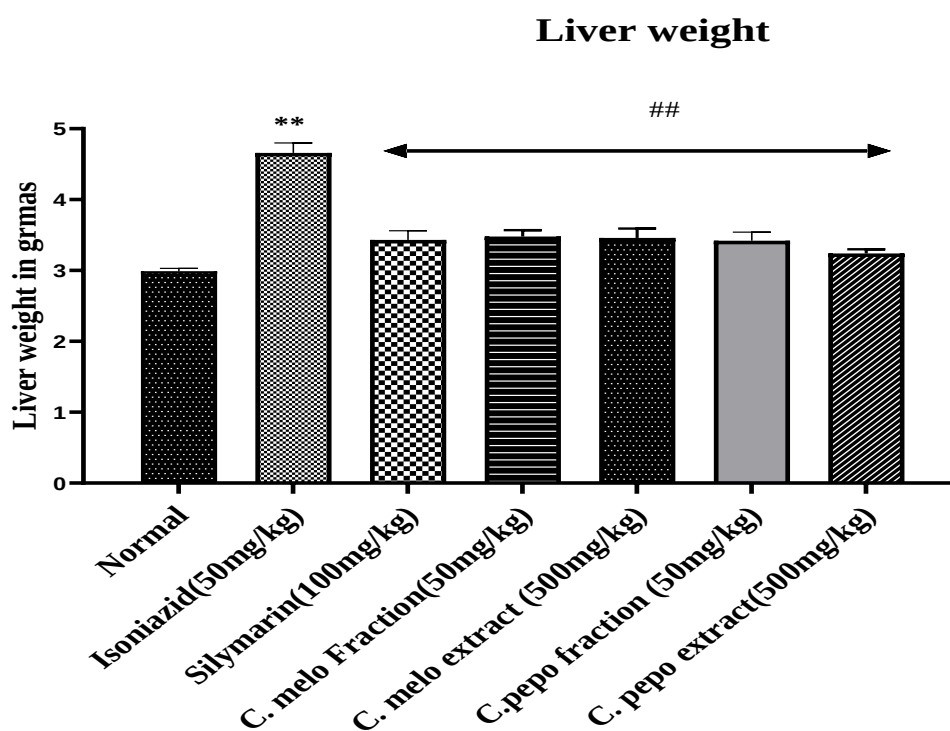


Figure 34: Effect of *C. melo* and *C. pepo* of liver weight

Effect of *C. pepo* and *C. melo* on liver enzymes

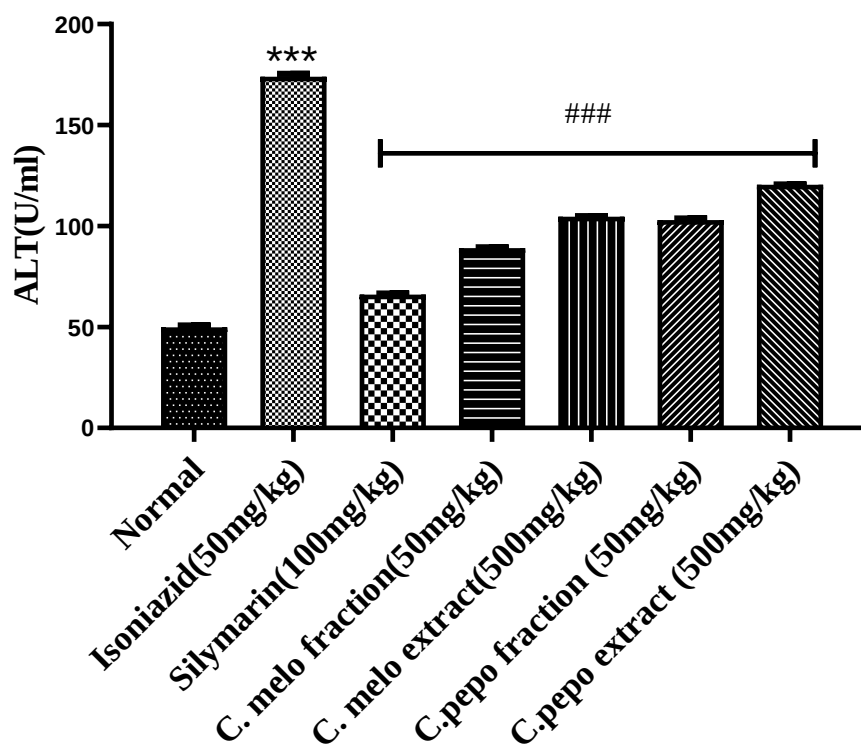
ALT, AST, and ALP were significantly increased ($p < 0.001$; 174, 234, 209.4U/ml) in the INH group compared to normal group (49.90, 77.85, 93.23U/ml). Treatment with *C. melo* (89.06, 122.28, 109.32U/ml for fraction and 104.60, 154.4, 122.38U/ml for extract) and *C. pepo* (102.90, 152.8, 109.69U/ml for fraction and 120.40, 164.6, 124.34U/ml for extract) was significantly ($p < 0.01$ to $p < 0.001$) reversed in all the treated groups compared to INH group. Similarly, total bilirubin and serum bilirubin were significantly increased ($p < 0.001$; 1.06, 4.64mg/dL) in the INH group compared to normal (0.47, 1.68mg/dL). Treatment with *C. melo* and *C. pepo* was significantly ($p < 0.01$ to $p < 0.001$) reversed in all the treated groups compared to INH group (Table 20 and Figure 35A-E).

Table 20: Effect of *C. melo* and *C. pepo* on ALT, AST, ALP, serum bilirubin and Total bilirubin in INH -induced hepatotoxicity.

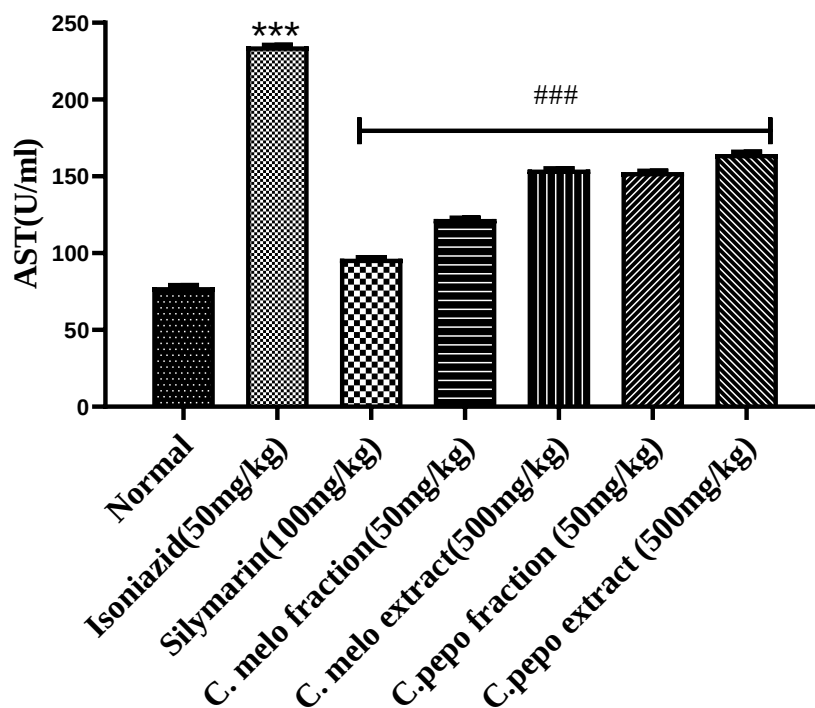
GROUPS	Serum Biochemical Parameters				
	ALT (U/ml)	AST (U/ml)	ALP (U/ml)	Serum Bilirubin (mg/dL)	Total Bilirubin (mg/dL)
I (Normal)	49.90±0.82	77.85±0.88	93.23±1.24	0.47±0.0045	1.68±0.0024
II (INH control; 50 mg/kg, p.o)	174.00±1.34***	234.60±0.52***	209.4±2.74***	1.06±0.0055***	4.64±0.019***
III (Standard; 50 mg/kg, p.o)	66.04±0.50 ^{###}	96.46±0.42 ^{###}	107.00±0.60 ^{###}	0.52±0.0062 ^{###}	1.89±0.0036 ^{##}
IV (<i>C. melo</i> fraction; 50 mg/kg, p.o.)	89.06±0.40 ^{###}	122.28±0.38 ^{###}	109.32±0.20 ^{###}	0.59±0.0042 ^{###}	2.48±0.0024 ^{##}
V (<i>C. melo</i> Extract; 500mg/kg p.o.)	104.60±0.32 ^{###}	154.4±0.28 ^{###}	122.38±0.24 ^{##}	0.88±0.0024 ^{##}	2.79±0.0043 ^{###}
VI (<i>C. pepo</i> fraction; 50 mg/kg, p.o.)	102.90±0.84 ^{###}	152.8±0.34 ^{###}	109.69±0.64 ^{##}	0.62±0.0034 ^{##}	2.62±0.0089 ^{###}
VII (<i>C. pepo</i> extract; 500mg/kg p.o.)	120.40±0.24 ^{##}	164.6±0.89 ^{##}	124.34±0.22 [#]	0.92±0.0069 [#]	2.94±0.0019 ^{##}

Data are presented as Mean±SEM (n=6), one way ANOVA followed by Tukey's t test. ***p < 0.01 compared with normal group. #p < 0.05, ##p < 0.01, ###P < 0.001 compared with INH control group.

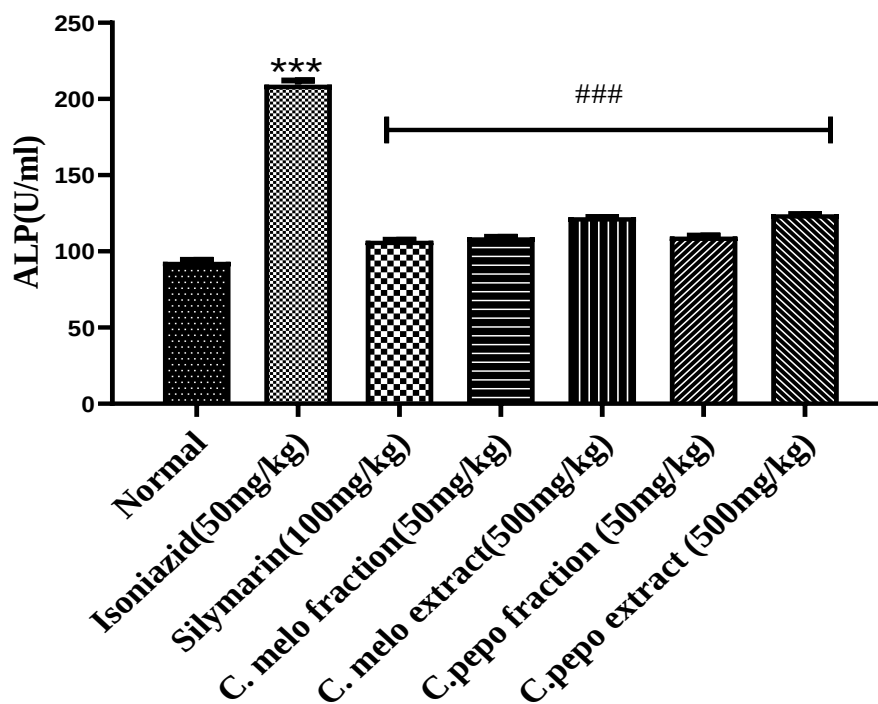
Alanine aminotransferase

A: Effect of *C. melo* and *C. pepo* on ALT.

Aspartate transferase

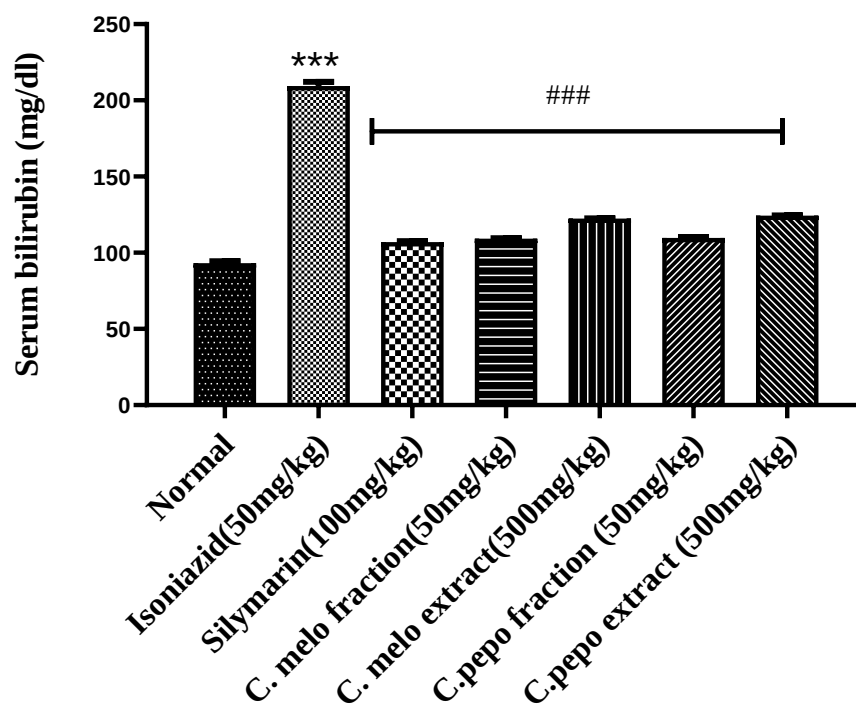
B: Effect of *C. melo* and *C. pepo* on AST.

Alkaline Phosphotase

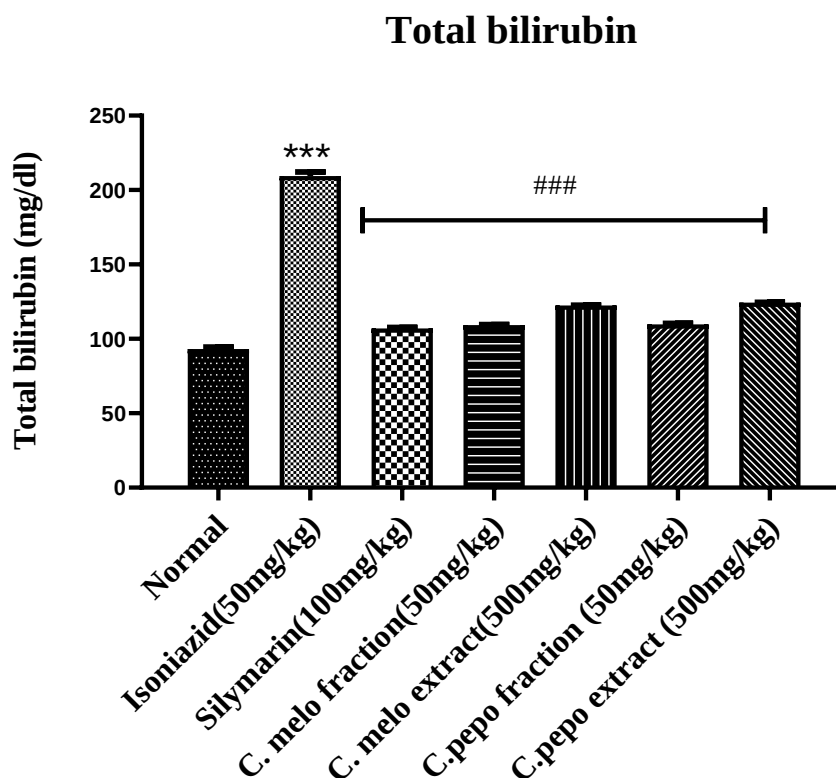


C: Effect of *C. melo* and *C. pepo* on ALP.

Serum bilirubin



D: Effect of *C. melo* and *C. pepo* on Serum Bilirubin.



E: Effect of *C. melo* and *C. pepo* on Total bilirubin.

Figure 35: Effect of *C. melo* and *C. pepo* on A) ALT, B) AST, C) ALP, D) Serum Bilirubin, and E) Total Bilirubin. ** $P < 0.01$ and * $P < 0.001$ compared to normal; ### $P < 0.01$ and ### $P < 0.001$ compared to INH**

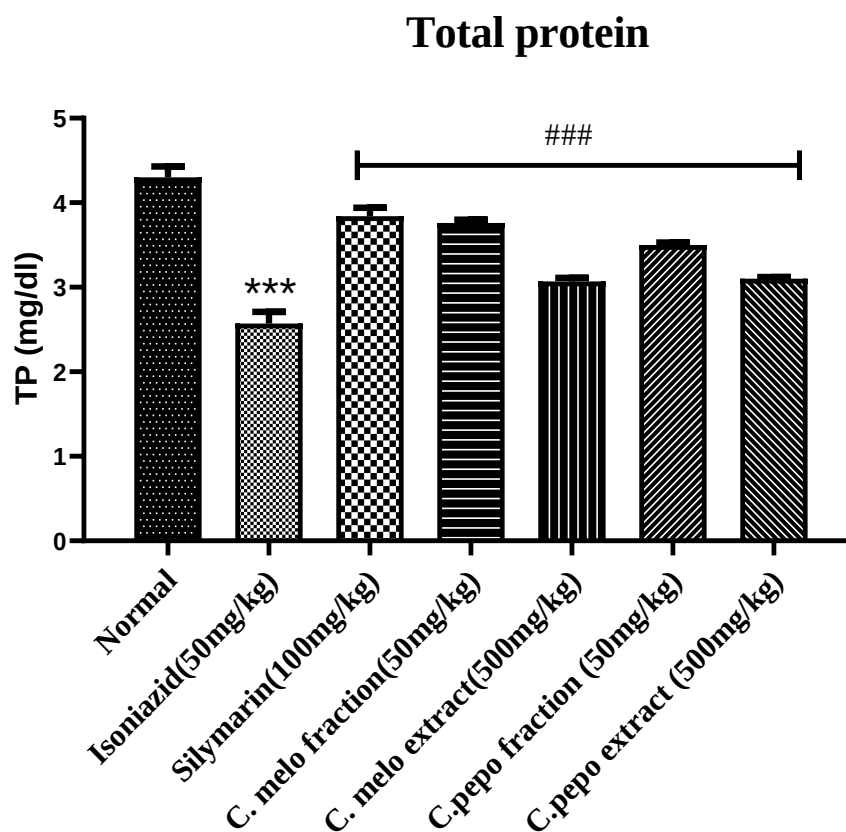
Effect of *C. pepo* and *C. melo* on lipid profile

INH treated group showed significant increase ($p < 0.001$) in the TC, TG, VLDL, and LDL (123.84, 119.80, 25.52, 74.76mg/d, respectively) compared to normal (95.15, 85.23, 18.43, 38.35mg/dL, respectively). And treatment with *C. melo* and *C. pepo* extract and fraction reversed to normal ($p < 0.001$; found towards normal). Whereas INH treated group showed significant decrease ($p < 0.001$) in the TP and HDL (2.57 and 22.65mg/dL, respectively) compared to normal (4.3 and 40.53mg/dL, respectively) and treatment with *C. melo* and *C. pepo* extract and fraction reversed to normal ($p < 0.001$; found towards normal) (Table 21 and Figure 36A-F).

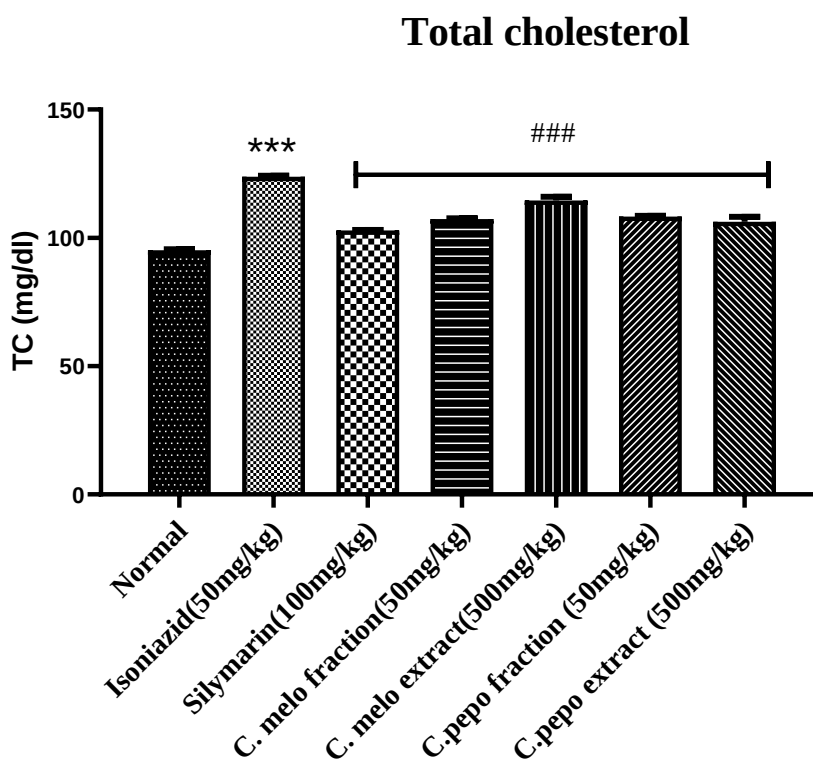
Table 21: Effect of *C. melo* and *C. pepo* on lipid profile in INH induced hepatotoxicity.

Groups	Serum Biochemical Parameters (mg/dl)					
	TC	TP	TG	HDL	VLDL	LDL
Normal	95.15±0.36	4.3±0.13	85.23±0.4	40.53±0.16	18.43±0.18	38.35±0.09
Isoniazid (50mg/kg)	123.84±0.32***	2.57±0.14***	119.80±0.20***	22.65±0.15***	25.52±0.12***	74.76±0.23***
Silymarin (100mg/kg) + Isoniazid	102.92±0.16 ^{###}	3.84±0.10 ^{###}	96.25±0.27 ^{###}	32.59±0.10 ^{##}	20.67±0.20 ^{##}	52.85±0.35 ^{##}
<i>C. melo</i> fraction (50mg/kg) + Isoniazid	107.28±0.319 ^{###}	3.76±0.04 ^{###}	99.52±0.14 ^{###}	31.09±0.28 ^{##}	21.94±0.18 ^{##}	57.85±0.64 ^{##}
<i>C. melo</i> extract (500mg/kg) + Isoniazid	114.58±1.42 ^{##}	3.07±0.04 ^{##}	104.04±0.56 ^{##}	28.73±0.71 [#]	22.19±0.25 ^{##}	60.30±0.41 ^{##}
<i>C. pepo</i> fraction (50mg/kg) + Isoniazid	108.24±0.312 ^{###}	3.50±0.03 ^{###}	102.62±0.15 ^{##}	29.06±0.58 ^{##}	22.6±0.12 ^{##}	59.36±0.27 ^{##}
<i>C. pepo</i> extract (500mg/kg) + Isoniazid	106.24±1.95 ^{##}	3.10±0.02 ^{##}	109.03±0.77 [#]	29.73±0.64 [#]	24.43±0.21 [#]	61.60±0.21 ^{##}

Data are presented as Mean±SEM (n=6), one-way ANOVA followed by Tuckey's multiple comparison test. **p < 0.05, ***p < 0.01 compared with normal group, ^{##}p < 0.05, ^{###}p < 0.01 compared with Isoniazid

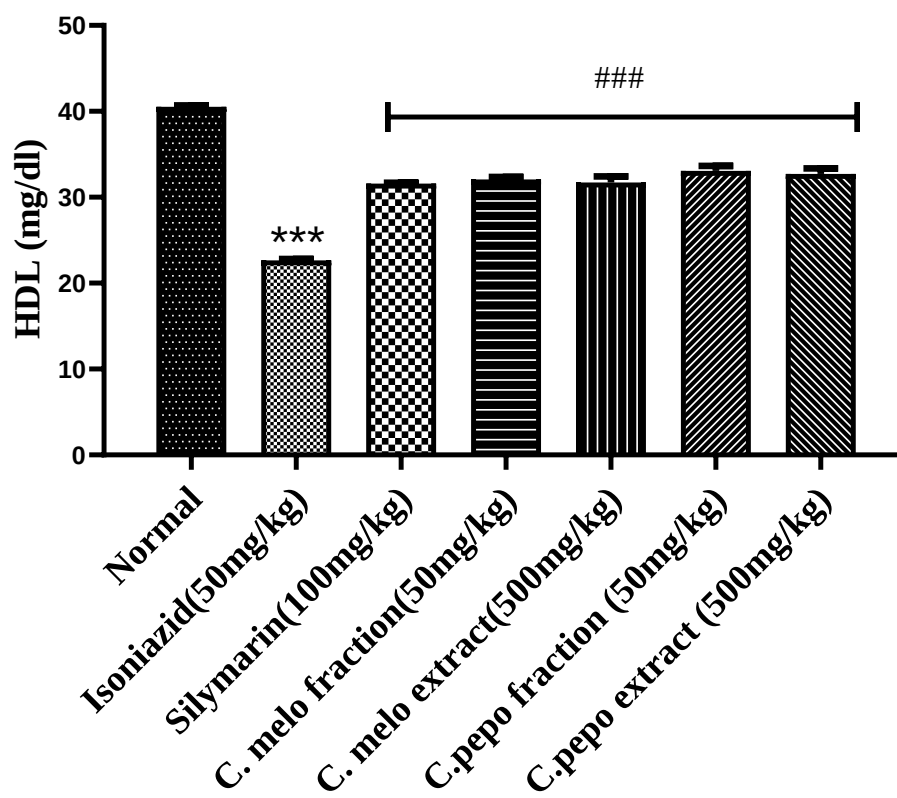


A: Effect of *C. melo* and *C. pepo* on TP.



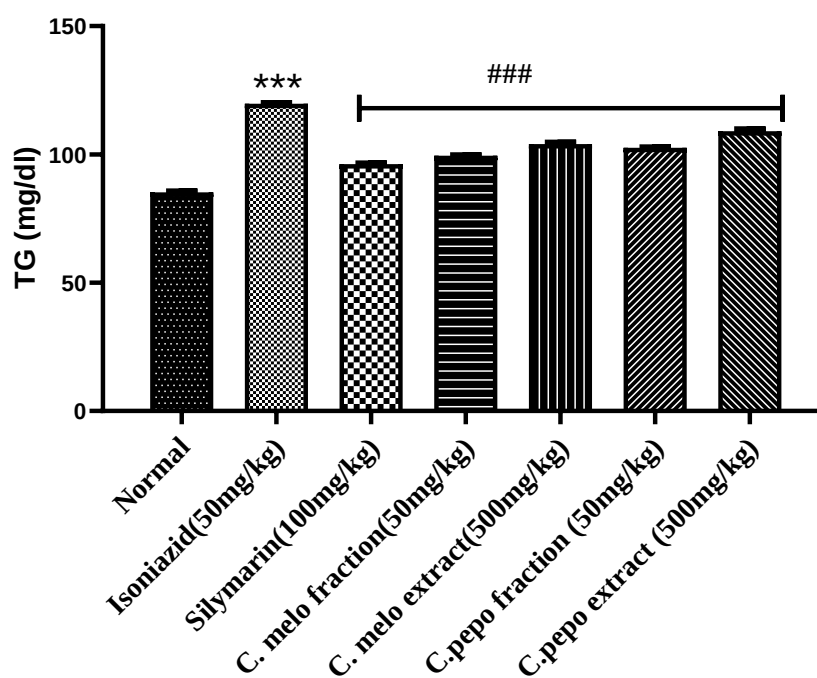
B: Effect of *C. melo* and *C. pepo* on TC.

High density lipoprotein

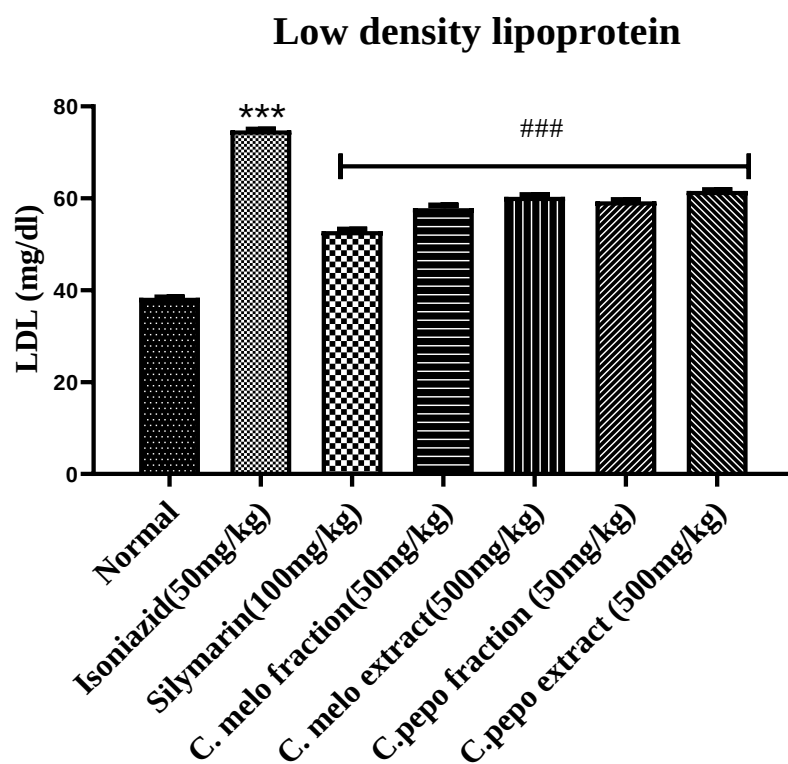


C: Effect of *C. melo* and *C. pepo* on HDL.

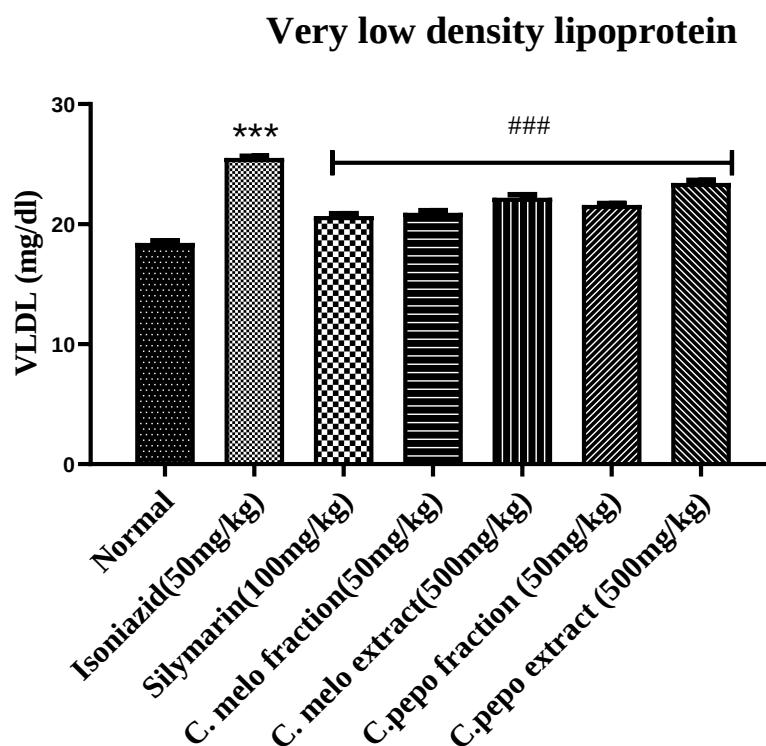
Triglyceride



D: Effect of *C. melo* and *C. pepo* on TG.



E: Effect of *C. melo* and *C. pepo* on LDL.



F: Effect of *C. melo* and *C. pepo* on VLDL.

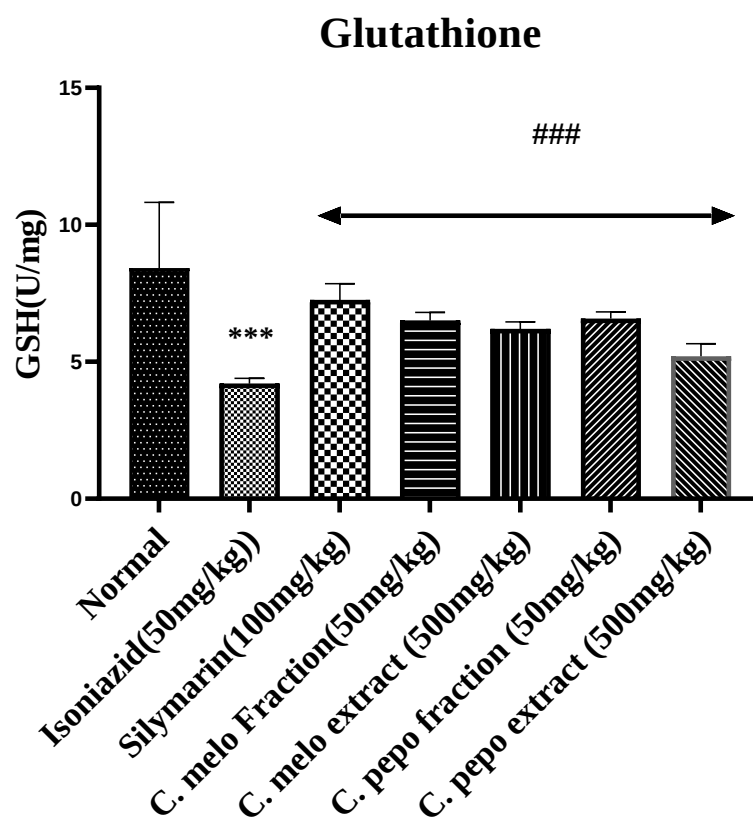
Figure 36: Effect of *C. melo* and *C. pepo* on A) TP, B) TC, C) HDL, D) TG, E) LDL and F) VLDL. ***P < 0.001 compared to normal; ###P < 0.001 compared to INH.

Effect of *C. pepo* and *C. melo* on antioxidant markers

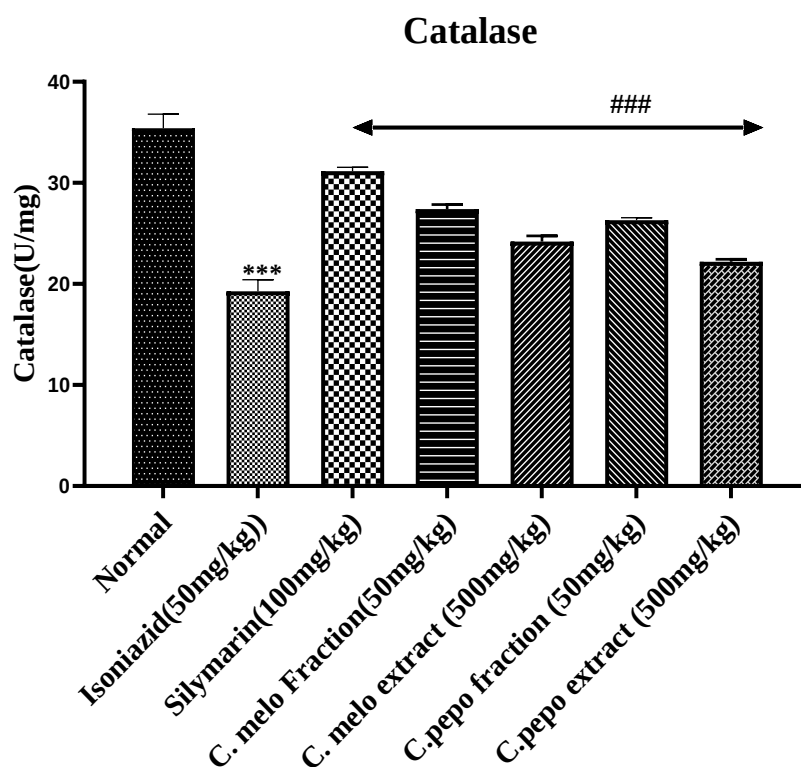
INH treated group showed significant decrease ($p < 0.001$) in the catalase (19.28U), SOD (5.40U), GSH (4.20 $\mu\text{M}/\text{mg}$) and increase ($p < 0.001$) in the LPO (22.50nM/mg) activity and treatment with Silymarin, *C. melo* fraction, extract, *C. pepo* fraction and extract, reverse (Catalase, SOD, GSH and LPO) elevated parameters. (Table 221 and Figure 37A-D). Among them Silymarin, *C.melo* fraction, and *C.pepo* fraction significantly increase ($p < 0.001$) in the catalase (31.15U, 27.40U, 26.30U), SOD (8.00U, 7.20U, 6.80U) GSH (7.25 $\mu\text{M}/\text{mg}$, 6.50 $\mu\text{M}/\text{mg}$, 6.51 $\mu\text{M}/\text{mg}$) levels. And significant decrease in LPO (12.30nM, 14.20nM, 15.20nM) level.

Table 22: Effect of *C. melo* and *C. pepo* on antioxidant markers in INH induced hepatotoxicity.

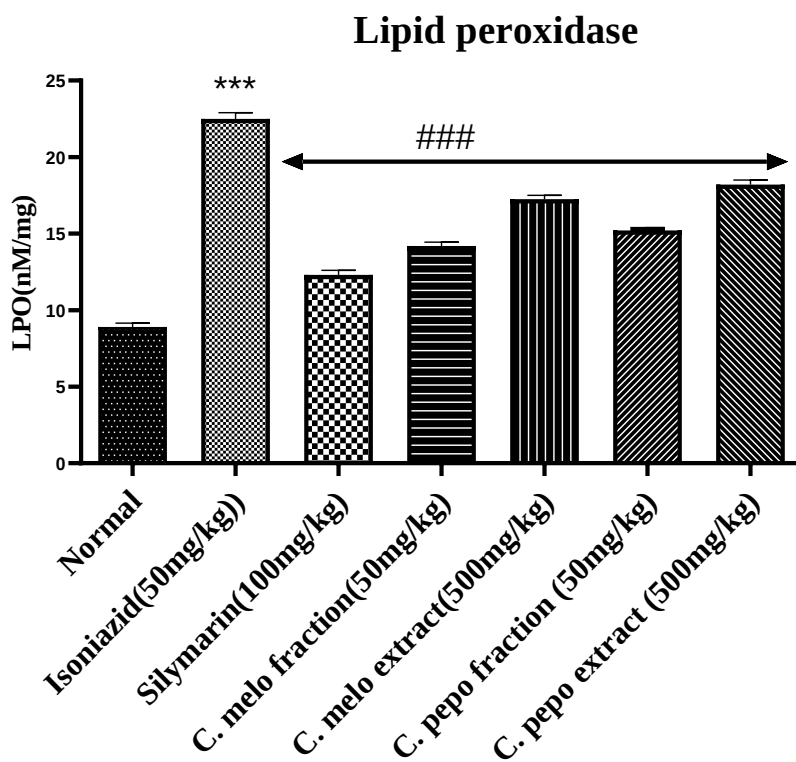
Group	Catalase (Units/mg of protein)	SOD (Units/mg of protein)	GSH (μ M/mg protein)	LPO (nM /mg protein)
Normal	35.40 ± 1.40	9.00 ± 0.40	8.42 ± 2.40	8.90 ± 0.25
Isoniazid (50mg/kg)	$19.28 \pm 1.14^{***}$	$5.40 \pm 0.20^{***}$	$4.20 \pm 0.20^{***}$	$22.50 \pm 0.40^{***}$
Silymarin(100mg/kg)+Isoniazid	$31.15 \pm 0.40^{###}$	$8.00 \pm 0.15^{###}$	$7.25 \pm 0.60^{###}$	$12.30 \pm 0.30^{###}$
<i>C. melo</i> fraction(50mg/kg)+ Isoniazid	$27.40 \pm 0.45^{###}$	$7.20 \pm 0.20^{##}$	$6.50 \pm 0.30^{##}$	$14.20 \pm 0.24^{###}$
<i>C. melo</i> extract(500mg/kg)+ Isoniazid	$24.20 \pm 0.55^{##}$	6.00 ± 0.15	$6.19 \pm 0.18^{##}$	$17.25 \pm 0.25^{##}$
<i>C. pepo</i> fraction(50mg/kg)+ Isoniazid	$26.30 \pm 0.25^{##}$	$6.80 \pm 0.50^{##}$	$6.51 \pm 0.24^{##}$	$15.20 \pm 0.10^{###}$
<i>C. pepo</i> extract(500mg/kg)+ Isoniazid	$22.18 \pm 0.26^{\#}$	$5.50 \pm 0.15^{\#}$	$5.20 \pm 0.40^{\#}$	$18.20 \pm 0.30^{\#}$



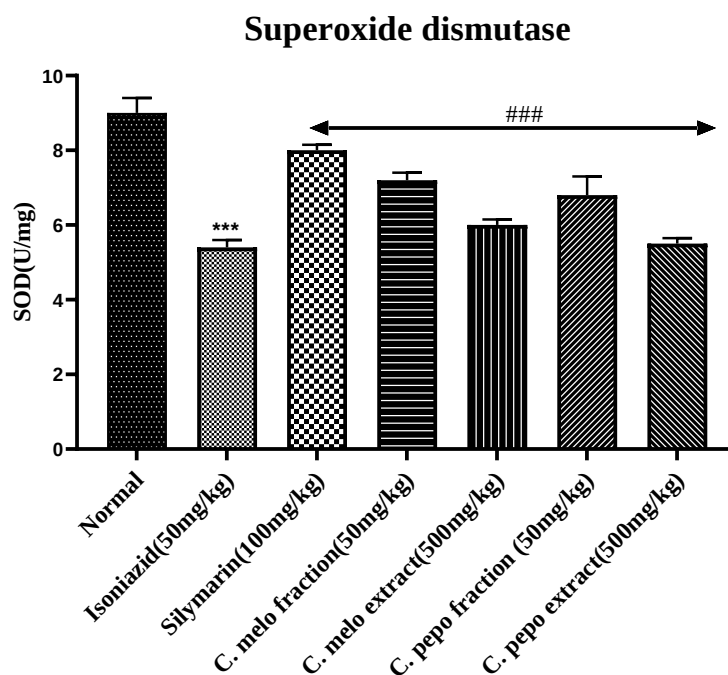
A: Effect of *C. melo* and *C. pepo* on GSH.



B: Effect of *C. melo* and *C. pepo* on Catalase.



C: Effect of *C. melo* and *C. pepo* on LPO.



D: Effect of *C. melo* and *C. pepo* on SOD.

Figure 37: Effect of *C. melo* and *C. pepo* on A) GSH, B) Catalase, C) LPO, and D) SOD; ***P < 0.001 compared to normal; ###P < 0.001 compared to INH

Effect of *C. pepo* and *C. melo* on HFD induced hepatotoxicity

Effect on body and liver weight

The disease control group (HFD) (38.39 ± 3.45) exhibited a remarkable ($p < 0.001$) rise in the body weight compared to the normal group (21.36 ± 2.36). Positive control groups such as the Silymarin (25.34 ± 2.84), *C. melo* fraction group, *C. melo* extract, *C. pepo* extract and *C. pepo* fraction group exhibited a decline in body weight. Among them *C. melo* fraction (28.44 ± 1.59), and *C. pepo* fraction (29.94 ± 2.78) exhibit significant decrease in liver weight compared to the disease control group (38.39 ± 3.45) (refer Figure 38 for BW and Figure 39 for liver weight). Table 23 shows the effect on BW and liver weight.

Table 23: Effect of *C. melo* and *C. pepo* on body weight and liver weight

Groups	Body weight (g)	Liver weight (g)
Normal	21.36 ± 2.36	1.66 ± 0.007
HFD (10ml/kg)	$38.39 \pm 3.45^{***}$	$3.45 \pm 0.005^{***}$
Silymarin (100mg/kg) + HFD	$25.34 \pm 2.84^{###}$	$1.98 \pm 0.001^{###}$
<i>C. melo</i> fraction (50mg/kg) + HFD	$28.44 \pm 1.59^{###}$	$2.24 \pm 0.001^{###}$
<i>C. melo</i> extract (500mg/kg) + HFD	$30.12 \pm 2.56^{##}$	$2.85 \pm 0.002^{##}$
<i>C. pepo</i> fraction (50mg/kg) + HFD	$29.94 \pm 2.78^{###}$	$2.56 \pm 0.003^{###}$
<i>C. pepo</i> extract (500mg/kg) + HFD	$30.65 \pm 2.78^{##}$	$2.94 \pm 0.002^{##}$

Data were expressed as Mean $\pm$ SEM (n=6), one-way ANOVA followed by Tuckey's multiple comparison test. ***p < 0.001 compared with normal group, ##p < 0.01, ###p < 0.001 compared with HFD group

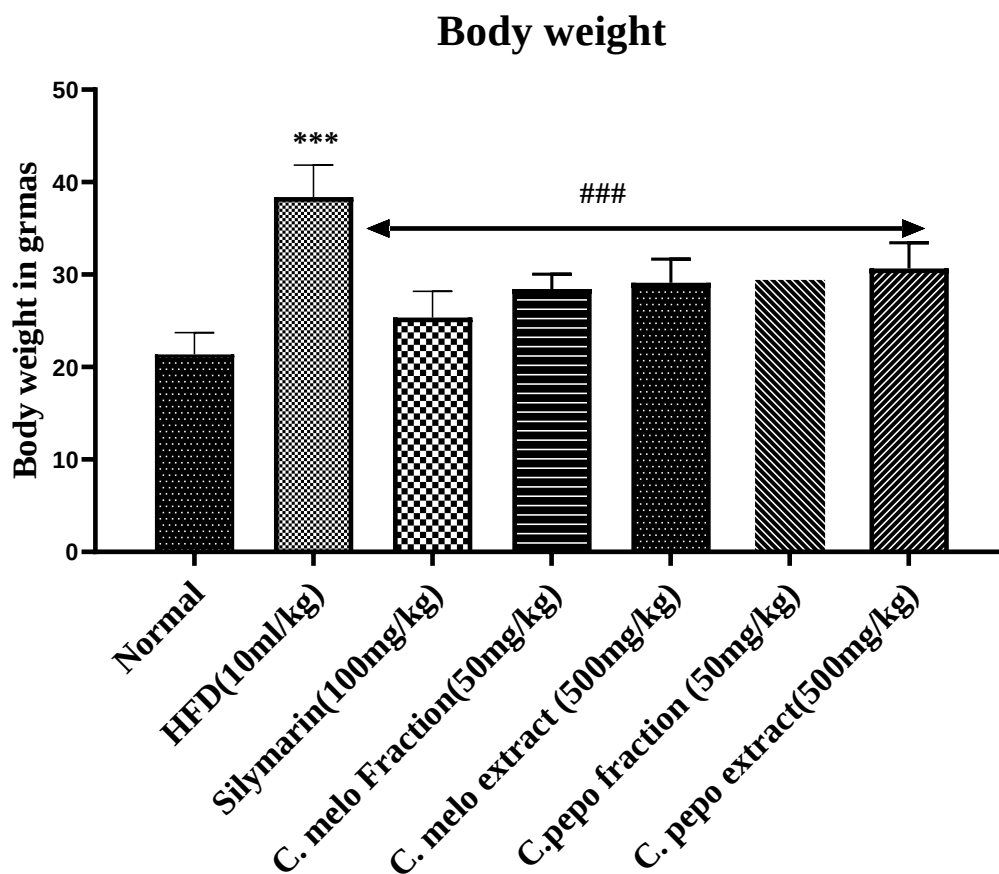


Figure 38: Effect of *C. melo* and *C. pepo* on body weight.

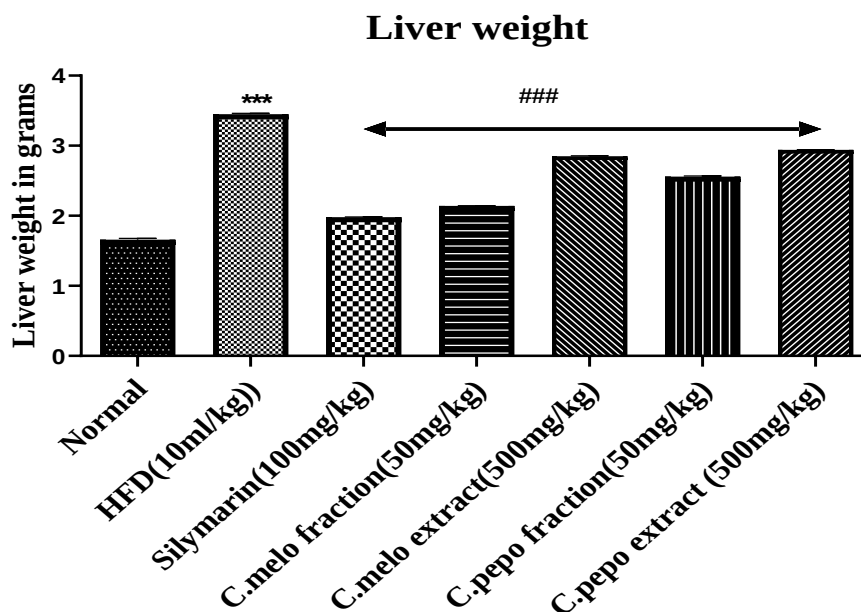


Figure 39: Effect of *C. melo* and *C. pepo* on liver weight.

Effect on liver enzymes

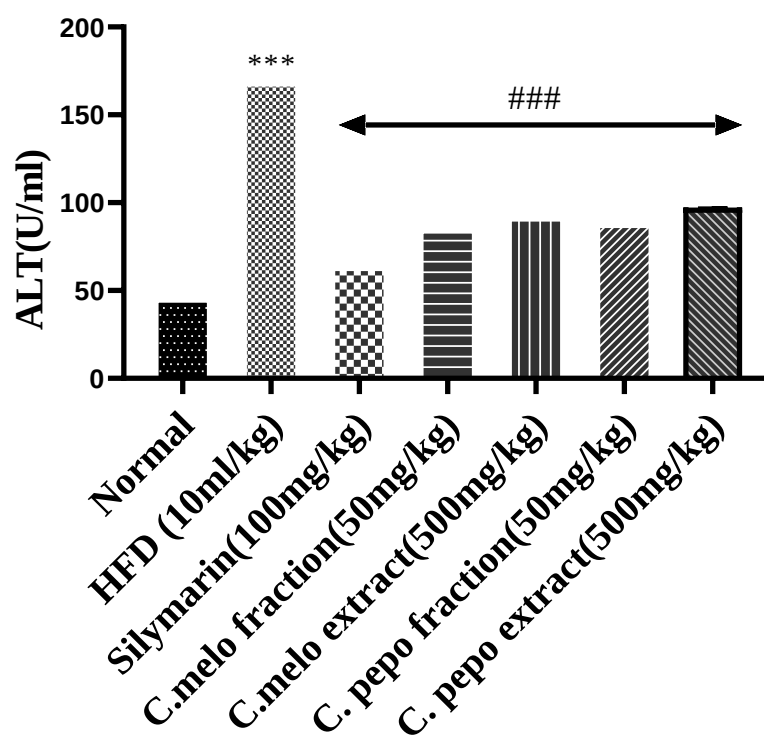
ALT, AST and ALP were significantly increased ($p < 0.001$; 169.09, 182.43, and 191.30U/ml, respectively) in the HFD group compared to normal group (46.15, 74.33, and 92.53U/ml, respectively). Treatment with Silymarin (64.09, 92.56, 102.63U/ml, respectively). *C. melo* fraction (85.34, 101.99, 112.67, respectively) and *C. pepo* fraction (88.65, 108.34, 114.49U/ml, respectively) was significantly ($p < 0.01$ to $p < 0.001$) reversed in all the treated groups compared to HFD group. Similarly, total bilirubin and serum bilirubin were significantly increased ($p < 0.001$) in the HFD group (0.42mg/dL and 1.74mg/dL, respectively) compared to normal (1.025mg/dL and 5.11mg/dL, respectively). Treatment with *C. melo* fraction (0.64mg/dL and 2.20mg/dL, respectively) and *C. pepo* fraction (0.69mg/dL and 2.90mg/dL, respectively) was significantly ($p < 0.01$ to $p < 0.001$) reversed in all the treated groups compared to HFD group. Whereas, Sylimarín treated group (0.531mg/dL and 2.11mg/dL, respectively) also significantly reversed the abnormal level of liver enzyme levels in the HFD treated groups ($p < 0.001$) Table 24 and Figure 40A-E).

Table 24: Effect of *C. melo* and *C. pepo* on ALT, AST, ALP, serum bilirubin and Total bilirubin against HFD induced hepatotoxicity in albino mice.

Groups	SERUM BIOCHEMICAL PARAMETERS				
	ALT (U/ml)	AST (U/ml)	ALP (U/ml)	Serum Bilirubin (mg/dL)	Total Bilirubin (mg/dL)
Normal	46.15±0.28	74.33±0.25	92.53±0.08	0.42±0.006	1.74±0.002
HFD (10ml/kg)	169.09±0.29***	182.43±0.12***	191.30±0.20***	1.025±0.003**	5.11±0.005***
Silymarin (100mg/kg)+HFD	64.09±0.29 ^{###}	92.56±0.07 ^{###}	102.63±0.06 ^{###}	0.531±0.004 ^{###}	2.11±0.003 ^{###}
<i>C. melo</i> fraction (50mg/kg)+ HFD	85.34±0.28 ^{###}	101.99±0.24 ^{###}	112.67±0.20 ^{##}	0.640±0.002 ^{###}	2.20±0.002 ^{###}
<i>C. melo</i> extract (500mg/kg)+ HFD	92.29±0.32 ^{##}	105.99±0.24 ^{###}	119.46±0.20 ^{##}	0.682±0.002 ^{##}	2.42±0.002 ^{###}
<i>C. pepo</i> fraction (50mg/kg)+ HFD	88.65±0.22 ^{###}	108.34±0.89 ^{###}	114.49±0.40 ^{##}	0.690±0.001 ^{###}	2.90±0.001 ^{###}
<i>C. pepo</i> extract (500mg/kg)+ HFD	97.26±0.14 ^{##}	119.29±0.12 ^{##}	120.85±0.12 ^{##}	0.820±0.002 [#]	3.24±0.002 ^{##}

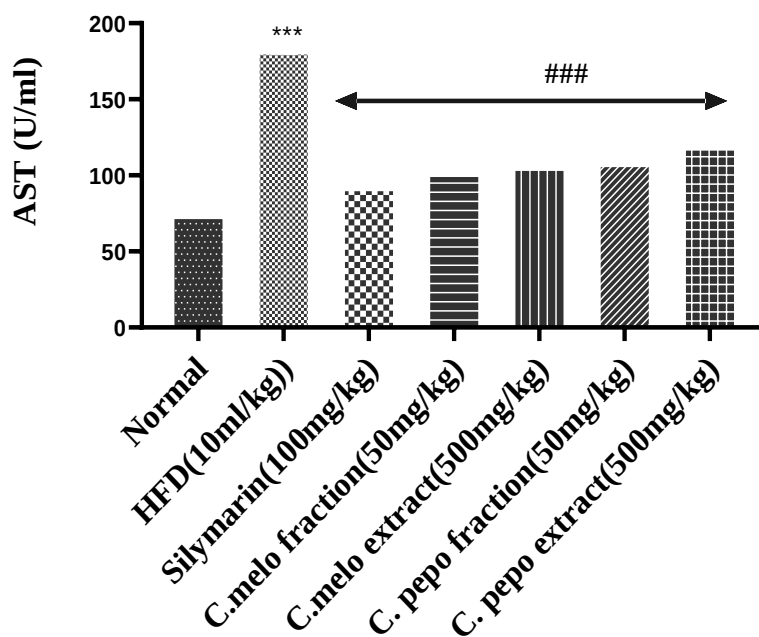
Data were expressed as Mean ± SEM (n=6), one-way ANOVA followed by Tuckey's multiple comparison test. ***p < 0.001 compared with normal group, ^{##}p < 0.01, ^{###}p < 0.001 compared with HFD group.

Alanine aminotransferase

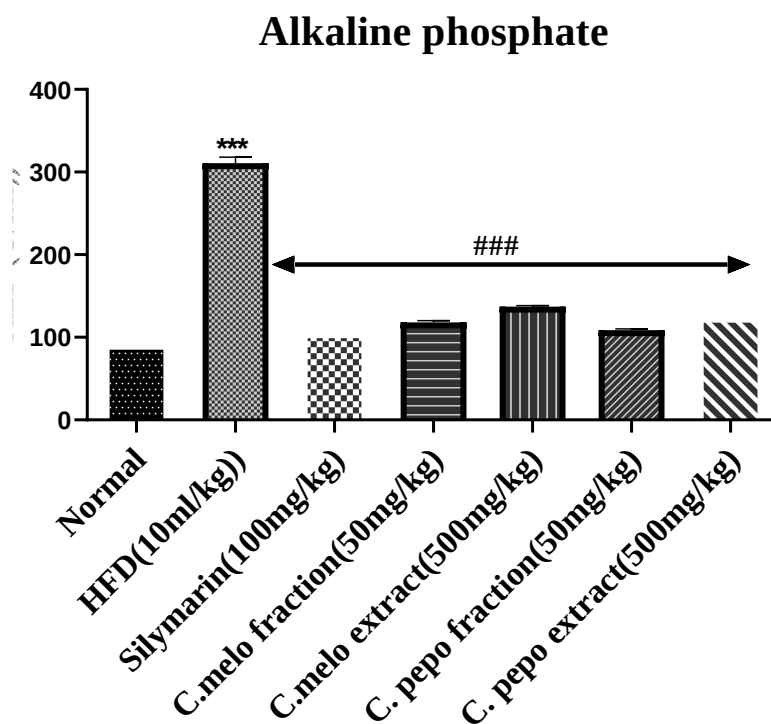


40A: Effect of *C. melo* and *C. pepo* on ALT.

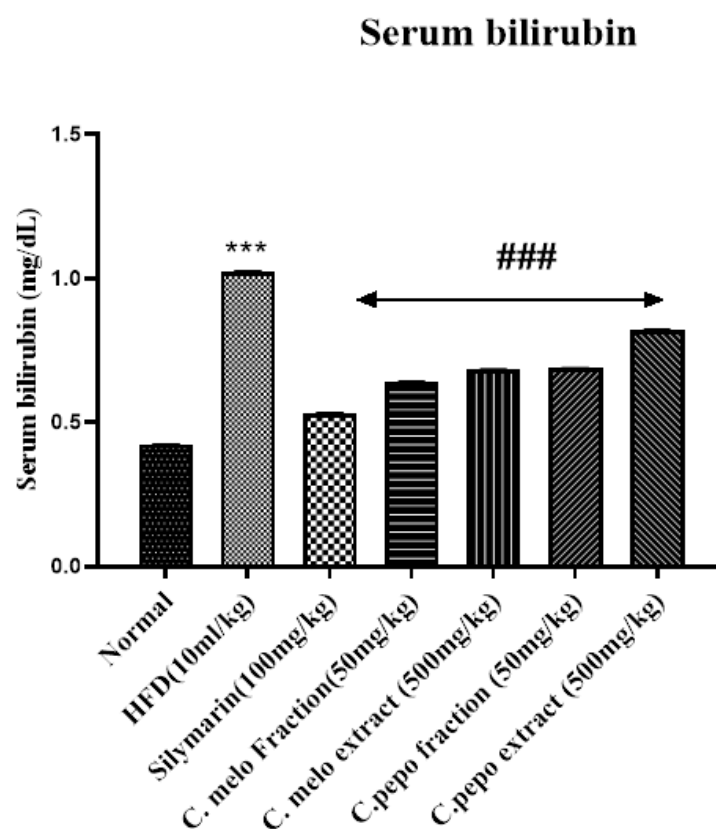
Aspartate aminotransferase



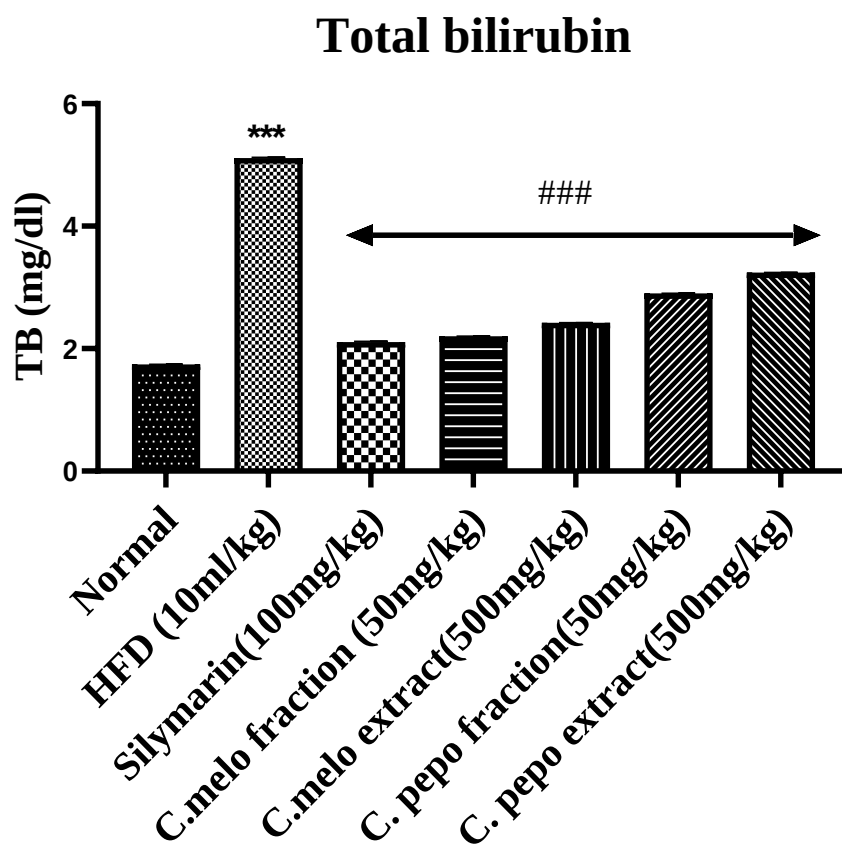
40B: Effect of *C. melo* and *C. pepo* on AST.



40C: Effect of *C. melo* and *C. pepo* on ALP.



40D: Effect of *C. melo* and *C. pepo* on Serum bilirubin.



40E: Effect of *C. melo* and *C. pepo* on Total bilirubin.

Figure 40: Effect of *C. melo* and *C. pepo* on ALT, AST, ALP, Serum Bilirubin, and Total Bilirubin. ** $p < 0.01$ and *** $p < 0.001$ compared to normal; ## $p < 0.01$ and ### $p < 0.001$ compared to HFD.

Effect on antioxidant markers

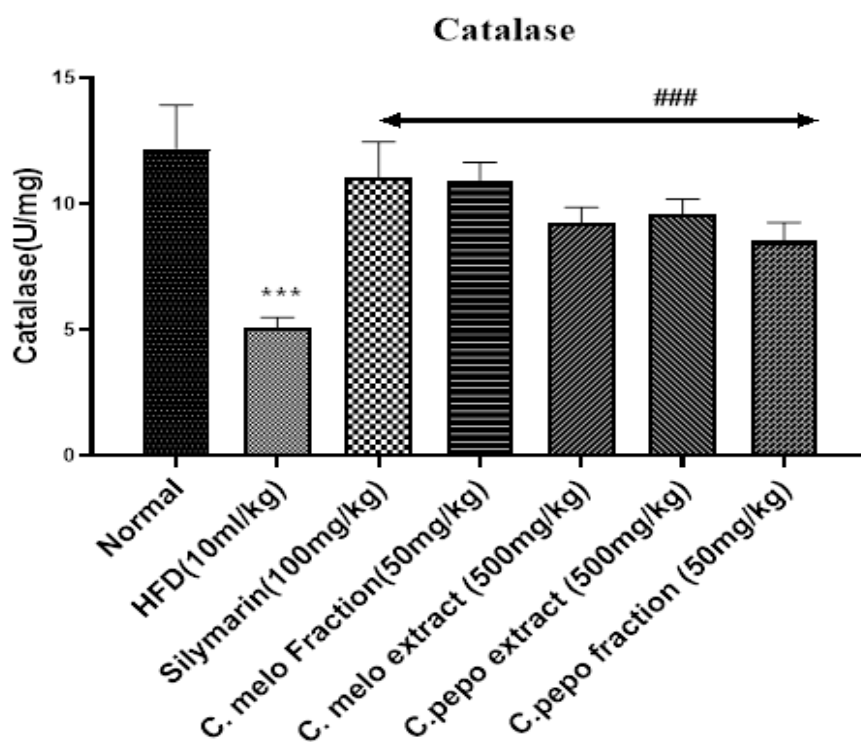
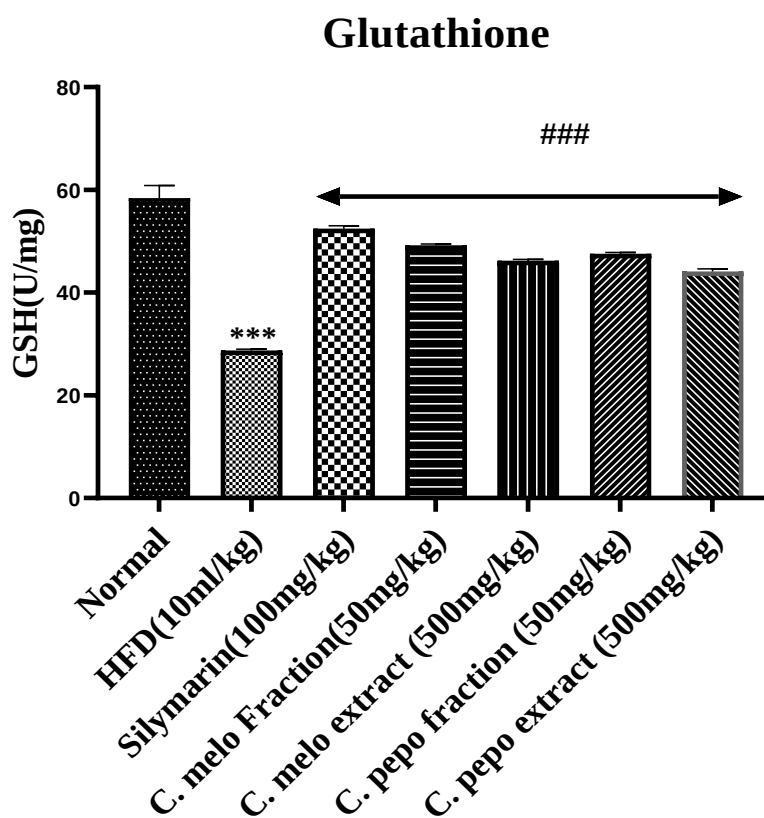
Catalase ($p < 0.001$; 5.094U/mg), SOD ($p < 0.001$; 9.245U/mg), GSH ($p < 0.001$; 28.67 μ M/mg), and nitrate ($p < 0.01$; 16.22ng/mg) were significantly decreased in the HFD treated groups compared to normal (12.20U/mg, 22.45U/mg, 58.42 μ M/mg, and 30.55ng/mg, respectively). Treatment with *C. melo* fraction (10.93U/mg, 18.29U/mg, 49.14 μ M/mg, and 25.34ng/mg, respectively) and *C. pepo* fraction (9.59U/mg, 17.34U/mg, 47.58 μ M/mg, and 24.39ng/mg, respectively) was significantly ($p < 0.01$ to $p < 0.001$) increased towards normal and in extract and Silymarin treated groups also compared to HFD group. Likewise, LPO (524.4nM/mg) was significantly increased in the HFD treated groups compared to normal (245.9nM/mg). Treatment with *C. melo* fraction ($p < 0.01$; 344nM/mg), *C. melo* extract ($p < 0.05$; 379.6nM/mg), and *C. pepo* fraction ($p < 0.01$; 365.2nM/mg) and extract ($p < 0.05$; 365.2nM/mg) significantly decreased towards normal in all the treated groups compared to HFD group. Whereas, Silymarin (324.2nM/mg) treated group also significantly reversed the abnormal level of antioxidant levels in the HFD treated groups ($p < 0.01$ to $p < 0.001$) (Table 25 and Figure 41A-E).

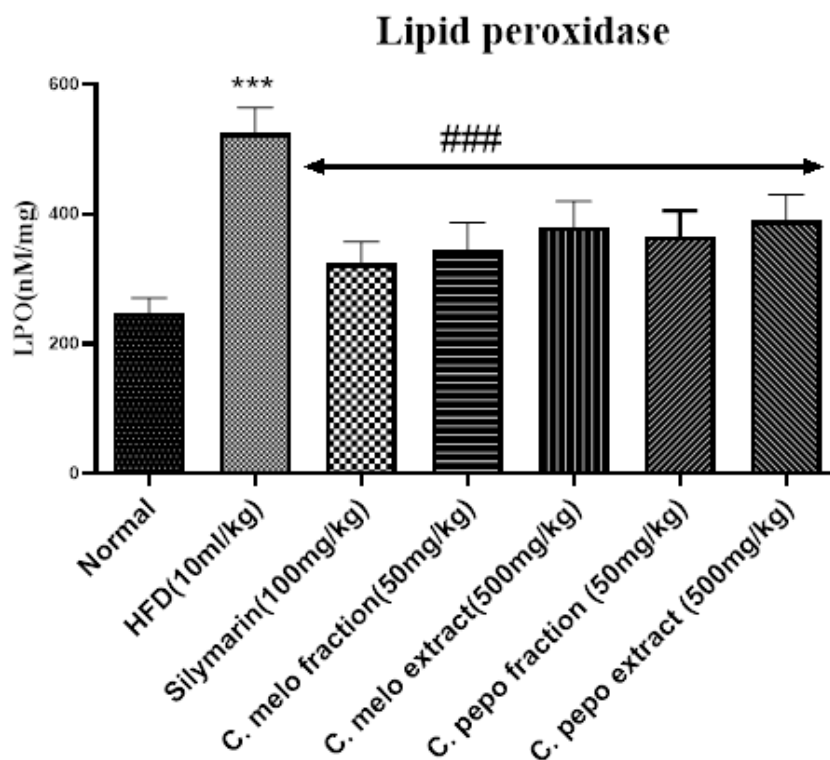
Table 25: Effect of *C. melo* and *C. pepo* on antioxidant markers in HFD induced hepatotoxicity.

Groups	Catalase (units/mg of protein)	SOD (Units/mg of protein)	GSH (μ M/mg protein)	Nitrite (ng/mg protein)	LPO (nM /mg protein)
Normal	12.20 $\pm$ 1.72	22.45 $\pm$ 2.84	58.42 $\pm$ 2.424	30.55 $\pm$ 2.182	245.9 $\pm$ 24.74
HFD (10ml/kg)	5.094 $\pm$ 0.40***	9.245 $\pm$ 0.422***	28.67 $\pm$ 0.3204***	16.22 $\pm$ 1.445**	524.4 $\pm$ 39.99***
Silymarin (100mg/kg)+HFD	11.04 $\pm$ 1.40###	20.24 $\pm$ 1.42###	52.40 $\pm$ 0.6307###	27.46 $\pm$ 0.5226##	324.2 $\pm$ 33.08###
<i>C. melo</i> fraction (50mg/kg)+ HFD	10.933 $\pm$ 0.70###	18.29 $\pm$ 1.41##	49.14 $\pm$ 0.3299##	25.34 $\pm$ 0.7719##	344.9 $\pm$ 42.01##
<i>C. melo</i> extract (500mg/kg)+ HFD	9.233 $\pm$ 0.70##	17.34 $\pm$ 1.32##	46.19 $\pm$ 0.2664##	19.54 $\pm$ 0.6617#	379.6 $\pm$ 40.02#
<i>C. pepo</i> fraction (50mg/kg)+ HFD	9.59 $\pm$ 0.62##	17.34 $\pm$ 1.32##	47.58 $\pm$ 0.2445##	24.39 $\pm$ 0.3555##	365.2 $\pm$ 40.04#
<i>C. pepo</i> extract (500mg/kg)+ HFD	8.52 $\pm$ 0.74#	16.82 $\pm$ 1.62#	44.14 $\pm$ 0.4556#	22.54 $\pm$ 0.6549##	389.9 $\pm$ 40.02#

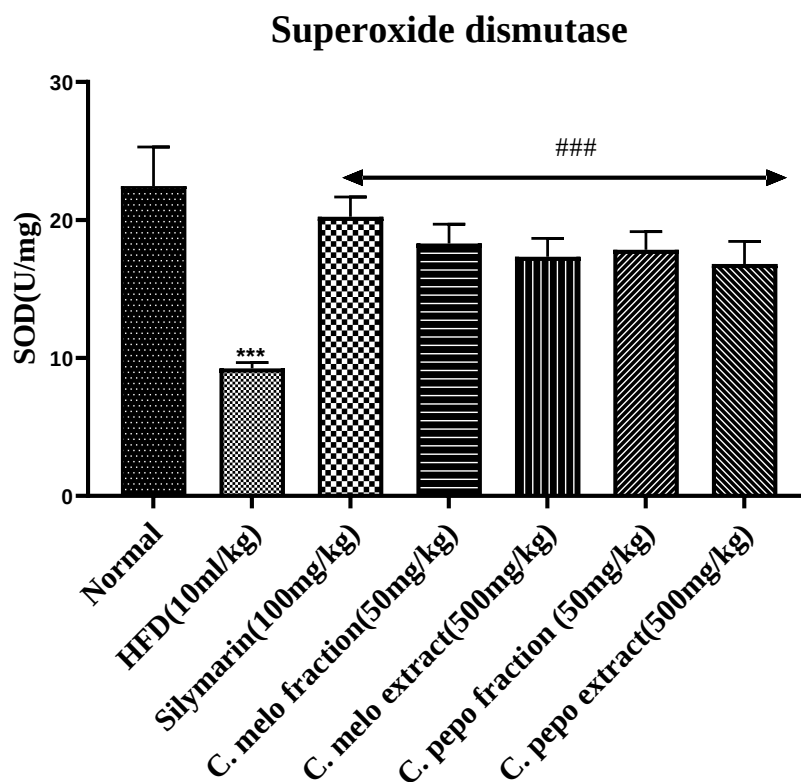
Data were expressed as Mean $\pm$ SEM (n=6), one-way ANOVA followed by Tuckey's multiple comparison test. **p < 0.01, ***p < 0.001

compared with normal group, #p < 0.05, ##p < 0.01, ###p < 0.001 compared with HFD group.

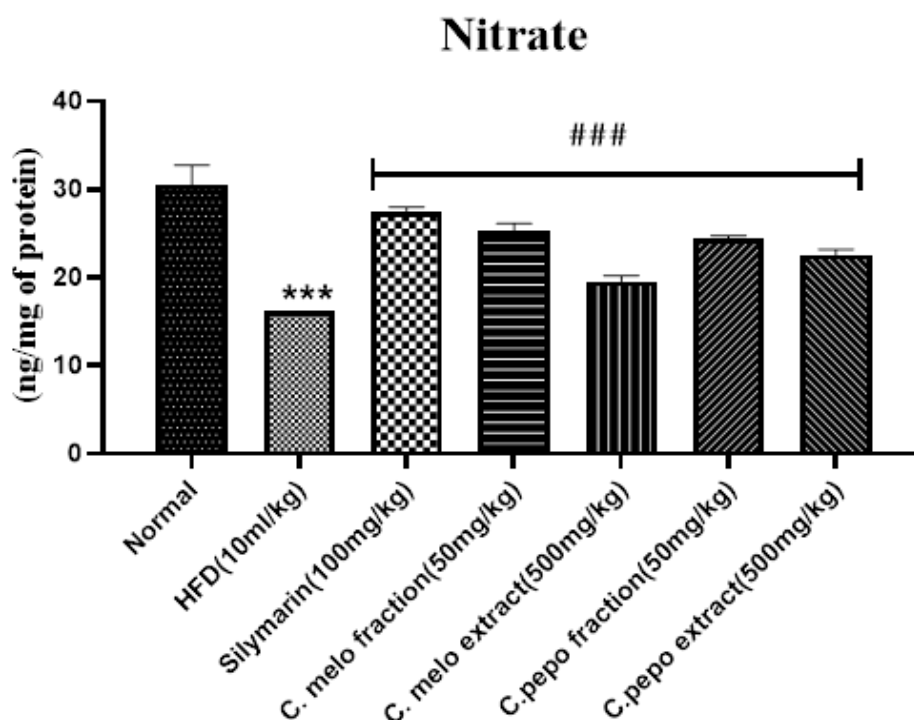
41A: Effect of *C. melo* and *C. pepo* on Catalase.41B: Effect of *C. melo* and *C. pepo* on Glutathione.



41C: Effect of *C. melo* and *C. pepo* on LPO.



41D: Effect of *C. melo* and *C. pepo* on SOD.



41E: Effect of *C. melo* and *C. pepo* on Nitrate.

Figure 41: Effect of *C. melo* and *C. pepo* on GSH, Catalase, LPO, SOD and Nitrite; * $p < 0.001$ compared to normal; ### $p < 0.001$ compared to HFD.**

Effect on lipid profile

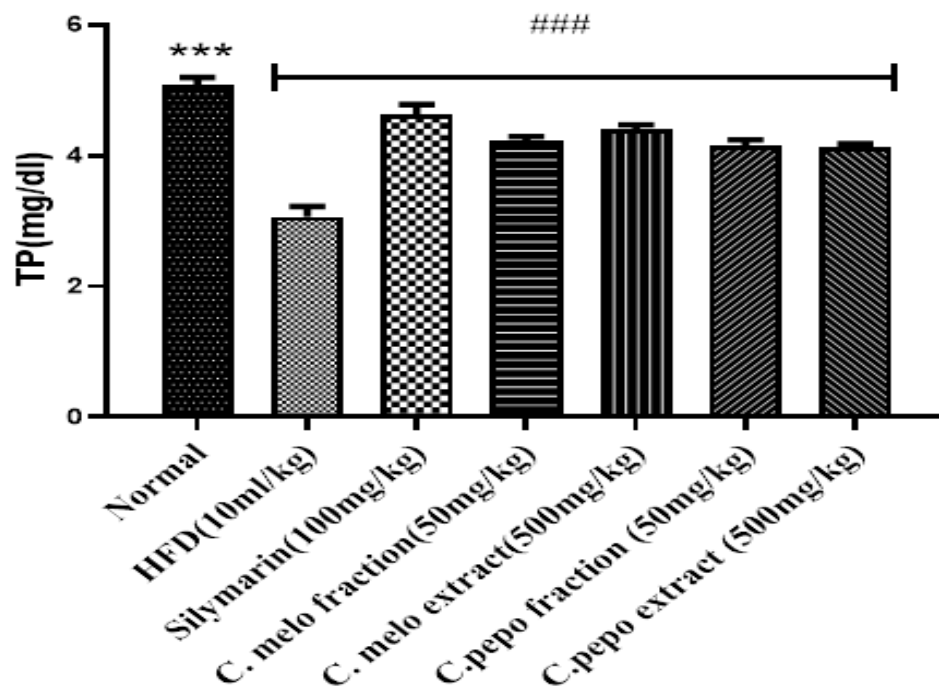
TC, TG, LDL, and VLDL were significantly increased ($p < 0.001$; 141, 126.26, 84.12, 28.745mg/dL) in the HFD group compared to normal group (91.32, 81.77, 32.25, and 19.61mg/dL). Treatment with *C. melo* and *C. pepo* was significantly ($p < 0.01$ to $p < 0.001$) reversed (found towards normal range) in all the treated groups compared to HFD group. Similarly, TP and HDL level were significantly decreased ($p < 0.001$; 3.06 and 20.41mg/dL, respectively) in the HFD group compared to normal group (5.08 and 42.41mg/dL, respectively). Treatment with *C. melo* and *C. pepo* was significantly ($p < 0.01$ to $p < 0.001$) reversed (found towards normal) in all the treated groups compared to HFD group. Whereas, Silymarin treated group also significantly reversed the abnormal level of lipid profile levels in the HFD treated groups ($p < 0.001$) (Table 26 and Figure 42A-F).

Table 26: Effect of *C. melo* and *C. pepo* on lipid profile in HFD induced hepatotoxicity.

Groups	Serum Biochemical Parameters (mg/dL)					
	TC	TP	TG	HDL	VLDL	LDL
Normal	91.32±0.18	5.08±0.11	81.77±0.33	42.41±0.07	19.61±0.07	32.25±0.04
HFD (10ml/kg)	141.2±0.29***	3.06±0.16***	126.26±0.14***	20.41±0.10***	28.745±0.08***	84.12±0.24***
Silymarin (100mg/kg) + HFD	105.44±0.11 ^{###}	4.63±0.15 ^{###}	94.43±0.10 ^{###}	39.15±0.06 ^{###}	21.51±0.03 ^{###}	44.25±0.14 ^{###}
<i>C. melo</i> fraction (50mg/kg) + HFD	109.59±0.11 ^{###}	4.23±0.06 ^{###}	99.84±0.13 ^{##}	35.395±0.08 ^{###}	22.24±0.01 ^{###}	52.55±0.21 ^{###}
<i>C. melo</i> extract (500mg/kg) + HFD	114.80±0.30 ^{##}	4.41±0.06 ^{###}	102.55±0.194 ^{##}	32.69±0.13 ^{##}	25.95±0.09 ^{ns}	60.25±0.54 ^{##}
<i>C. pepo</i> fraction (50mg/kg) + HFD	112.98±0.43 ^{###}	4.15±0.09 ^{###}	98.74±0.11 ^{##}	33.28±0.14 ^{##}	21.27±0.19 ^{###}	59.66±0.52 ^{###}
<i>C. pepo</i> extract (500mg/kg) + HFD	119.98±0.65 [#]	4.12±0.06 ^{##}	110.35±0.25 [#]	25.02±0.68 [#]	27.85±0.04 ^{ns}	64.24±0.32 ^{##}

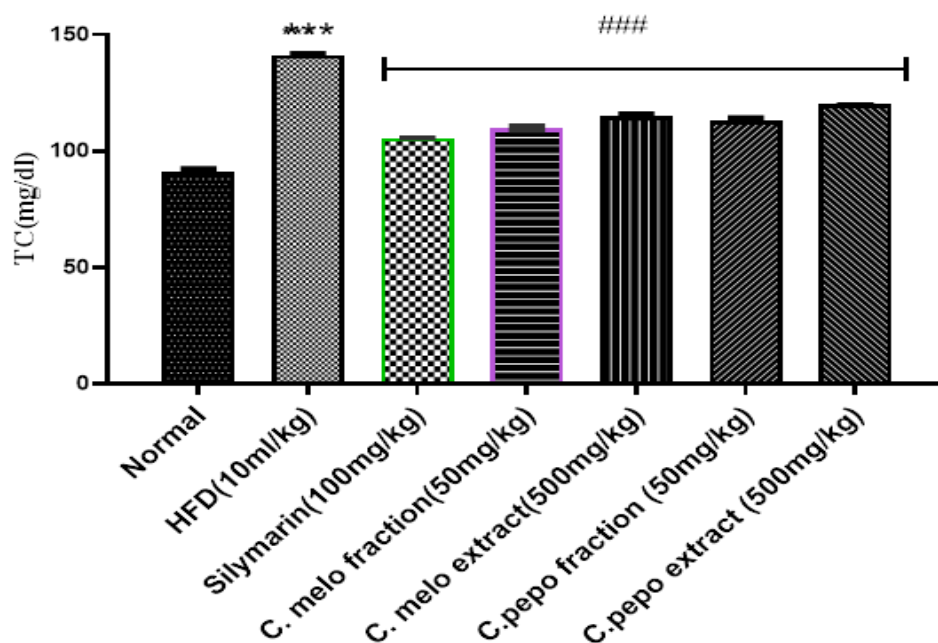
Data were expressed as Mean ± SEM (n=6), one-way ANOVA followed by Tuckey's multiple comparison test. ***p < 0.001 compared with normal group, #p < 0.05, ##p < 0.01, ###p < 0.001 compared with HFD group.

Total protein



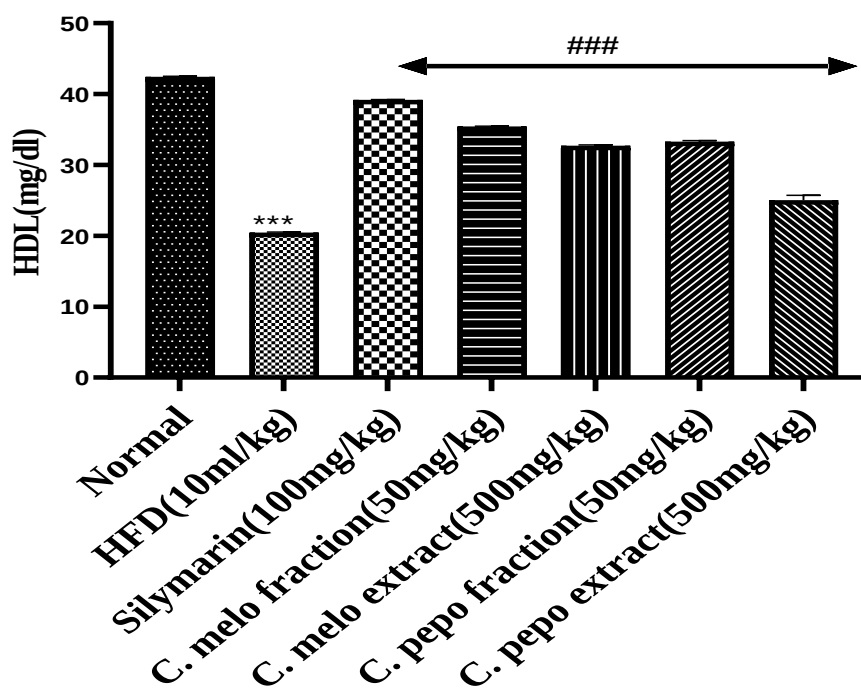
42A: Effect of *C. melo* and *C. pepo* on Total protein.

Total cholesterol



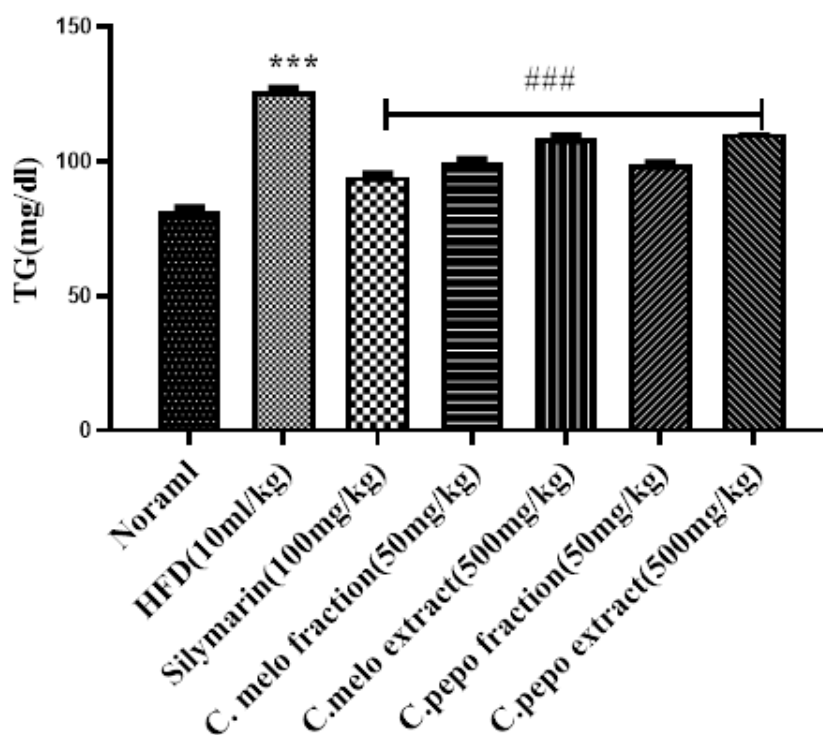
42B: Effect of *C. melo* and *C. pepo* on Total cholesterol.

High density lipoprotein

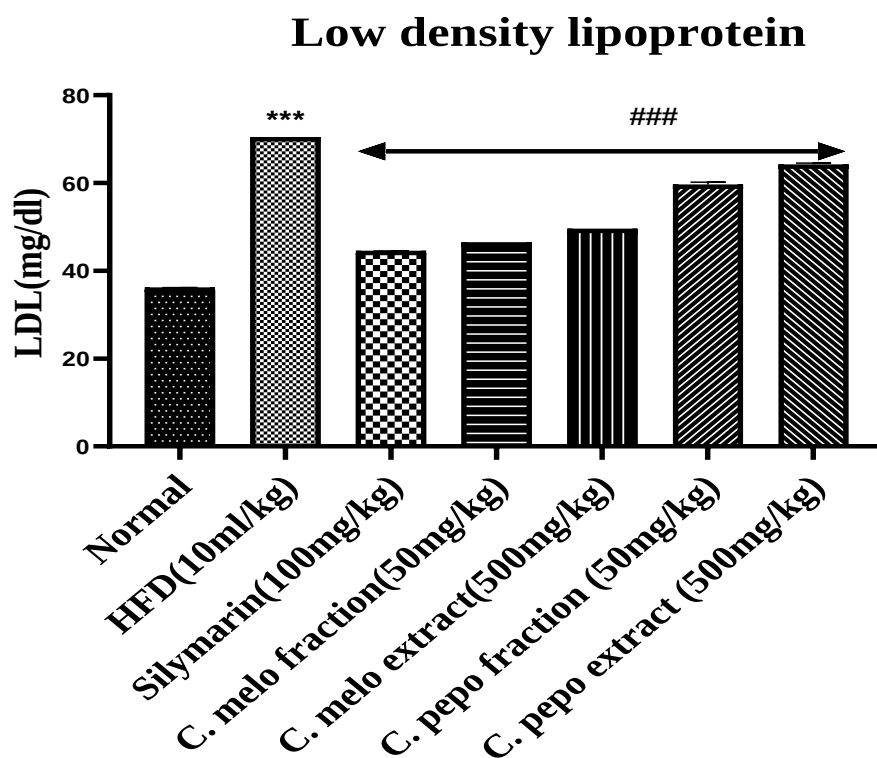


42C: Effect of *C. melo* and *C. pepo* on High density lipoprotein.

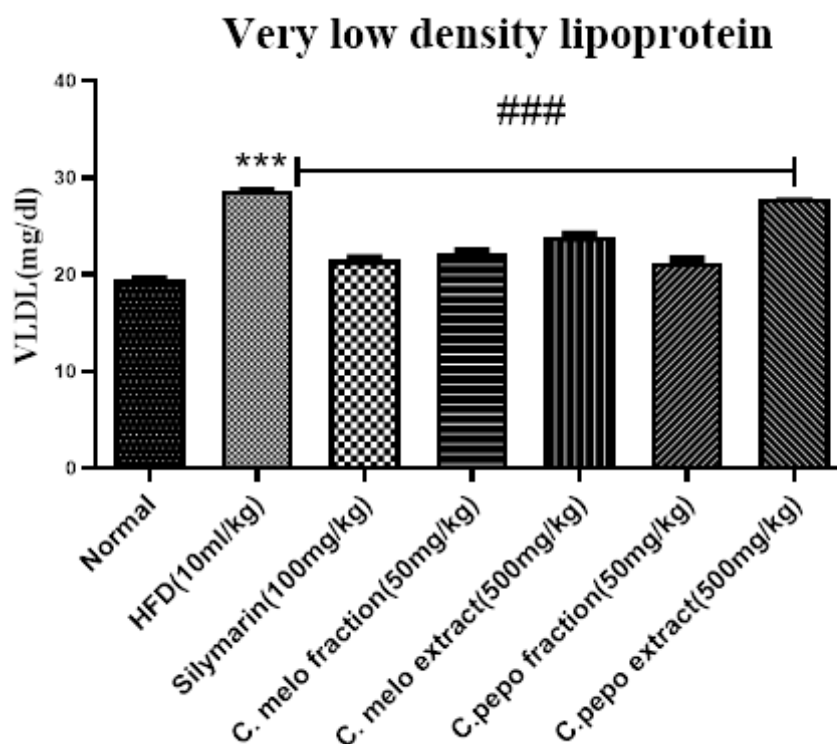
Triglyceride



42D: Effect of *C. melo* and *C. pepo* on Triglyceride.



42E: Effect of *C. melo* and *C. pepo* on Low density lipoprotein.



42F: Effect of *C. melo* and *C. pepo* on Very low density lipoprotein.

Figure 42: Effect of *C. melo* and *C. pepo* on TP, TC, HDL, TG, LDL, and VLDL.

***p < 0.001 compared to normal; ###p < 0.001 compared to HFD.

Histological examination of liver in HFD induced hepatotoxicity

The biochemical examinations were confirmed by histology of liver at 10x and 40X magnification. Vehicle treatment showed normal liver tissue of hepatic parenchyma with central vein (CV) in mice. Histological changes were ameliorated after HFD treatment indicating liver cirrhosis showed congested blood vessel, disintegrated tissue, fatty degeneration with abundant inflammation. These abnormalities were normalized with treatment of Silymarin, bioactive guided fraction and extract of *C. melo* and *C. pepo* (Figure 43).

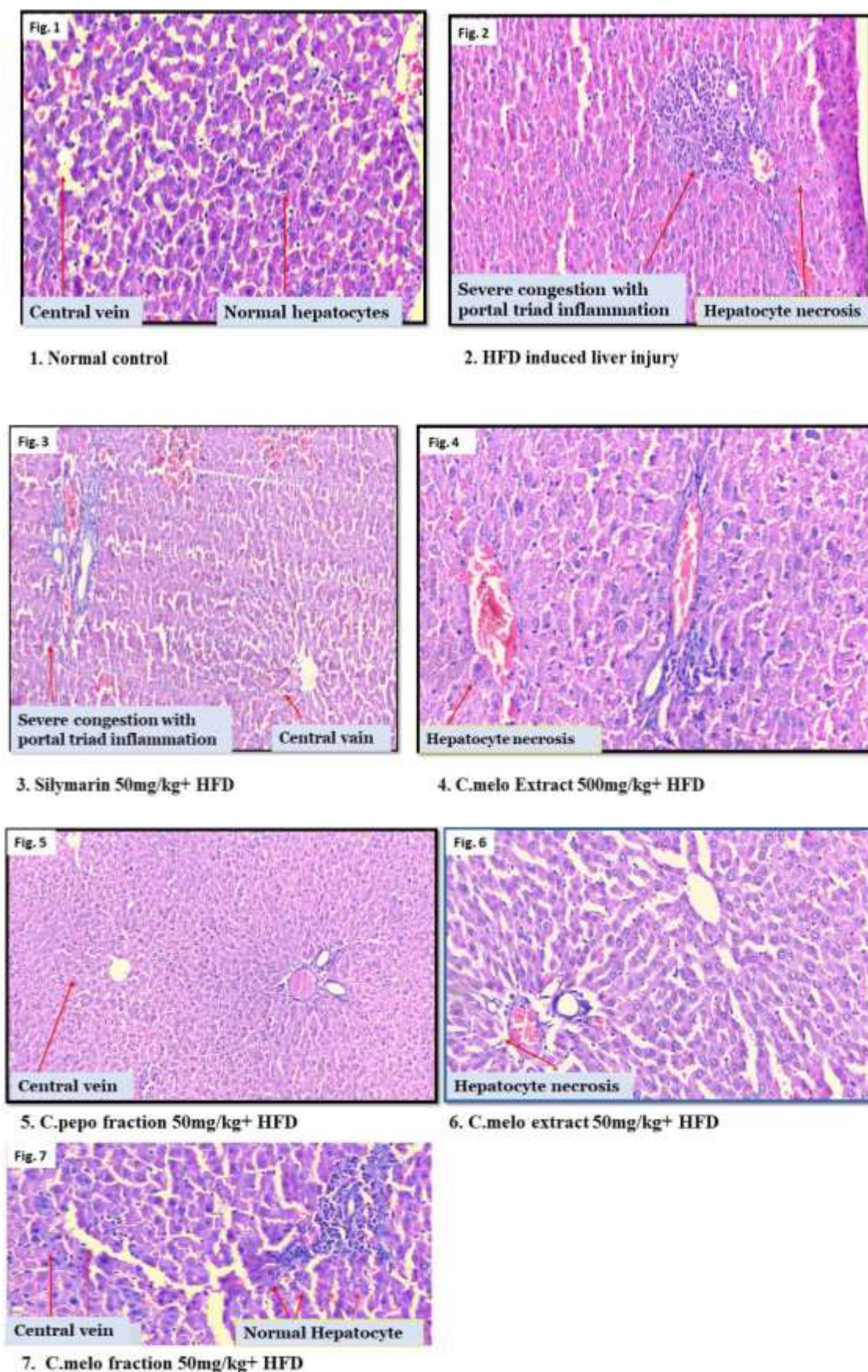


Figure 43: Histology of liver. (1): Normal (2) HFD group, (3) Silymarin group (4) *C. pepo* extract, (5) *C. melo* fraction, (6) *C. melo* extract, and (7) *C. melo* fraction.

DISCUSSION

In this study an attempt was made to elucidate the possible mechanisms of *C. pepo* and *C. melo* for their hepatoprotective properties in hepatotoxicity produced by INH and HFD via both computational and experimental studies. The hydroalcoholic extract of the *C. pepo* and *C. melo* are reported to be rich in nutrients such as minerals, fibers, carotenoids, vitamin C and majorly flavonoids. The compounds from these plants possess spectrum of biological activities including hepatoprotective and antioxidant properties. In previous investigations, it is reported that the wide range of phytoconstituents such as phenols, flavonoids, alkaloids, tannins, saponins, terpenoids, glycosides, steroids, sugar, protein, essential and volatile oils from different plants such as "*Ficus benghalensis*, *Ficus religiosa*, *Silybum marianum*, *Glycyrrhiza glabra*, *Phyllanthus amarus*, *Salvia miltiorrhiza*, *Astragalus membranaceus*, *Capparis spinosa*, *Cichorium intybus*, *Solanum nigrum*, *Sapindus mukorossi* Gaertn., *Ginkgo biloba*, *Woodfordia fruticosa*, *Vitex trifolia*, *Schisandra chinensis*, *Cuscuta chinensis*, *Lycium barbarum*, *Angelica sinensis*, *Litsea coreana* and many more have been reported as immunomodulation, stimulation of hepatic DNA synthesis, stimulation of superoxide dismutase and glutathione reductase to inhibit oxidation in hepatocytes, reduction of intracellular reactive oxygen species by enhancing levels of antioxidants, lipid accumulation, inhibition of nucleic acid polymerases to downregulate viral mRNA transcription, translation, free radical scavenging and reduction of hepatic fibrosis by decreasing the levels of TGF beta-1 and collagen synthesis in hepatic cells.¹³⁸ However their exact molecular mechanism is unclear, there are many gaps in the literature on INH and HFD induced liver injury mechanisms and the effects of natural medicinal ingredients. therefore, this investigation attempted that steroids and carotenoids from *C. melo* and *C. pepo* managing liver injury by modulating NR1H3,

HMGCR and *FXR* proteins mainly through *PPAR* signaling pathway, also normalizing biochemical and histopathological parameters. This study firstly elucidated the possible molecular mechanisms of *C. pepo* and *C. melo* for their hepato-protection and elucidated each reported bioactive moieties for their regulation of biological pathways in the human *via* computational tools.

***In silico* studies**

In silico* studies on *C. pepo

From the *In silico* study, it is inferred that the phytosterols from *C. pepo* as potent modulator of major genes and pathways involved in as liver protective agents in liver cirrhosis by preventing oxidative damage and inflammation caused by INH drug. Claims from the traditional system and the available literature, seeds of the *C. pepo* plant are used to treat liver disease. However, there is a lack of data on the molecular mechanism of *C. pepo* for the treatment of liver illness. Hence, this study was designed to identify the potential bioactives and their molecular mechanisms for hepatoprotective activity. LXR is a major nuclear regulatory transcription gene of cholesterol sensing in liver. LXRs are made up of two closely similar receptor isoforms, LXR beta (NR1H2) and LXR alpha (NR1H3), which are expressed by different genes. LXR may help treat drug-induced liver disease as well as non-alcoholic fatty liver disease (NAFLD). Once it gets activated, the other genes are highly regulated by binding to different cholesterol molecules and plays significant role in inflammation, immune regulation, lipid and glucose metabolism. Some selective LXR activators such as desmosterol¹³⁹, the intestine-specific ligand GW6340 and the LXR-623, shows adverse effects. Oxysterols and intermediates from the cholesterol derivatives¹⁴⁰ activate NR1H3 signaling. Another enzyme, *HMG-CoA reductase* is the rate-controlling enzyme of the mevalonate pathway in cholesterol

synthesis and its regulation is *via* a negative feedback mechanism by sterols and non-sterol isoprenoids derived from mevalonate, and the reaction is catalysed by reductase.¹⁴¹ Cholesterol derivatives are major suppressor of this enzyme in mammalian cells and responsible for degradation of low-density lipoprotein (LDL) via the LDL receptor.¹⁴²

Phytochemicals–target network was constructed through network pharmacology and intermolecular interactions were elucidated and/or analysed by molecular docking. The underlying mechanism of network predicted hepatoprotective effect of *C. pepo* is a novel approach for the management of liver disease against INH induced hepatotoxicity. This research showed that the biological network method was more effective in illuminating the connections between illnesses and phytochemicals. In the current study, we relied on a dr. dukes and plethora of open data sources to mine the phytochemicals present in the *C. pepo* and the PASS server was used to pick the compounds with Pa > 0.5, or 50%. Therapeutic Target Database (TTD) and gene card was used to mind the target of liver disease, subjected to network construction. Based on the constructed network, we selected the highly modulated compounds from *C. pepo* and observed their interactions with highly modulated proteins. In docking, the lead hit molecule is determined by the three key points 1) BE, which reflect binding affinity, 2) the number of H-bond interactions, and 3) the H-bond residues.

Oxidative stress, mitochondrial dysfunction, LDH leakage and apoptosis are critical factor in drug induced hepatotoxicity and occurs due to the activation of the ROS system and metal ions transition, which modify homeostatic proteins is one prime basis for the pathogenesis of INH induced hepatotoxicity.¹⁴³ Phytosterols from *C. pepo* provide nutrition to the hepatocytes, contain high amount unsaturated fatty

acids,¹⁴⁴⁻¹⁴⁵ a hydrogen-donating property that can stabilise and delocalize unpaired electrons by creating a hydrogen bond with free radicals and stopping the Fenton reaction.¹⁴⁶ Finally, we constructed the network interaction between phytosterols of *C. pepo* their targets and probable pathways. The result reflects that phytocompounds mainly phytosteroids to regulate the fatty acids interactions with the multiple protein molecules involved in the pathogenesis of liver within the network.

In silico studies on C. melo

Initially, we traced the 160 reported phytocompounds in *C. melo* to propose a probable mechanism in hepato-protection. Out of 160 phytocompounds, 16 were predicted to show a positive druglikeness and were further predicted to target 27 protein molecules and these were involved in 65 pathways, of which 7 pathways were linked with HCC. The network was built between the phytocompounds-targets-pathways and analysed via 'edge count' parameter to predict the node having the highest interactions. Among 27 predicted targets, FXR and HMGCR were identified as a major modulated and therapeutic target involved in the pathogenesis of hepatotoxicity by modulating all 7 pathways within the network. FXR indirectly lowers intracellular FA levels, which in turn mitigates lipid-induced liver injury¹⁴⁷ and FXR was identified as being often overexpressed in a number of liver diseases; it is also a well-established therapeutic target in cases of hepatotoxicity.¹⁴⁷ It is highly expressed in liver and intestine which further standalone in activating varieties of inflammatory markers causing hepatocellular or cholestatic conditions.¹⁴⁷ Several tetracyclic triterpenes from fruits and vegetables have been reported to inhibit the production of FXR by blocking the inflammatory cellular signaling pathways primarily MAPK, PI3-Akt and NF-kB. The bioactive molecules from *C. melo* show strong anti-inflammatory and anti-colitis activity by reducing the FXR expression in

bile formation, TNF- α and IL-6 upregulations.¹⁴⁸ Furthermore, an *In vitro* and *In vivo* studies confirmed that euphol showed a both preventive or therapeutic treatment were effective in reducing the severity of inflammatory conditions and reducing intestinal smooth muscle with propulsion of faces by controlled acute inflammatory reactions. Also, it regulates the nitric oxide production, mucosal damage, and decreased in the component expression of inflammatory responses.¹⁴⁹ Therefore, the network analysis, and predicted affinity of phytochemicals of *Cucumis melo* towards the active site residues of FXR seems to be concurrence with these findings and provide the possible molecular mechanism of action of *Cucumis melo* as a potent hepato-protective nutraceutical. In network, non-alcoholic fatty liver diseases, AMPK, HIF-1, Insulin resistance, PPAR, hepatitis C and pathways in cancer were inferred to be highly modulated pathways *via* targeting multiple protein molecules and on looking into the non-alcoholic fatty liver diseases pathway and PPAR signaling pathways, these includes 9 potential targets i.e., FABP4, PPARG, FABP1, FABP5, PPARG, FABP3, PPARG and, FXR within the network. A previous study showed that *C. melo* exhibited a potent hepato-protective effects through modulation of PPAR signalling pathway by inhibiting the PPARG and PPARG proteins. The current study identifies the tetracyclic triterpene and triterpenoids to target PPAR signalling pathway by targeting the 9 protein targets, among which FXR is also present. Activation of PPAR signalling pathway modulate the lipid metabolism and energy homeostatic, a change that occur during liver injury causes in alteration of metabolic functionality, aggravate liver damage.^{150,151} In some conditions the impaired activity of PPAR signalling pathway might contribute to Hepatocellular carcinoma. Insulin resistance mechanism is a vital signaling pathway in which it binds to the FXR which further extends role in regulating lipid and cholesterol homeostatic. Activation of FXR reverses the insulin resistance and lipid abnormalities gaining its protection against liver toxicity.¹⁵²

Further, it is well reported that, phytochemicals from *C. melo* act as an anti-inflammatory,¹⁵³ anti-diabetic,¹⁵⁴ diuretic,¹⁵⁵ gastroprotective,¹⁵⁶ anti-microbial¹⁵⁷ and hepatoprotective activity¹⁵⁸ by inducing the antioxidant effect. Also, phytochemicals present in the *C. melo* via sitosterol, stigmasterol, codisterol, euphol, tirucallol, lupeol, cucurbitacin B. etc are reported in secretion of the expressed genes involved in hepatotoxicity.¹⁵⁹ Hence, the present investigation on hepato-protective activity of *C. melo* could be due to the modulation of PPAR, Insulin resistance, pathways in cancer, AMPK, NAFLD, hepatitis C and, HIF-1 signaling pathways. Additionally, our study provided an add-on molecular docking support to elucidate the probable role of action of phytochemicals from *C. melo* at the molecular level.¹⁶⁰ Moreover, docking was executed to identify the binding energy of selected phytochemicals against the FXR protein target to assess its hepatoprotective activity. A total of 17 phytochemicals i.e., stigmasterol, sitosterol, beta-sitosterol, 24-methylene cycloartenol, 22-dihydrobrassicasterol, cycloartenol, avenasterol, codisterol, campesterol, alpha-amyrin, beta-amyrin, taraxerol, mutliflorenol, euphol, Tirucallol, lupeol and, cucurbitacin b interacted with FXR target with a least of one hydrogen bond. In FXR, euphol scored a lowest binding energy of -11.1 kcal/mol having maximum active site interactions among others compared to standard silymarin -9.4kcal/mol. This proves the effective binding is present between the selected euphol with FXR protein target.¹⁶⁰

INH induced toxicity model

Hepato-renal damage is a potential major side effect of the anti-TB medication regimen that consists of INH, RIF, and PZA, which may cause toxicity. During the process of biotransformation in the liver, both endogenous and external chemicals undergo alterations and are eliminated by the kidneys.¹⁶¹ This results in the

production of various reactive metabolites that first affect the structural integrity of organs and subsequently their functions. Xeno-metabolism may contribute to hepatorenal toxicities due to the formation of several metabolites during drug metabolism, which may directly or indirectly lead to drug-induced oxidative stress. The process of INH metabolism involves the formation of acetyl hydrazine and diacetyl hydrazine via the activity of N-acetyltransferase 2 (NAT2).¹⁶² Furthermore, the removal of diacetyl hydrazine does not have any harmful impact on the host. However, isoniazid itself or the hydrolysis of acetyl hydrazine generates a reactive intermediate called isoniazid hydrazine. In addition, CYP2E1 catalyzes the oxidation of acetyl hydrazine to form N-hydroxy acetyl hydrazine, and the resulting dehydrated product is acetyl diazine. In addition, acetyl diazine and its decomposition products, namely acetyl onium ion, acetyl radical, and ketene, have the potential to function as reactive oxygen species (ROS).¹⁶³ These active metabolites form strong chemical bonds with different biological components in the liver and kidneys, which ultimately result in harmful effects on these organs. demonstrated that INH acts as a mechanism-based inactivator of human CYP1A2, 2A6, 2C19, and 3A4 enzymes. Intoxication with isoniazid (INH) results in extensive tissue death or long-term inflammation of the liver, and its clinical manifestations can resemble those of acute hepatitis caused by a virus. The typical indications of INH toxicity include loss of appetite, queasiness, retching, and tiredness. However, the occurrence of jaundice accompanied by black urine has been noted as the early signs of liver damage.¹⁶⁴⁻¹⁶⁵

In the current investigation, INH induced hepatotoxicity results from the modulation of numerous biological pathways and genes within the liver. A comprehensive analysis in this study identified 182 pathways that are affected by INH administration. Among these, the TNF and PPAR signaling pathways were notable

for having the highest gene count, indicating a substantial impact on liver function. Chronic activation of this pathway is a critical factor in the development of liver injury and fibrosis, making it a significant target for understanding INH-induced hepatotoxicity and INH-induced modulation of the PPAR pathway can disrupt lipid metabolism, leading to fatty liver (steatosis) and subsequent liver damage. This pathway's significant alteration highlights its importance in the context of INH-induced liver toxicity. Among the genes modulated by INH, GSK3B and PPARG were identified as hub genes within the network, meaning they have a high degree of connectivity and play critical roles in pathway regulation. Targeting the TNF and PPAR signaling pathways, as well as the hub genes GSK3B and PPARG, could offer potential therapeutic strategies to mitigate liver damage caused by INH. For instance, inhibitors of TNF-alpha or modulators of PPAR activity could help reduce inflammation and restore lipid metabolism balance in the liver.

The membrane damage and cellular necrosis causes the enzyme to be released into circulation; can be detected in the blood serum. An abnormal (increased/decreased) level of serum enzymes suggests cell membrane damage and cellular leakage of liver. The transformation of alanine into pyruvate and glutamate is catalysed by ALT. ALT is a superior metric for identifying liver impairment since it is more specific to liver damage.¹⁶⁶ Aspartate is converted to oxaloacetate and glutamate by AST. Elevations in AST levels have been linked to myocardial infarction, liver damage, and muscle injury. Serum ALP is associated with the liver and the bones, whereas bilirubin is associated with the liver and the blood. In the current investigation, INH therapy markedly raised the levels of ALT, AST, and ALP and *C. pepo*, *C. melo* both extract and fraction, and standard drug significantly decreased the ALT, AST and ALP. The synthesis of serum protein relies on a well-functioning liver,

which is responsible for a significant portion of protein metabolism. When the liver becomes injured/infected, protein synthesis slows dramatically, leading to low blood protein levels. Albumin contributes in limiting intravascular fluid loss and our immune system relies heavily on globulins. Patients with liver disease tend to have elevated blood total protein levels.¹⁶⁷ When red blood cells perish, they produce a substance called bilirubin, which is then transported to the liver. Bilirubin is secreted by the liver in a fluid known as bile. If the liver fails to perform its task properly, bilirubin won't be cleared out of the body. One of the most sensitive tests used for hepatic illness detection is serum bilirubin. The biliary system was blocked, leading to hyperbilirubinemia caused by an excess of heme breakdown. When the biliary duct is blocked, the conjugation process is drastically slowed, and unconjugated bilirubin is released from injured and dead hepatocytes. In the present study, a significant increase in the bilirubin and total protein was seen in the INH group, and treatment with *C. pepo*, *C. melo* both extract and fraction and standard drug significantly reversed to normal range of bilirubin and total protein levels.

The application of antioxidant nutraceuticals to lower oxidative stress and liver inflammation is one way to lessen drug-induced hepatotoxicity. Plant secondary metabolites exhibit remarkable pharmacological action in the treatment of a range of illnesses, including diabetes, hypertension, cancer, hepatotoxicity, and allergies. They inhibit the oxidation of these free radicals, thus they act as free radical scavenging activity. Researchers have been concentrating more on fruit materials. They are regarded as abundant antioxidant sources. Secondary plant metabolites, such as terpenoids (carotenoids), phytosterols, and polyphenols (phenolic acids, flavonoids). Rich in unsaturated fatty acids, antioxidants, and fiber, pumpkin seeds (*Cucurbita pepo* L.) are also known to have hepatoprotective and anti-atherogenic properties¹⁶⁸

From the result its also found that *C. pepo*, *C. melo* extracts, fractions and standard drug significantly normalise the elevated antioxidants (Catalase, GSH, SOD, LPO and nitrite) especially glutathione and lipid parameters (TP, TC, TG, HDL, LDL and VLDL) levels, by suppressing lipid peroxidation and increasing level of antioxidants

High fat diet model

In the current study, initially the study traced the molecular mechanisms of HFD induced hepatotoxicity. HFD may alter multiple protein targets and biological pathways which results in the complications like diabetes, obesity, hepatitis, etc. About 171 genes were obtained for HFD-induced hepatotoxicity using gene cards and were found to involve in 186 pathways of which 14 pathways were majorly enriched for HFD-induced hepatotoxicity, diabetes, obesity etc. The genes were found to potentially modulate PI3K-Akt, IL-17, TNF, JAK-STAT, MAPK, p53, PPAR, NF-kappa B signaling pathway and are responsible for hepatocellular carcinoma, insulin resistance, etc. A wide range of cellular processes, including the survival and death of many cell types, are regulated by the PI3K/Akt signalling. Activation of this signaling pathway is essential for the initiation of the adipogenic transition and adipocyte hyperplasia.¹⁶⁹ The histologic characteristic of NAFLD that is most important is hepatocyte apoptosis, which is correlated with increasing inflammation and fibrosis. Although the exact molecular mechanisms causing hepatocyte death are unknown, NAFLD development significantly involves mitochondrial dysfunction. Recent research has shown that mitochondria have a role in controlling apoptosis as well as cell division and proliferation, suggesting that they may act as intermediaries of the PI3K/Akt signaling pathway. Though many lines of evidence have shown a strong connection between insulin-induced signaling and mitochondrial functioning, it is still unknown how the PI3K/Akt signaling pathway and the abnormalities in the

mitochondria that cause NAFLD may be related. Hence, the modulation of PI3K/Akt signaling pathway by HFD could be majorly due to alteration of 32 genes viz., MAPK1, HGF, CSF3, NFKB1, IL2, CCND1, IL4, EPO, MDM2, MAPK3, KDR, EGF, TP53, ERBB2, EGFR, IFNA1, NOS3, PRL, INSR, IRS1, CASP9, MTOR, FASLG, MCL1, IL2RA, IGF1, INS, BCL2, CDKN1A, IL6, IGF2, and PIK3CG. Also, high fat diet induced liver lipogenesis and hepatic steatosis are also majorly due to the modulation of IL-17, TNF, JAK-STAT, MAPK, p53, PPAR, NF-kappa B signaling pathways and were identified in this study.¹⁷⁰

On the other hand, phytochemicals from *C. pepo* and *C. melo* were found to modulate MAPK, PI3-Akt and NF-kB and may inhibit the progression of HFD induced hepatotoxicity. In order to check this, *C. pepo* and *C. melo* extract and fractions were tested on HFD induced hepatotoxicity in HepG2 cell line. Initially, *C. melo* and *C. pepo* were collected and its hydroalcoholic extracts were prepared from the maceration technique. The extract was further subjected to fractionation to obtain a steroid enriched fraction (Dichloromethane>Petroleum ether>methanol). Further, the obtained fraction was screened for cytotoxicity evaluation in HepG2 cell line to obtain the possible biological effect on hepatocytes. Here, both extract and fraction showed significant cell viability compared to HFD treated groups. In HFD animals, elevated hepatic fatty acid metabolism results into cholesterol and triglycerides accumulation as well as elevated liver inflammation and liver damage.¹⁷¹ This will also contribute to the other pathological conditions such as cardiovascular disorders. On peer literature of this, we found that intake of HFD leading to the alteration of biological markers viz., catalase, nitrate, GSH, LPO, AST, ALT, ALP, TG, HDL, TC, LDL, VLDL albumin, bilirubin, etc which are an essential mediator in the regulation of biological oxidation, lipid metabolism, adipogenesis, etc. A fatty liver may advance to NASH and cirrhosis when there is inadequate lipid oxidation due to failure of lipid

metabolism. This leads to the hepatocytes accumulating excess lipids.¹⁷² An excessive development of adipose tissue mass, mostly brought on by the excessive accumulation of TG inside adipocytes, is the hallmark of obesity arising from over-eating on a low-fat diet. It is believed that saturated fatty acids are linked to lipogenesis and a higher risk of lipid-induced diseases, such as fatty liver. Fast food consumption contributes to elevated ALT levels and body weight gain because it contains high levels of trans fatty acid in the form of butter and frying oils. Furthermore, a diet heavy in fat and cholesterol may contribute to the buildup of lipids in the liver, and IR is a potent oxidative stressor that lowers the levels of naturally occurring antioxidants in cells by producing too many ROS. Lipid peroxidation, which is brought on by ROS, activates the immune system's response in cells including hepatic stellate and Kupffer cells, leading to inflammation. Anomalies in lipid metabolism, such as elevated blood TG, TC, and LDL levels and decreased HDL levels, are indicative of dyslipidemia. Lipid peroxidation, stellate cell activation, steatosis, and ultimately precirrhotic steatohepatitis occur in the liver as a result of dyslipidemia.¹⁷²

The extract (500mg/kg. p.o.) and fraction (50mg/kg. p.o.) was screened for its hepatoprotection in albino mice. In HFD treated groups, a remarkable rise in the liver weight ($p < 0.001$) could be due to the accumulation of fatty material, and which was reversed in the treatment groups. This suggests that phytosterols may have a function in controlling lipid metabolism. Several studies have shown that eating too much fat raises blood levels of free fatty acids, which in turn causes oxidative stress and chronic liver inflammation. In the present study, HFD treated animal showed abnormal level of antioxidant markers TP, GSH, LPO, SOD, nitrate, and catalase, levels and were reversed in the *C. melo* extract and fraction groups. Similarly, the lipid markers like HDL, LDL, VLDL, TG and TC were also abnormal in the HFD

treated animal and were found to be towards normal in *C. melo* extract and fraction groups. Serum ALP, AST, ALT, and bilirubin is considered as a specific marker for hepatic damage and were significantly elevated in the HFD treated animals and were found to be towards normal in *C. melo* extract and fraction groups. Histology of the liver showed a remarkable histological change after treatment. In HFD treated animals shows a healthy liver tissue sample and normal liver architecture. The liver cells (hepatocytes) appear healthy, with uniform size and shape, and are neatly arranged in cords radiating from the central vein. The section represents a typical healthy liver with no signs of damage or disease. Whereas, HFD animals showed liver damage. There is notable congestion in the blood vessels, along with inflammation in the portal triad areas (regions where the hepatic artery, portal vein, and bile duct are located). Presence of fat droplets within hepatocytes, indicating steatosis, a common consequence of high-fat diets leading to NAFLD. Areas of cell death, suggesting severe liver injury and potential progression to more serious liver conditions like fibrosis or cirrhosis. And treatment with Silymarin, *C. pepo* and *C. melo* reversed these changes and normal liver architecture is observed with slight fibrotic state.

CONCLUSION

The current study employed computational approaches combined with multiple *In vitro* and *In vivo* studies to trace the hepatoprotective potential of *C. pepo* and *C. melo*. Firstly, from the computational study, the study traced the phytochemicals of both *C. pepo* and *C. melo* and identified the probable modulated genes by each compound. Further, the gene set enrichment and network analysis revealed that the phytochemicals from *C. pepo* (18 compounds) to hit 26 targets involved in 11 pathways associated with liver injury, liver fibrosis, and cirrhosis viz., PPAR signaling pathway, metabolic and adipocytokine signaling pathway, Th17 cell differentiation, AMPK signaling, arachidonic acid metabolism, etc. These compounds were predicted to interact with active site of NR1H3 and HMGCR protein targets, identified as the hub genes within the network. Likewise, phytochemicals from *C. melo* (16 compounds) to hit 20 targets involved in 16 pathways associated with liver injury, liver fibrosis, and cirrhosis viz., PPAR signaling pathway, AMPK signaling pathway, Pathways in cancer, NAFLD, and Hepatitis C related pathways. These compounds were predicted to interact with active site of FXR protein target, identified as a hub gene within the network. Additionally, these traced compounds pose druggability. Majorly, the phytochemicals from *C. pepo* and *C. melo* were found to target the MAPK, PPAR, and NF- κ B signalling pathway which were altered by the INH and HFD.

Secondly, from the cytotoxicity assay, it is identified that both *C. pepo* and *C. melo* extract and crude fraction to be non-toxic in HepG2 cell line and possess hepatoprotective activity against INH and HFD induced hepatic injury via reducing LDH, ALT, and AST levels. Also, *C. pepo* and *C. melo* extracts and crude fractions possess antioxidant activity which confirmed via DPPH scavenging assay. The effect

of *C. pepo* and *C. melo* extract and their fractions were further tested for their hepatoprotective activity in INH- and HFD-induced liver injury (the model is used for chronic hepatitis). We observed a significant alteration of the liver function and lipid profile by INH and HFD treated animals and treatment with *C. pepo* and *C. melo* extracts and crude fractions reversed the level of ALT, AST, ALP, bilirubin, TC, TP, TG, HDL VLDL and LDL levels towards normal levels. Additionally, both the plant extracts and fractions showed antioxidant potency via regulating the catalase, SOD, GSH, nitrite, and MDA levels and also reversed histological restoration of the liver damaged by INH and HFD. Theoretical knowledge combined with predicted and validated results of this study confirms *C. pepo* and *C. melo* extract and their crude fraction as valuable resources for the management of hepatitis.

LIMITATION & PROSPECTIVE

For the first time, the hepatitis targets were mapped onto the bioactives from *C. pepo* and *C. melo* in order to integrate results with experimental pharmacology and determine the mechanism of action in preventing hepatitis produced by INH and HFD. However, the results of system biology are only mapped using functional biomarkers, which may be the results of shared homologous proteins. Further cross-mapping using particular molecular biomarkers associated with a particular gene is required. The study primarily focused on *In vitro* and animal model experiments. Clinical trials involving human subjects are necessary to validate the efficacy and safety of these plant extracts and steroid fractions for hepatitis management. Also, investigating the synergistic effects of combining *C. pepo* and *C. melo* extracts with conventional therapies or other natural compounds could lead to novel treatment strategies for hepatitis.

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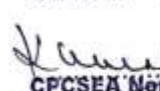
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ANNEXURE – I - INFORMED CONSENT FORM

	KLE College of Pharmacy A Constituent Unit of KLE Academy of Higher Education and Research (Deemed to be University) DEPARTMENT OF PHARMACOLOGY <i>[NMC Campus, Nehru Nagar, Belagavi - 590 010, Karnataka, India]</i> <u>INSTITUTIONAL ANIMAL ETHICS COMMITTEE</u> <i>Reg.No.221/Po/Re/S/2000/CPCSEA</i>	
Date: 21/01/2022		
CERTIFICATE		
This is to certify that the project proposal no ¹⁰...entitled," In silico docking based Pharmacological screening for the hepatoprotective activity of Cucumis melo and Cucurbita pepo in Experimental animals", submitted by Mr. / ~~Ms.~~ Sanjay Ugare under the guidance of Dr. N.A. Khatib has been approved/recommended by the IAEC of KLE College of Pharmacy, Belagavi, Reg.No.221/Po/Re/S/2000/CPCSEA in its meeting dated 21/1/2022, resolution No. 32 has been sanctioned <u>42 each</u> ☒ Rats/ ☒ Mice/ ☒ Rabbits/ ☒ Guinea pig (animals) sex <u>either</u> under this proposal for a duration of next.....months.		
You are hereby informed to strictly adhere to the protocol submitted for approval. Further you are required to keep the account of animals used for the project in specified Performa, Form D.		
Authorized by	Name	Signature
Member Secretary:	Dr. N.A. Khatib	 22/01/2022
MEMBER SECRETARY Institutional Animal Ethics Committee, KLE's College of Pharmacy, BELAGAVI		
Main Nominee of		
CPCSEA:	Dr. Vinod Kumar CS	 22/01/2022
CPCSEA Nominee Institutional Animal Ethics Committee KLE's College of Pharmacy, BELGAUM.		

SHRI B.M.K. AYURVEDA MAHAVIDYALAYA
 A constituent unit KLE Academy of Higher Education & Research
 Deemed-to-be-University
Central Research Facility
DRUG AUTHENTICATION REPORT



Submitted By: Sanjay Ugare

Submitted Date: 17/06/2020

Date of Issue: 22/06/2020

S N	Sample Name	Scientific Name	Family	Part submitted	CRF Code	Authenticated as		
						Ayurvedic Name	Scientific Name	Family Authenticated
1	Chirbhita	<i>Cucumis melo</i> L.	Cucurbitaceae	Seeds	CRF/Auth / 2020/01	Chirbhita	<i>Cucumis melo</i> L.	Cucurbitaceae Seeds
2	Kushmandi	<i>Cucurbita pepo</i> L.	Cucurbitaceae	Seeds	CRF/Auth / 2020/02	Kushmandi	<i>Cucurbita pepo</i> L.	Cucurbitaceae Seeds



Signature: *Dr. Divya Khare*
 Authentication Expert Name: Dr. Divya Khare
 Date: 22/06/2020

[Signature]
 Signature of Coordinator
 ASU Drug Testing Laboratory

ANNEXURE – II - RESEARCH PUBLICATIONS

List of Research publications

1. Ugare SR, Khatib NA, Patil VS, Patil D, Dodakallanavar J. Computational and experimental pharmacology reveals hepatoprotective effect of Cucurbita pepo in isoniazid-induced liver cirrhosis. Journal of Applied Pharmaceutical Science. 2024 Jan 4;14(1):177-88.
2. The Effectiveness of Hydroalcoholic Extract and Fraction of Cucumis melo on Amelioration of High Fat Diet Induced hepatotoxicity: A Comprehensive Approach Integrating Computational and Preclinical Validation, 2024 Jun.

List of poster and oral presentations

Sr.no.	Title	Name of the conference	Organized by
1.	Screening of Cucurbita pepo against therapeutic targets of liver cirrhosis. <i>An in silico and in viro study</i>	9 th International congress of Society for Ethnopharmacology, India	JSS academy of Higher Education & Research, Mysuru. From 22 to 24 th April 2022
2.	Network Pharmacology and Bioinformatics approach: Exploring molecular mechanism of <i>Cucumis melo</i> in liver cirrhosis fallowed by experimental validation	7 th Annual International conference on IPR Current Glabal Trends in IPR: what we do/ should know !!	Goa college of Pharmacy, Panaji-Goa from November 15-16 2022
3.	Network Pharmacology Approach to understand molecular mechanism action of Traditional medicine	Faculty Development programme	G. Pulla Reddy College, Hyderabad. From 13-17 th February 2023

4	<i>Insilico</i> Pharmacology Approach to find natural phytocompounds & targets	National level workshop	Appasaheb Birnale College of Pharmacy, Sangli on 16 the January 2024
Patent			
	Title of Invention	Application /Granted No.	Date
1.	Formulation and evaluation of Phytosomes loaded with <i>Tinospora cordifolia</i> on Ethylene glycol induced Urolithiasis.	20244103799 A	31/05/2024
Book published			
A Text book of Human Anatomy and Physiology. Dr. N.S. Vyawahare, Dr. Bhupalam Pradeepkumar, Dr. Karishma Rathi, Mr. Sanjay R. Ugare			

Book published:

A Text book of Human Anatomy and Physiology. Dr. N.S. Vyawahare, Dr. Bhupalam Pradeepkumar, Dr. Karishma Rathi, Mr. Sanjay R. Ugare

Publications (other than Ph.D work):

1. Tubachi SS, Rasal VP, Ugare SR, Khatib NA, Ojha PS, Patil VS. Evaluation of Ylang Ylang essential oil on alcohol induced hepatotoxicity in rats. *Advances in Traditional Medicine*. 2023 Jun;23(2):575-88.
2. Rasal VP, Patel V, Ugare SR. Hepatoprotective potential of ethanolic extract of solanum melongena leaves against isoniazid (inh) induced liver damage in animal models.

3. Sridhanya V, Rajeshwari K, Ugare SR, molecular mechanism of Nishamalaki as Rasayana in Diabetes Mellitus. International Journal of Ayurvedic medicine 2024 June; 15(2): 375-382
4. Krishika M. UgareSR, Hiremath KV, Probable mode of action of *Sida Cordifolia* in Dyspareunia an *insilico* network Pharmacological approach.
5. Jose J, Khot KB, Thomas SP, Chopra H, Gopan G, Bandiwadekar A, Babu A, Ugare SR, Ashraf GM, Dhyani A, Vora V. Advances in microneedles-based drug delivery system on promoting wound healing. Journal of Drug Delivery Science and Technology. 2023 Nov 14:105163.
6. Jose J, Khot KB, Shastry P, Gopan G, Bandiwadekar A, Thomas SP, Muhammad ST, Ugare SR, Chopra H, Choudhary OP. Recent advancements in microneedle-based vaccine delivery. International Journal of Surgery. 2022 Nov 1;107:106973.
7. Magaduma P, Khatib N, Ugare S. Attenuation of salt induced cardiac remodeling by garcinia indica fruit extract in rats. Indian drugs. 2021 may 1;58(5).



Computational and experimental pharmacology reveals hepatoprotective effect of *Cucurbita pepo* in isoniazid-induced liver cirrhosis

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Cucurbita pepo,
 hepatoprotective, network
 pharmacology, molecular
 dynamics, isoniazid.

ABSTRACT

Cucurbita pepo is widely utilized as a vegetable and is reported to possess hepatoprotective activity. However, its hepatoprotective molecular mechanisms are not yet elucidated. The current study utilized multiple *in silico* approaches to identify the mechanisms of active biomolecules of *C. pepo*. Additionally, the *C. pepo* effect on Isoniazid (INH) induced liver cirrhosis was evaluated in experimental animals. First, *in silico* studies, viz., genes pathways and network analysis, hub gene molecular docking and dynamics were employed. Secondly, *C. pepo* steroid fraction was subjected to liquid chromatography-mass spectrometry analysis and thirdly, hepatoprotective activity of both hydroalcoholic extract and steroid fraction was evaluated in INH-induced liver cirrhosis in rats. Out of 135 compounds, 18 (mainly phytosteroids) were identified to modulate 4 main pathways in liver disease. Among the predicted targets, NR1H3 and 3-hydroxy-3-methylglutaryl-CoA reductase were identified as a hub genes. Schottenol and Alpha-Spinasterol formed stable contacts with NR1H3 throughout 30 ns molecular dynamics simulation. INH (50 mg/kg/day) treatment elevated aspartate aminotransferase, alanine transaminase, alkaline phosphatase, serum bilirubin, total bilirubin, total protein, total cholesterol, triglyceride, high-density lipoprotein, low-density lipoprotein, very low density lipoprotein levels in blood serum and 500 mg/kg/day extract and 50 mg/kg/day of steroidal fraction reversed liver biomarkers altered by INH in rats. *In silico* combined with experimental study identified the possible molecular mechanisms of *C. pepo* for the management of liver cirrhosis.

INTRODUCTION

The epidemiology and etiology of liver injury vary among the population of different countries. The risk of drug-induced liver damage is low in the general population but higher in hospitalized patients and liver cirrhosis is often treated with pharmaceuticals, nutritional supplements, and botanical medicines. Recent evidence showed that oxidative stress has been

linked to the major pathogenic mechanism of chemical-induced liver injury resulting in various disorders such as hepatotoxicity, cancer, diabetes, hypertension, various neurological disorders, etc. [1]. Hepatotoxicity is usually caused by a viral infection, excess alcohol consumption, non-alcoholic fatty liver disease (NAFLD), and drug toxicity [2]. Among the modulated genes in liver disease, Liver X receptors (LXRs) is a nuclear receptor that regulates lipid equilibrium and cytoplasmic cholesterol homeostasis. They have a key regulatory role in the immune system including *de novo* lipogenesis and inflammation. The binding of synthetic cholesterol derivatives, endogenous and certain oxysterols to LXR are widely recognized as the biologically active LXR ligands [3]. Two isoforms of LXR viz., NR1H3 and NR1H2, in which NR1H3 is highly expressed in liver tissue, with a lower level in the kidney, spleen, adipose tissue, and intestine [4,5]. The promoter region in LXR α conforms to the regulation of LXR α and LXR β . Specific

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oxysterols or synthetic ligands modulate both LXR isoforms [6,7]. Expanding knowledge of LXR function in fatty acid and cholesterol homeostasis has been considered a major precursor from the pharmaceutical point of view.

Isoniazid (INH) is considered a first-line drug for the treatment of tuberculosis. Several cases of INH-induced hepatotoxicity have been detected in different patients. INH is metabolized in the liver by N-acetyl transferase 2 into acetyl-INH. A number of secondary metabolites, including monoacetyl hydrazine (MAH) and diacetyl hydrazine are produced during the metabolism of acetyl-INH. A covalent connection between acetyl hydrazine and liver macromolecules and the formation of free radicals from reactive metabolites of MAH have both been hypothesized as mechanisms by which INH metabolites may impact the rate of INH-induced hepatotoxicity.

To combat the drug-induced hepatotoxicity includes the utilization of antioxidant nutraceuticals to target liver inflammation triggered by oxidative stress. From the plants, secondary metabolites have eminent pharmacological activity for the management of multiple ailments such as diabetes, hypertension, cancer, hepatotoxicity, allergy, etc., and these secondary metabolites protect the cells from free radicals, and thus they act as free radical scavenging activity. Recently researchers are more focussing on fruit materials. Which are considered a rich source of natural antioxidants, plant secondary metabolites such as phytosterols, terpenoids (carotenoids), polyphenols (phenolic acids, flavonoids). *Cucurbita pepo* seeds (*C. pepo* L.) are a rich source of unsaturated antioxidants, fatty acids, and fibers and known to have hepatoprotective and anti-atherogenic activities. *Cucurbita pepo* is widely used as functional food or medicine because of a rich source of carotenoids, polyunsaturated fatty acids. It has high contents of alkaloids, flavonoids, terpenoids, proteins, lipids, carbohydrates, minerals, and phytosterols. In general, hepatoprotective plants contains a variety of phytochemicals, such as phenols, glycosides, alkaloids, flavonoids, carotenoids coumarins, steroids, lignans, essential oils, monoterpenes, organic acids, carotenoids, xanthines, triterpenoids, etc. As such, on the basis of the traditional use, the present investigation was carried out to evaluate the molecular mechanism of *C. pepo* by network pharmacology approach supported by *in vivo* hepatoprotective studies.

MATERIALS AND METHODS

Computational approach

Retrieval of phytochemicals and proteins involved in liver disease

The reported phytochemicals from *C. pepo* were collected from phytochemical databases, and PubChem was employed to retrieve structural data on those compounds from several open-source libraries. The potential probable protein targets of phytochemicals for liver cirrhosis were then identified. Throughout the dataset's construction, duplicate phytochemicals and proteins related to liver cirrhosis were removed. PubChem database was employed to retrieve each phytochemical's canonical simplified molecular-input line-entry system (SMILES) [8]. SMILES were queried in SwissTargetPrediction [9] for the target prediction at a percentage similarity of >0.1%.

With respect to the existing targets of liver disorders included in the therapeutic target database, the proteins implicated in liver cirrhosis were located [10]. Each protein identified as the cause of liver cirrhosis has its gene ID acquired from UniProt [11] and phytochemicals acting on liver targets were identified.

G-Profiler web server

The study of biological data frequently involves looking at the number of genes associated with different diseases. G: Profiler software server is frequently used to determine biological categories that are enriched in gene lists (<https://biit.cs.ut.ee/profiler>). GOST uses g: Convert to map gene/protein IDs across different namespaces, g: Orth to map orthologous genes across species, and g: SNPense to map human single nucleotide polymorphisms identifiers to genes to perform the enrichment analysis of single or multiple gene lists [12].

Set of pathway and network analysis of *C. pepo*

STRING database was used to identify a set of proteins involved in liver disease [13] and enrichment analysis of gene was employed to infer the related pathways that are altered by the phytochemical. Detailed examination of the pathways implicated in liver pathogenesis was found using the KEGG database. The network linking phytochemicals, protein molecules, and identified pathways was built using Cytoscape 3.7.2 [14].

Docking studies

Protein retrieval and preparation

PubChem online database was utilized to retrieve the 3D structure of all phytochemicals and minimized using MMFF94 forcefield [15]. A literature search and Research Collaboratory for Structural Bioinformatics database (<https://www.rcsb.org/>) were used to identify the protein and Protein Data Bank ID for NR1H3 protein (1UHL) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) protein (2R4F). Protein was prepared via protein preparation wizard panel of Schrodinger's (2021-2), where water beyond 5 Å from Het groups was removed, zero bond order to metals was created, polar hydrogen was assigned Methionine residues were restored, and Restrain energy minimization was carried out using optimized potential for liquid simulation (OPLS3e) force field [16,17].

Ligand preparation

The ligprep panel of Schrodinger's (2021-2) was used to generate possible states of ligands at the target pH using the Epik module. Chirality was retained for the selected ligand and at most one stereoisomer was generated for the given ligand, finally, energy was minimized using the OPLS3e force field [18].

Grid generation

The glide module of Schrodinger containing a receptor grid generation panel was utilized to create a grid at the binding site. The site of the co-crystal ligand was considered a binding site and a grid was generated.

Ligand docking

The glide module of Schrodinger's was utilized for ligand docking and Maestro 12.3 was used to visualize the

binding interactions. Extra precision mode for docking was carried out to understand the binding energy and orientation of ligands within the binding pocket. To visualize the interaction between the ligand and the protein following molecular docking, the posture scoring the lowest BE was chosen. The nuclear receptor superfamily's NR1 subfamily includes the NR1H3 protein, which is encoded by the gene. NR1 family members are the primary modulators, controlling transcriptional regulation in the maintenance of lipid homeostasis and inflammation. As demonstrated, experimental research in mice lacking this gene suggests that it may be crucial for controlling cholesterol homeostasis [19].

Molecular dynamics

To evaluate the stability of complex formation, a 30 ns molecular dynamics (MD) simulation was performed on ligand-protein complexes using the Desmond software. Using the simple point charge water model, the system in a cubical box with $10 \text{ \AA} \times 10 \text{ \AA} \times 10 \text{ \AA}$ periodic boundary conditions was solvated. Na^+/Cl^- counter ions were also added, which further neutralized the solution. Bond angles and lengths of heavy atoms geometry were restrained by the SHAKE algorithm. Long-range interactions were calculated by "particle mesh Ewald" method. The cut-off value for "Lennard-Jones interactions" was set at $10.0 \text{ \AA}$. Additionally, the system was loosened up or minimized using default settings. A Nose-Hoover chain thermostat with a 1.0 ps relaxation time and the Martyna-Tobias-Klein barostat method with a 2.0 ps relaxation time were then used for the final 30 ns of the production run, with a Coulombic short range cutoff radius set to $9.0 \text{ \AA}$ and the temperature and pressure being set to 300 K and 1.01325 bar , respectively. To infer the structural stability, root-mean-square fluctuation (RMSF) of residues, root-mean-square deviation (RMSD) of complex, and protein radius of gyration were analyzed. In order to infer stable interactions within the ligand-protein throughout MD simulation, the ligand-protein percentage fraction interactions were analyzed during 30 ns simulation.

In vitro studies

Collection and authentication of *C. pepo*

The ripe fruits of *C. pepo* were purchased locally in June month of 2020 from the local area of Belagavi region, Karnataka then plant seeds were authenticated by Central Research Facility B.M.K. Ayurveda Mahavidyalaya, Belagavi. With voucher specimen number CRF/Auth/2020/1 and CRF/Auth/2020/2.

Preparation of plant extract and fraction

The *C. pepo* fruits were peeled, the seeds were carefully hand-removed from the pulp, cleaned, rinsed, and the damaged seeds were separated. The remaining seeds were then dried in an oven at 40°C for 24 hours. Dried seeds were then ground in an electrical grinder. 70% v/v ethanol was used as a solvent for the maceration of dried seeds and 5% chloroform (preservative) for 72 hours. The extract was filtered and the raw material that remained in the maceration was subjected to soxhlation (70% v/v ethanol). Filtrates of maceration and soxhlation were concentrated at 40°C under reduced pressure

using a rotary evaporator which yield total extract. Finally, the extract was subjected to fractionation. Fractionation was performed as explained by Cos et al. [20]. The hydroalcoholic extract was dissolved in water and the dichloromethane mixture and dichloromethane fraction were further mixed with methanol (90%) to obtain the sterols fraction.

Yield of the extract (%) = (weight of extract/weight of dried seeds) $\times 100$. The percentage yield of extract/ 100 g of powder was found to be 14% and the percentage yield of fraction using 20 g of hydroalcoholic extract of *C. pepo* was found to be 4%.

Liquid chromatography-mass spectrometry (LC-MS) profile of the hydroalcoholic extract of *C. pepo*

When using the LC-MS 2010A (Shimadzu Japan) to run the sample, the following requirements were upheld. Methanol and water were employed in a 90:10 v/v ratio as the mobile phase, with a flow rate of 200 $\mu\text{l}/\text{minute}$. The C18 column served as the stationary phase. The sample was injected (μl) after being dissolved in the mobile phase. The chemicals in the sample were identified using the electrospray ionization peaks [21].

Assessment of hepatoprotective activity of *C. pepo* by in vivo

The Institutional Animal Ethics Committee (Reg. No. 221/Po/Re/S/2000/CPCSEA at its meeting date of 21/01/2022) was evaluated the protocol of the study. Albino-Wistar rats weighing 160 to 180 g were housed in standard laboratory conditions of temperature ($25^\circ\text{C} \pm 2^\circ\text{C}$), 12 hours in light and dark places, and with food and water ad libitum. INH (50 mg/kg p.o.) was administered daily for 28 days in drug-induced liver toxicity model, to all animals except group I. Silymarin (50 mg/kg p.o.) was used as standard. The animals were segregated into seven groups of six each as follows [22]. Group-I: received food and water. Group-II: (control) received INH (50 mg/kg) p.o. up to 28 days. Group III: received Silymarin 50 mg/kg orally and INH (50 mg/kg) p.o. up to 28 days. Group IV: received 500 mg/kg of *C. pepo* extract and INH (50 mg/kg). Group V: received 50 mg/kg of *C. pepo* steroid fraction and INH (50 mg/kg). Under ether anaesthesia, blood was collected from each rat via the retro-orbital plexus for biochemical analysis, including aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), bilirubin estimate, high-density lipoprotein (HDL) low density lipoprotein (LDL), very low density lipoprotein (VLDL), triglyceride (TG) and total cholesterol (TC). The blood serum was separated by centrifugation at 2,500 rpm for 15 minutes after the blood had been allowed to coagulate at 37°C for 30 minutes. The animals were euthanized and the liver was promptly removed and processed for histological analysis.

Statistical analysis

Network analysis results were expressed in gene count and false discovery rate. Docking binding energy is expressed in kcal/mol. During MD analysis, RMSD and RMSF were considered for evaluation. In vivo experiments results were expressed as Mean $\pm$ SEM and the difference among the mean was evaluated using one-way ANOVA followed by Tukey's test, using GraphPad Prism version 8.0.

Table 1. Pathways modulated by *C. pepo* compounds.

KEGG ID	Pathway name	Gene count	FDR	Genes set within the pathway
hsa03320	PPAR signaling pathway	10	1.40E-14	PPARG, FABP1, FABP3, FABP4, PPARG, RXRG, RXRB, PPARG, RXRA, NR1H3
hsa04931	Insulin resistance	5	4.68E-05	NR1H2, PTPRF, PPARG, IL6, NR1H3
hsa01100	Metabolic pathways	11	0.00013	CYP26B1, CYP51A1, CYP24A1, CYP26A1, CYP2C9, HMGCR, CYP11B1, CYP11B2, CYP17A1, CYP2C19, CYP19A1
hsa04920	Adipocytokine signaling pathway	4	0.00013	RXRG, RXRB, PPARG, RXRA
hsa04659	Th17 cell differentiation	4	0.00037	RXRG, RXRB, IL6, RXRA
hsa04932	Non-alcoholic fatty liver disease	4	0.0014	PPARG, IL6, RXRA, NR1H3
hsa04976	Bile secretion	3	0.0021	HMGCR, RXRA, NR1H4
hsa00591	Linoleic acid metabolism	2	0.0067	CYP2C9, CYP2C19
hsa04152	AMPK signaling pathway	3	0.0067	PPARG, HMGCR, HNF4A
hsa00590	Arachidonic acid metabolism	2	0.0219	CYP2C9, CYP2C19
hsa00982	Drug-metabolism cytochrome P450	2	0.0233	CYP2C9, CYP2C19

Table 2. Druggability and rule of five drug-likeness score of phytochemicals.

Compounds		Lipinski rule of five				Side effects			
		Molecular weight (g/mol)	HBA	HBD	Log P	Drug likeness score	Pa	PI	Side effect
S. No	Acceptable values	<500	<5	<5	<10	0 to 2			
1	Linoleic acid	280.4	2	1	5.88	0.09	0.304	0.152	Nephrotoxicity
2	Cucurbitic acid	212.28	3	2	1.82	-0.14			Nephrotoxicity
3	Octadecanoic acid	284.5	2	1	7.65	-0.54	0.500	0.050	Hepatotoxicity
4	Schottenol	414.7	1	1	8.58	0.18	0.322	0.135	Nephrotoxicity
5	Alpha-Spinasterol	412.7	3	1	2.68	0.29	0.254	0.212	Nephrotoxicity
6	Chondrillastanol	412.7	1	1	7.87	-0.05	0.254	0.212	Nephrotoxicity
7	(+)-Dehydrovomifolol	222.28	1	3	1.38	-1.14	Nil	Nil	Nil
8	Clenosterol	412.7	1	1	8.58	0.68	Nil	Nil	Nil
9	Codisterol	398.7	1	1	7.70	0.43	Nil	Nil	Nil
10	Isofacosterol	412.7	1	1	7.82	0.85	Nil	Nil	Nil
11	Cucurbitacin-D	516.7	7	4	2.61	0.39	Nil	Nil	Nil
12	Cucurbitacin-I	514.6	7	4	2.86	0.26	Nil	Nil	Nil
13	22-Dihydrobrassicasterol	400.37	1	1	7.87	0.59	Nil	Nil	Nil
14	Cryptoxanthin	552.9	1	1	12.83	0.78	0.7	0.012	Myocardial infarction
15	Eicosadienoic acid	308.5	2	1	-7.61	-0.30	0.304	0.152	Nephrotoxicity
16	Delta-7-Ergosterol	400.7	1	1	8.00	-0.03	0.28	0.179	Nephrotoxicity
17	Abscisic acid	264.32	4	2	2.56	-0.86	0.244	0.159	Nephrotoxicity
18	7 beta, 13-Dihydroxykaurenoide	552.20	4	2	2.12	-0.34	0.312	0.294	Nephrotoxicity

Pa: Probable activity; Pi: Probable inactivity.

Leu260, Ala261, Ser264, Ile339, Phe326, Phe335, Leu331, Arg305, Thr302, Glu301, Met298, Ile295, Trp443, Thr314, Ile313, Tyr321 (Fig. 2) and Alpha-Spinasterol has the highest H-bond interaction with HMGCR i.e. 10 interactions viz., Lys691, Asp690, Met655, Met657, Asn658, Ser661, Lys662, Glu665, Arg590, Val683 in Figure 3.

Molecular dynamics

Stability of Schottenol with NR1H3

The RMSD of both backbone and complex was found to be stable throughout 30 ns MD simulation (Fig. 4). The average RMSD of the backbone was found to be ~2.0 Å and the complex

Table 3. The ADME profile of each phytochemicals of *C. pepo* by Swiss ADME.

Compounds	GI absorption	BBB permeant	P-gp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	LogKp (skin permeation)
Linoleic acid	High	Yes	No	Yes	No	Yes	No	No	-3.05 cm/second
Cucurbitic acid	High	Yes	No	No	No	No	No	No	-6.26 cm/second
Octadecanoic acid	High	No	No	Yes	No	No	No	No	-2.19 cm/second
Schottenol	Low	No	No	No	No	No	No	No	-2.38 cm/second
Alpha-Spinasterol	Low	No	No	No	No	No	No	No	-2.92 cm/second
Chondrillasterol	Low	No	No	No	No	No	No	No	-2.92 cm/second
(+)-Dehydrovomifoliol	High	Yes	No	No	No	No	No	No	-7.30 cm/second
Clerosterol	Low	No	No	No	No	Yes	No	No	-2.24 cm/second
Codisterol	Low	No	No	No	No	Yes	No	No	-2.53 cm/second
Isocuposterol	Low	No	No	No	No	No	No	No	-2.53 cm/second
Cucurbitacin-D	High	No	Yes	No	No	No	No	Yes	-7.99 cm/second
Cucurbitacin-I	Low	No	Yes	No	No	No	No	Yes	-7.54 cm/second
22-Dihydrobrassicasterol	Low	No	No	No	No	No	No	No	-2.50 cm/second
Cryptoxanthin	Low	No	Yes	No	No	No	No	No	-0.98 cm/second
Eicosadienoic acid	High	No	No	Yes	No	Yes	No	No	-2.47 cm/second
Delta-7-Ergosterol	Low	No	No	No	No	Yes	No	No	-3.44 cm/second
Abseicic acid	Low	No	No	No	No	No	No	No	-2.24 cm/second
7 beta,13-Dihydroxykaurenolide	High	No	No	Yes	No	No	No	No	-2.45 cm/second

Table 4. Binding energy and mode of interaction of each phytochemicals against NR1H3.

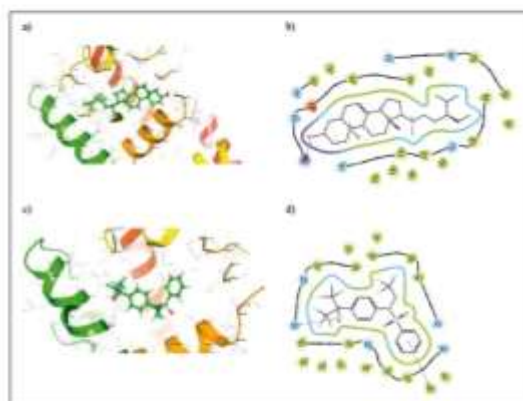
Ligand	Binding energy (kcal/mol)	NHB	Hydrogen bond residue
Linolenic-acid	-4.8	6	Val400, Asn377, Leu422, Leu425, Val426, Arg429
Cucurbitic acid	-6.7	8	Leu453, Leu449, Phe271, Thr316, Phe329, Met312, Ala275, Trp457
Octadecanoic acid	-7.5	6	Trp457, His322, Tyr320, Arg361, Phe329, Arg319
Schottenol	-14.2	24	His421, Gln424, Val425, Leu428, Leu439, Phe234, Phe257, Thr238, Leu260, Ala261, Ser264, Ile339, Phe326, Phe335, Leu331, Arg305, Thr302, Gln301, Met298, Ile295, Trp443, Thr314, Ile313, Tyr321
Alpha-Spinasterol	-7.6	6	Gln280, Val279, Leu452, Ile456, Ile301, Leu304
Chondrillasterol	-8.9	6	Asn239, Phe329, Leu345, His435, Val439, Trp457
Dehydrovomifoliol	-5.8	1	Lys305
Clerosterol	-7.5	3	Leu452, Ile301, Val283
Codisterol	-7.5	4	Leu452, Val279, Ile456, Ile301
Isocuposterol	-7.5	4	Gln280, Val279, Leu452, Ile301
Cucurbitacin-D	-7.4	6	Gln323, His322, Tyr320, Arg361, Leu365, Arg319
Cucurbitacin-I	-7	3	Asp367, Arg318, Arg361
Dihydrobrassicasterol	-7.4	4	Gln280, Ile301, Leu301, Val279
Cryptoxanthin	-7.8	3	Leu330, Arg319, Arg316
Eicosadienoic acid	-6.4	3	His322, Arg316, Trp457
Delta-7-Ergosterol	-7.5	5	Gln280, Leu452, Val279, Ile456, Ile301
Abseicic acid	-6.8	5	Leu452, Val279, Arg361, Phe329, Met312,
7-beta,13-Dihydroxykaurenolide	-5.8	6	Val279, Leu452, Met312, Phe243, Arg316, Trp457

was ~2.2 Å. The residues involving the ligand interaction, i.e., Phe329, Phe245, Leu274, Phe271, Ala275, Trp457, Met312 showed the least fluctuation compared to the N- and C- terminal

residues. The compound "Schottenol" was found to interact with Phe329, Phe245, Leu274, Phe271, and Ala275, Trp457, and Met312 of NR1H3 throughout 30ns MD simulation.

Table 5. Binding energy and mode of interaction of each phytochemicals against HMGCR.

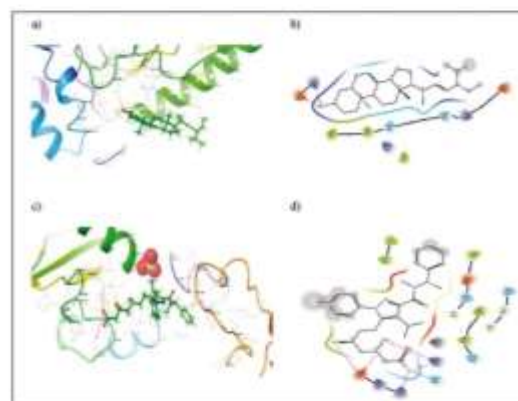
Ligand	Binding energy (Kcal/mol)	NHB	Hydrogen bond residue
Linolenic-acid	-9.4	8	Leu345, His435, Val439, Trp457, Val279, Ile456, Ile301, Arg318
Alpha-Spinasterol	-12.4	10	Lys691, Asp690, Met655, Met657, Asn658, Ser661, Lys662, Glu663, Arg590, Val683
Chondrillasterol	-11.2	7	Pro813, Tyr517, Ile456, Ile301, His322, Tyr320, Arg361
Clerosterol	-9.9	6	Leu452, Ile301, Trp457, Val279, Ile456, Gln280, Leu452,
Codisterol	-10.4	6	Val279, Ile456, Ile301, His322, Tyr320, Arg318
Cryptoxanthin	-9.6	4	Leu811, Cys527, Gln280, Leu452,
Cucurbitic acid	-10.5	5	Ile301, His322, Tyr320, Arg318
Cucurbitacin-D	-12.4	6	Cys527, Val530, Tyr519, Tyr533, Cys527, Val530
Cucurbitacin-I	-10.5	7	Cys527, Gln280, Leu452, His322, Tyr320, Arg361, Phe329
Dehydromomifolol	-8.9	8	Phe271, Thr316, Phe329, Leu449, Phe329, Phe271, Tyr320, Arg318
Delta-7-Ergosterol	-9.4	9	Val279, Leu452, Ile301, Val279, Ile456, Gln280, Leu452, Tyr320, Arg318
Fucoesterol	-14.4	6	Arg361, Phe329, His322, Tyr320, Arg318.
Schottenol	-12.6	8	His322, Tyr320, Arg361, Phe329, His322, Tyr320, Arg318, Val530.
Octadecanoic acid	-11.5	6	Phe329, Leu449, Phe329, Phe271, Tyr320, Arg318
Dihydrobrassicasterol	-13.8	3	Phe329, Arg318, Gln280
Abcisic acid	-11.9	3	Arg319, Gln280, Phe243
7-beta,13-Dihydroxykaweenolide	-5.2	4	Met312, Arg316, Trp457, Gln280, Leu449

**Figure 2.** (a) 3D and (b) 2D binding orientation of Schottenol ligand in NR1H3 binding pocket. (c) 3D and (d) 2D binding orientation of cocystal ligand in NR1H3 binding pocket.

Experimental wet lab studies

Cucurbita pepo phytochemicals profile

Physico-chemical investigations of *C. pepo* were represented in Table S1. The phytochemical screening of *C. pepo* showed the presence of alkaloids, glycosides, saponins, steroids, tannins, and flavonoids (Table S2). LC-MS profile

**Figure 3.** (a) 3D and (b) 2D binding orientation of Alpha-Spinasterol ligand in HMGCR binding pocket. (c) 3D and (d) 2D binding orientation of cocystal ligand in HMGCR binding pocket.

showed alpha-linolenic-acid, alpha-spinasterol, chondrillasterol, clerosterol, codisterol, isofucoesterol, dihydrobrassicasterol, and cryptoxanthin (Table S3 and Fig. S1).

In vivo experimental validation

The hepatoprotective effect of hydroalcoholic extract and steroid fraction of *C. pepo* studied on INH (50 mg/kg, p.o)

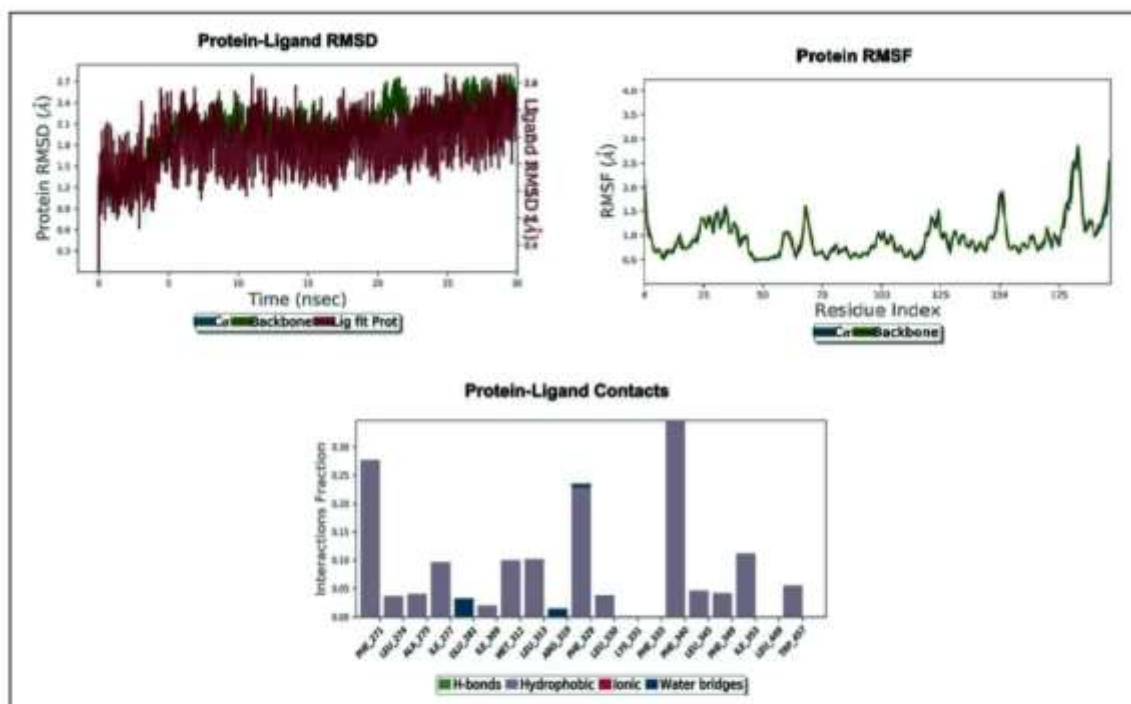


Figure 4. Stability of Schottanol with NR1H3.

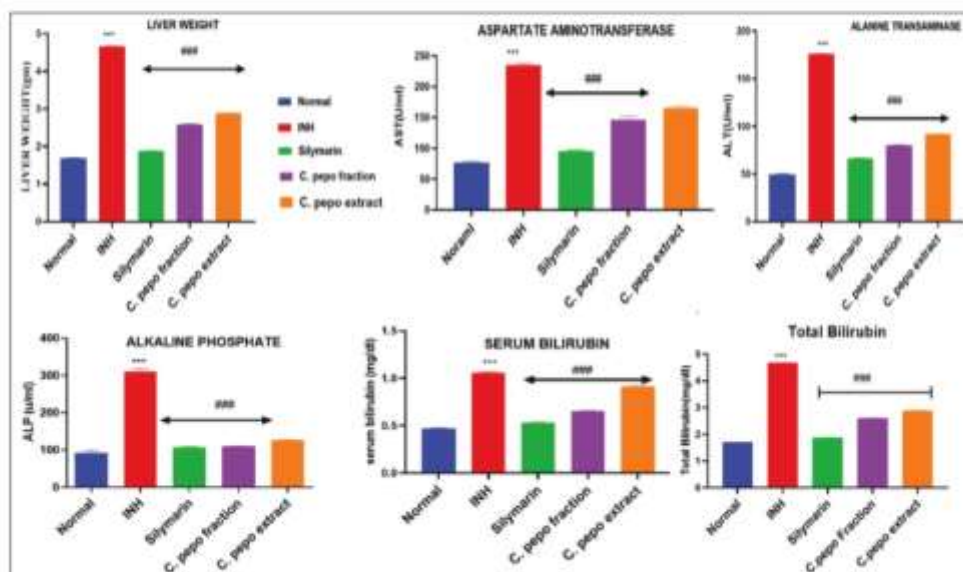


Figure 5. Effect of *C. pepo* on liver weight, AST, ALT, ALP, serum bilirubin, and total bilirubin. Data are presented as Mean $\pm$ SEM ($n = 6$), one way ANOVA followed by Tukey's test was applied. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with normal group, $\Delta p < 0.05$, $\Delta\Delta p < 0.01$, $\Delta\Delta\Delta p < 0.001$ compared with INH treated group.

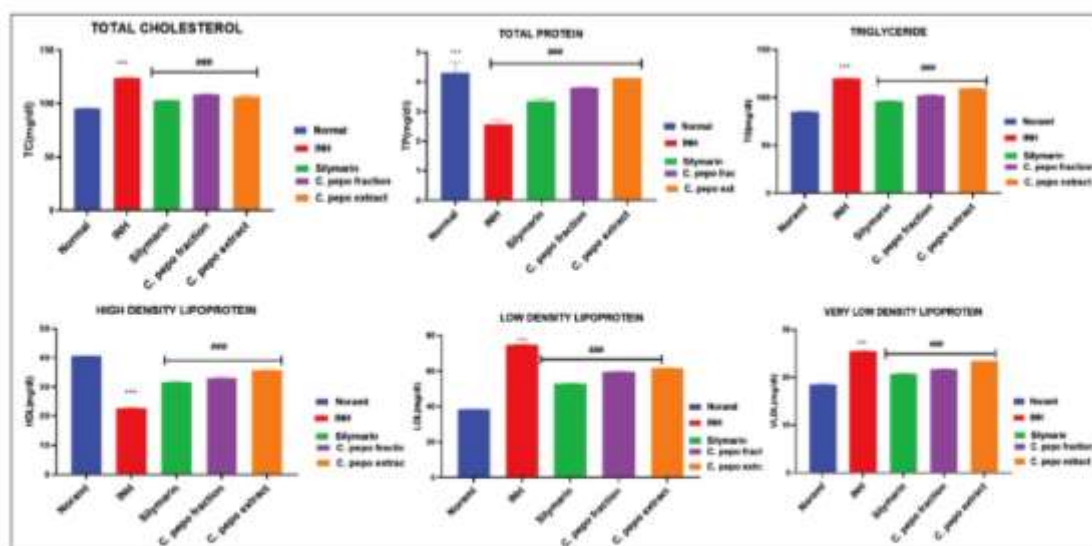


Figure 6. Effect of *C. pepo* on total cholesterol, total protein, TG, HDL, LDL, and VLDL. Data are presented as Mean $\pm$ SEM ($n = 6$), one way ANOVA followed by Tukey's test was applied. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with normal group, $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ compared with INH treated group.

induced hepatotoxicity in Wistar rats. Initially, body weight and liver weight of the animals were examined. As a result, INH showed no significant effect on the rat body weight compared to normal and treated groups. However, a significant increase in the liver weight was observed in INH treated group and a slight decrease in the liver weight after treatment with Silymarin and *C. pepo* (Table S4). Hepatoprotective activity was monitored by estimating the serum transaminases such as ALP, serum bilirubin, and lipid profile in the livers of experimental rats. Pre-treatment of rats with different concentrations of test drugs and standard leads to normalization of INH-induced changes in the levels of all the biochemical parameters (Fig. 5 and Table S5). Further, the effect of *C. pepo* on cholesterol also revealed normalization of their level, in which enriched fraction was found to reduce cholesterol levels significantly compared to INH group (Fig. 6 and Table S6). According to the current investigation, fraction was shown to be more efficient than extract at preventing liver damage brought on by INH-induced oxidative stress. The results of this study give evidence in favor of the therapeutic usefulness of medications that can be used to treat liver damage caused by INH. In comparison to the extract, the bioactive directed fraction exhibits superior impact.

DISCUSSION

The present work described phytosterols from *C. pepo* can be preferred as liver protective agents in liver cirrhosis by preventing oxidative damage and inflammation caused by AntiTB drugs. Claims from the traditional system and the available literature, seeds of the *C. pepo* plant are used to treat liver disease. However, there is a lack of data on the molecular mechanism of *C. pepo* for the treatment of liver illness.

Hence, the present study was designed to identify the potential bioactives and their molecular mechanisms for hepatoprotective activity. This study will extend from *In silico* research to wet-lab evaluations to determine the hepatoprotective activity of the secondary metabolites from *C. pepo*. LXR is a major nuclear regulatory transcription gene of cholesterol sensing in liver. Once it gets activated, the other genes are highly regulated by binding to different cholesterol molecules and play a significant role in inflammation, immune regulation, lipid, and glucose metabolism. Some selective LXR activators such as desmosterol [23], the intestine-specific ligand GW6340, and the LXR-623, show adverse effects. Oxysterols and intermediates from the cholesterol derivatives [24] activate NR1H3 signaling. The other enzyme, HMG-CoA reductase is the rate-controlling enzyme of the mevalonate pathway in cholesterol synthesis and its regulation is via a negative feedback mechanism by sterols and non-sterol isoprenoids derived from mevalonate, and the reaction is catalyzed by reductase. Cholesterol derivatives are major suppressors of this enzyme in mammalian cells and are responsible for the degradation of LDL via the LDL receptor.

Phytochemicals-target network was constructed through network pharmacology and analyzed by molecular docking. The underlying mechanism of network-predicted hepatoprotective effect of *C. pepo* is a novel approach for the management of liver disease against INH-induced hepatotoxicity. This research showed that the biological network method was more effective in illuminating the connections between illnesses and phytochemicals.

In the current study, we relied on a plethora of publicly available data sources to mine the phytochemicals present in the *C. pepo* and the PASS server was used to pick the compounds

with $P_i > 0.5$, or 50%. Based on the constructed network, we selected the most highly modulated compounds from *C. pepo* and observed their interactions with highly modulated proteins. In docking, the lead hit molecule is determined by the three key points one) BE, which reflects binding affinity, 2) the number of H-bond interactions, and 3) the H-bond residues.

Oxidative stress, mitochondrial dysfunction, LDH leakage and apoptosis are critical factors in drug-induced hepatotoxicity and occurs due to the activation of the reactive oxygen species system and metal ions transition, which modify homeostatic proteins is one prime basis for the pathogenesis of INH-induced hepatotoxicity [25]. Phytosterols from *C. pepo* provide nutrition to the hepatocytes and contain a high amount of unsaturated fatty acids, a hydrogen-donating property that can stabilize and delocalize unpaired electrons by creating a hydrogen bond with free radicals and stopping the Fenton reaction. Finally, we constructed the network interaction between phytosterols of *C. pepo* their targets and probable pathways. The result reflects that phytocompounds mainly phytosteroids to regulate the fatty acids interactions with the multiple protein molecules involved in the pathogenesis of liver within the network.

CONCLUSION

The present research proposes that, *C. pepo* steroidal fraction may be utilized as a hepatoprotective agent. The presence of secondary metabolites in this fraction may operate synergistically to have a potential therapeutic impact by targeting multiple proteins and modulating different pathways implicated in liver pathogenesis by reducing oxidative stress and cytotoxicity. This research provides strong *in silico* support for *in vivo* experiments investigating INH-induced hepatotoxicity. More specifically, it provides direction for promoting clinical studies involving human participants.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

The Institutional Animal Ethics Committee (Reg. No. 221/Po/Re/S/2000/CPCSEA at its meeting date of 21/01/2022) was approved the protocol of the study.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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SUPPLEMENTARY MATERIAL

Supplementary data can be downloaded from the
link [https://japsonline.com/admin/php/uploadss/4112_pdf.pdf]

The Effectiveness of Hydroalcoholic Extract and Fraction of *Cucumis melo* on Amelioration of High Fat Diet Induced hepatotoxicity: A Comprehensive Approach Integrating Computational and Preclinical Validation

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ABSTRACT

Background: Non-Alcoholic Fatty Liver Disease (NAFLD) is most prevalent global health problem, develops due to accumulation lipids in the hepatocytes in the form of Triglycerides (TG) and Free Fatty Acids (FFA). Traditional healers recommend seeds of *Cucumis melo* Linn as a liver tonic as well as for its treatment of liver cirrhosis, however its hepatoprotective mechanism have not been explored. **Objectives:** To investigate significant molecular mechanism of *C. melo* against liver cirrhosis via in vivo analysis followed by in silico. **Materials and Methods:** HFD was used for the induction of hepatotoxicity in mice and Silymarin as a standard control. The physical parameters were measured throughout the study along with antioxidant, serum biomarkers and histology of liver. Further, System biology tools were used to predict the possible mechanism of action. Docking studies were carried out with modulated phytochemicals against FXR protein target. **Results:** *C. melo* Extract and fraction ameliorated the HFD induced oxidative stress and histological changes. Additionally, improved liver biochemical parameters such as serum bilirubin, total bilirubin, LDL, VLDL, TC and TP were seen remarkable significant effects. Furthermore, System biology revealed the 16 phytochemicals of *C. melo* Linn, potentially regulate the PPAR signaling. Among Euphol phytochemical were predicted to interact with secreted FXR and may contribute to reduced hepatotoxicity. **Conclusion:** The results suggests *C. melo* as a promising therapeutic agent for hepatotoxicity by reducing the symptoms and stress associated with diseases condition which may be due to the regulation of multiple protein via multiple phytochemicals.

Keywords: *Cucumis melo*, High Fat Diet, in silico, Silymarin.

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INTRODUCTION

Non-Alcoholic Fatty Liver Disease (NAFLD) presents a significant worldwide health concern characterized by hepatic dysfunction and persistent dyslipidemia.^{1,2} This condition results in the abnormal accumulation of fat and cholesterol within liver tissue, devoid of inflammatory processes and is driven by a complex interplay of signaling mechanisms. Metabolic disorders such as obesity, type 2 diabetes, dyslipidemia and hepatic steatosis are

major precursors for the pathogenesis of the liver.^{1,2} Studies suggest that mitochondrial dysfunction is a major precursor pathogenesis of the liver. As per previous reports, prolonged consumption of HFD is responsible for abnormal lipid accumulation in the form of Free Fatty Acid (FFA) and Triglyceride (TG) in the serum/liver, increasing the weight of the liver, oxidative stress liable to steatohepatitis, cirrhosis, and Hepatocellular Carcinoma (HCC).^{1,2} Cholesterol major precursor of the hepatic cells, impairment in the regulation of cholesterol level, metabolism of lipoprotein, dietary uptake, endogenous synthesis excretion,³ elevated plasma levels of Total Cholesterol (TC), Low-Density Lipoprotein Cholesterol (LDL-C), Triglyceride Concentration (TG), decrease in plasma High-Density Lipoprotein cholesterol level (HDL-C) are highly manifested leading to dyslipidemia.



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The Nuclear Receptor (NR) is the biggest family ligand-modulated of transcriptional regulators, which has 49 members in mice and 48 in humans and provides a framework for a better knowledge of liver physiology and pathobiology. Endogenous and exogenous substances such as hormones, Fatty Acids (FAs), Bile Acids (BAs), medicines, toxins and intermediary molecules in metabolism are examples of ligands for NRs.⁴ These occurrences play a pivotal role in governing signal transduction pathways, both in normal physiological states and during pathological conditions.⁵ Over time, Numerous steroid hormone Receptors (NRs) have been identified, including the Farnesoid X Receptor (FXR), Liver X Receptor (LXR), Pregnane X Receptor (PXR), Peroxisome Proliferator-Activated Receptor (PPAR) and Retinoid X Receptor (RXR). Prior to their discovery, the natural ligands and functions of these receptors remained elusive. However, their regulation has been extensively researched and identified to regulate lipid and glucose metabolism, BA homeostasis, drug disposition, reproduction, inflammation, cell differentiation and various aspects of tissue repair including liver regeneration, fibrosis and, finally, tumour formation.⁶

The current investigation aims to evaluate the potential beneficial effects of *Cucumis melo* Linn in managing the HFD- induced hepatotoxicity in mice. *C. melo* is known for its traditional medicinal consideration in India, exhibits protection against hepatic damage, dyslipidaemia and inflammation. The seeds and fruit used traditionally for multiple purpose such as obesity, cough, flatulence, jaundice. The flesh of the fruit is a considerable source of protein, fatty acids, ascorbic acid, folic acid, carotenoids, terpenes and steroidal glycosides.⁷

MATERIALS AND METHODS

Experimental Analysis

Plant collection, authentication and extraction

The fresh fruits of *C. melo* were harvested in the Belagavi region of Karnataka. By comparing morphological traits, the Central Research Facility B.M.K. Ayurveda Mahavidyalaya, Belagavi, authenticated with accession number CRF/Auth./2020/1. Initially, Soxhlet extraction technique was used for 100 g of *C. melo* seeds with solvent as 95% v/v ethanol and the obtained extract was subjected to bio-guided fractionation as shown in Figure 1.

DPPH free radical scavenging activity

The DPPH assay of *C. melo* extract and Ascorbic acid was performed as per the method of Tubachi *et al.*,⁸ and the IC₅₀ was determined by,

$$\% \text{ Inhibition (IC}_{50}) = \frac{\text{Abs of Control} - \text{Abs of Sample}}{\text{Abs of Control}} \times 100$$

In vitro cytotoxicity assay

HepG2 cells were seeded in 96-well plates at a density of 20000 cells/well and were incubated at 37°C for 24 hr with 95% humidity in a CO₂ incubator. After 24 hr, the cells were treated with test substance (*C. melo* extract and fraction), standard (Silymarin) and HFD and was incubated at 37°C and 5% CO₂. Cytotoxicity was evaluated after 24 hr by adding 20 µL of MTT solution (5 mg/mL in phosphate buffer saline) for 4 hr incubation followed by 100 µL DMSO for dissolving formazan crystals. The intensity of color was measured at 570 nm using an ELISA plate reader and CC₅₀ was determined.⁹

Animal studies

Animal selection and ethical approval

Healthy Swiss albino mice (15-20 g) were purchased from Shri Venkateshwara Enterprises. The ethical clearance was obtained at KLE College of Pharmacy, Belagavi IAEC (Reg No. 221/Po/Re/S/2000/CPCSEA).

Preparation of High-Fat Diet (HFD) and model for hepatoprotective activity of *C. melo*

High fat diet was prepared by mixing the powder pallet diet+5g of Ghee (34%)+1 g of Vanaspati (18%)+5 g of yeast powder (8%)+5 g of lard (12%)+1 mL of Coconut oil (6%), totally 78% of calories from fat and make up the volume up to 10 mL/kg. The percentage of fat is conformed according to the RM value.¹⁰ Albino mice were acclimatized for 7 days, then divided into six groups (n=6). Except for group I, all group animals received HFD daily for 28 days while Silymarin (50 mg/kg p.o) served as the standard. Group I: food and water. Group II: HFD (10 mL/kg).¹¹ Group III: Silymarin 50 mg/kg and HFD (10 mL/kg). Group IV: 50mg/kg of *C. melo* fraction and HFD (10 mL/kg), and lastly Group V: 500mg/kg of *C. melo* extract and HFD (10 mL/kg). 24 hr after the last dosing, a blood sample was collected through the retro-orbital puncture and animals were sacrificed by cervical dislocation under general anaesthesia. The liver was collected for estimation of biochemical parameters and liver histopathological examination.

In silico analysis

Collection of phytochemicals

Phytochemicals of *C. melo* were retrieved from reported literature and open database viz., Dr. Dukes DB (<https://www.nal.usda.gov/dr-dukes-database>), IMPPAT DB (<https://ch.imsc.res.in/impapat/>), Phytochemical integration DB (<https://www.genome.jp/db/pidb/>) and their structures with PubChem CID and SMILES were retrieved from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>).

Druggability and Toxicity analysis

Molsoft tool was used to predict the Druglikeness score for each phytocompound based upon Lipinski's rule of 5. Moreover, Molecular Weight (MW), Formula (MF), number of Hydrogen Bond Acceptors (HBA) and Donor (HBD), Surface area (MolPSA) and lipophilicity (MolLogP) of screened phytocompounds were obtained. The adverbPred database¹⁷ (<https://www.way2drug.com/adverbPred/>) was used to predict the side effects for the screened phytocompounds.

ADME profiling

We used ADMETlab 2.0 (<https://admetmesh.scbdd.com/>)¹⁸ to predict the ADME of the selected phytocompounds which is been widely recognized for their pharmacokinetics properties i.e., absorption, distribution, metabolism and, excretion.

Target analysis

SuperPred (<https://prediction.charite.de/subpages/targetprediction.php>)¹⁹ server, was used to predict targets of each phytocompounds with a *p*-value of ≥ 0.7 and their respective Gene ID were obtained from UniProtKB server (<https://www.uniprot.org/>).

Enrichment analysis and network construction

A set of predicted protein targets modulated by the phytocompounds targeting FXR were submitted to STRING¹⁵ ver 11.0 (<https://stringdb.org/>) and were used to identify the modulated pathways by a set of queried gene targets. Additionally, KEGG,¹⁶ genomes pathway database and published literature were also used to compile modulated genes and their related pathways in liver cirrhosis. Finally, using Cytoscape¹⁷ ver 3.6.1 a network was created between phytocompounds, targets and pathways using edge count as a topological parameter. For the network representation, the map node was set from low to high and small to big.

Molecular docking

3D structure of each phytocompounds and known standard inhibitor Silymarin were retrieved from PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) in "sdf" file format and converted into "pdb" format using Discovery Studio Visualizer (<https://discover.3ds.com/discoverstudio-visualizer-download>) ver 17.2. Similarly, FXR (PDB Id: 6HL1) was retrieved from Protein Data Bank¹⁸ (RCSB; www.rcsb.org). Molecular docking was performed using AutoDock Vina via executed through the POAP pipeline.¹⁹ Standard docking was performed and the grid was set around the active site with the box dimensions center $x=9.070$, $y=17.169$, $z=13.316$ and size $x=27.408$, $y=29.430$ and $z=23.349$ and exhaustiveness was set to 50. The binding results were further, analyzed for interaction between ligand and target and were visualized using 'BIOVIA Discovery studio visualizer 2019'.

Statistical analysis

All the data were expressed as mean $\pm$ SEM. The data was analyzed by One way ANOVA followed by Tukey's multiple comparison tests using Graph Pad Prism version 8 with $p < 0.05$ considered statistically significant and linear regression was used to analyze the inhibitory constant. Enrichment analyses were analyzed using the whole genome and edge count was defined to analyze the network representation, kcal/mol showed the least binding energy.

RESULTS

Experimental analysis

Extract yield and preliminary phytochemical and physiochemical analysis

The hydroalcoholic extract yield of *C. melo*, seeds per 100 g w/w of powder was found to be 16%. Additionally, the *C. melo* fraction yield for 16 g w/w of hydroalcoholic extract was calculated as 6% respectively.

In vitro antioxidant assay

The IC_{50} of ascorbic acid and *C. melo* was found to be 47.89 and 153.0 $\mu\text{g/mL}$, respectively as shown in (Figure 2).

In vitro cytotoxicity assay in HepG2 cell line

C. melo extract and its fraction (Conc. 12.5–100 $\mu\text{g/mL}$) showed the significant cell viability after 24 hr (Figure 3).

In vivo studies

Effect of *C. melo* on body weight and liver weight

Disease control group (HFD) exhibited remarkable raise in body weight and liver weight compared to normal group ($p < 0.001$). Positive control groups, *C. melo* fraction and extract group exhibited decline in body weight ($p < 0.001$) and liver weight ($p < 0.001$) compared to disease control group (Figure 4).

Effect of *C. melo* on antioxidant markers

Disease control group (HFD) exhibited remarkable decrease ($p < 0.001$) in Catalase, Nitrate, SOD and GSH level and increase in the LPO level compared to normal group. Whereas, treatment with Silymarin, *C. melo* fraction and *C. melo* extract reversed ($p < 0.001$) the same compared to disease control group. Among these, *C. melo* fraction exhibited potent antioxidant property (Figure 5).

Effect of *C. melo* on liver function

Disease control group (HFD) exhibited remarkable increase ($p < 0.001$) in ALP, ALT, AST, total and serum bilirubin compared to normal group. Whereas, treatment with Silymarin, *C. melo* fraction and *C. melo* extract showed significant decrease ($p < 0.001$) in the liver enzymes compared to disease control group. Among

these, *C. melo* fraction was found to be good hepatoprotective compared to extract (Figure 6).

Effect of *C. melo* on lipid profile

Disease control group (HFD) exhibited remarkable increase ($p < 0.001$) in LDL, VLDL, TC, TG and decrease ($p < 0.001$) in the HDL and TP compared to normal group. Whereas, treatment with Silymarin, *C. melo* fraction and *C. melo* extract showed significant reverse in the parameters ($p < 0.001$) compared to disease control group (Figure 7).

Histological examination

In mice receiving vehicle, liver tissue containing a Central Vein (CV) appeared unharmed. Histological abnormalities that suggested liver cirrhosis showed clogged blood vessels,

disintegrating tissue, fatty degeneration and excessive inflammation were improved after HFD treatment. Pretreatment with Silymarin, extract and fraction of *C. melo* anomalies was brought back to normal as shown in Figure 8.

In silico analysis

Mining of phytochemicals from *C. melo*

A total of 160 phytochemicals were reported to be present in the *C. melo*.

Druggability and druglikeness profile

Out of 160 phytochemicals, 16 phytochemicals were predicted to have a positive drug likeness score in which 24-methylenecycloartanol showed the highest molecular weight with 1 HBD and HBA of each, followed by Euphol with 426.7,

Table 1: Binding energy of ligands with FXR protein.

Compounds	BE kcal/mol	HBI	NHBI	Total number of residues	Number of interactions with active sites
* Silymarin	-9.4	Arg331...O, Arg331...OH, Tyr369...O	Met290, Ile352, Tyr369, Met265, Ile335	8	6
Euphol	-11.1	Tyr361...O, His447...O	Trp454, Ala291, His294, Met265 (3), Arg264, Val297, Met290 (3), Met328 (4), Leu348 (2), Leu287, Ile352 (2), Phe329, Met450, Tyr361 (2)	26	12

*Standard compound; BE: Binding energy; HBI: Hydrogen bond interaction (s); NHBI: Non- hydrogen bond interaction (s).

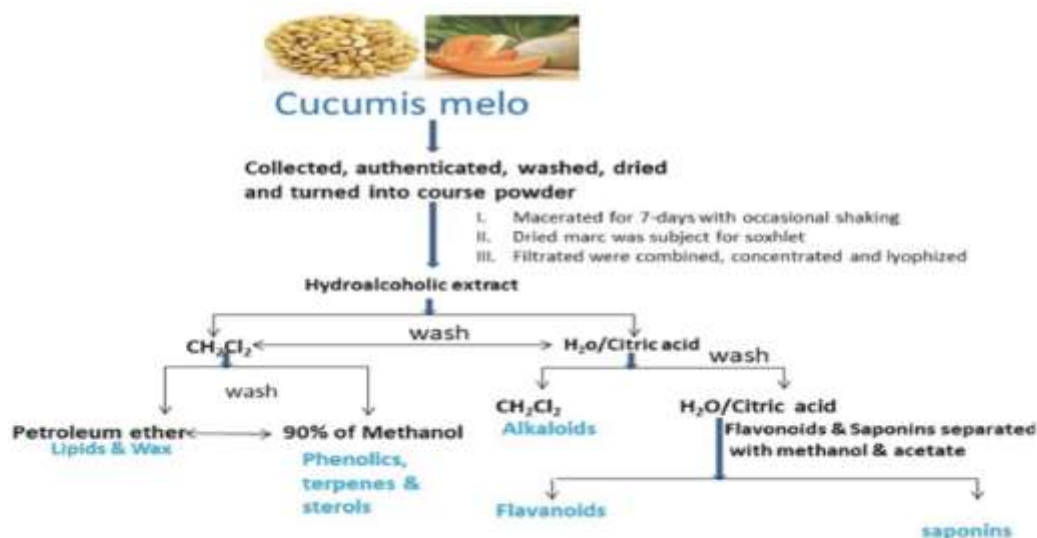


Figure 1: Hydroalcoholic extraction and crude fractionation of *Cucumis melo*.

Running title

the least was observed in Avenasterol and Stigmasterol with molecular weight 412.7. Moreover, none of the phytocompounds were identified to cross the blood-brain barrier as shown in Table S1.

Toxicity screening and ADME profiling

Among all the screened phytocompounds (as shown in Table S2 and Table S3), Avenasterol and Sitosterol were predicted to have toxicity, but were observed within the limits of Pa 0.246 and Pi 0.228.

Gene enrichment analysis and network construction

Gene enrichment analysis showed that 16 phytocompounds were predicted to modulate the 27 targets. Additionally, these 27 targets interacted with each other to modulate the 65 pathways in the KEGG database. Among them 7 pathways were identified to be associated with liver cirrhosis. As depicted in Table S4

and Table S5, the PPAR signaling pathway scored the lowest FDR of $1.95E-13$ by modulating the 8 protein targets, followed by insulin resistance and AMPK pathway. Furthermore, out of 16 phytocompounds, Euphol was found to highly modulated compound by regulating a maximum number of proteins (Figure 9).

Molecular docking

The active site residues of FXR, namely Ser259, Tyr260, Gln263, Arg264, Met265, Thr270, Ile273, Leu287, Thr288, Met290, Ala291, His294, Val297, Leu298, Phe301, Asn327, Met328, Arg331, Ser332, Ile335, Phe336, Lys338, Ser345, Leu348, Ile352, Ile357, Tyr361, Ile362, Met365, Phe366, Tyr369, Thr386, His447, Met450, Trp454 and Trp469 were taken from the crystal structure "6HLI.pdb". Among all the phytocompounds, Silymarin scored the lowest Binding Energy (BE) of -9.4kcal/mol via forming 3 hydrogen bonds and 5 non- hydrophobic interactions (Figure 10). Euphol scored the lowest binding energy among all others

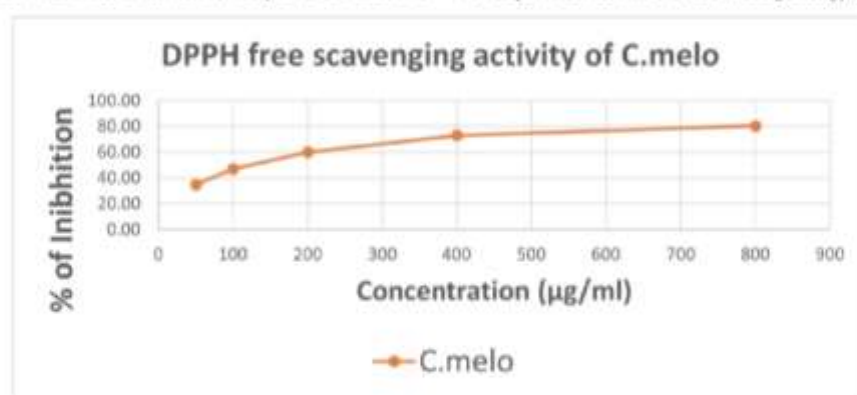


Figure 2: Antioxidant activity C. melo by DPPH assay.

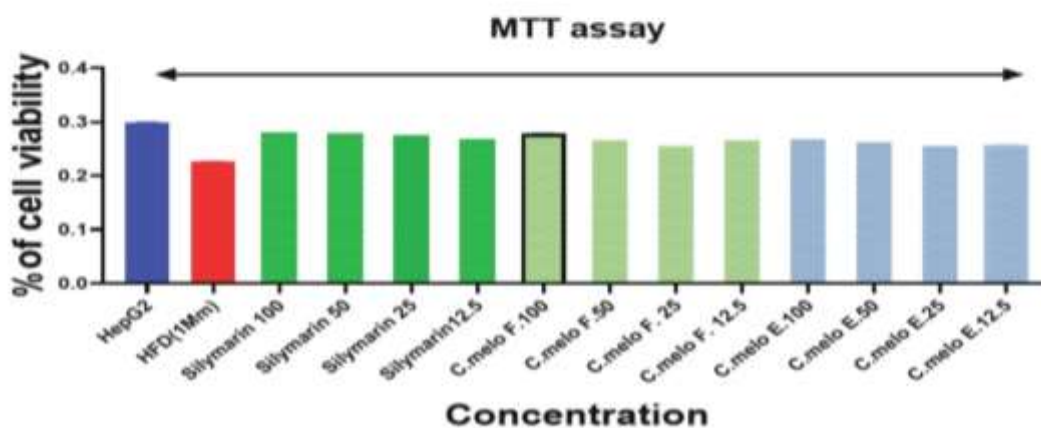


Figure 3: MTT cytotoxicity activity.

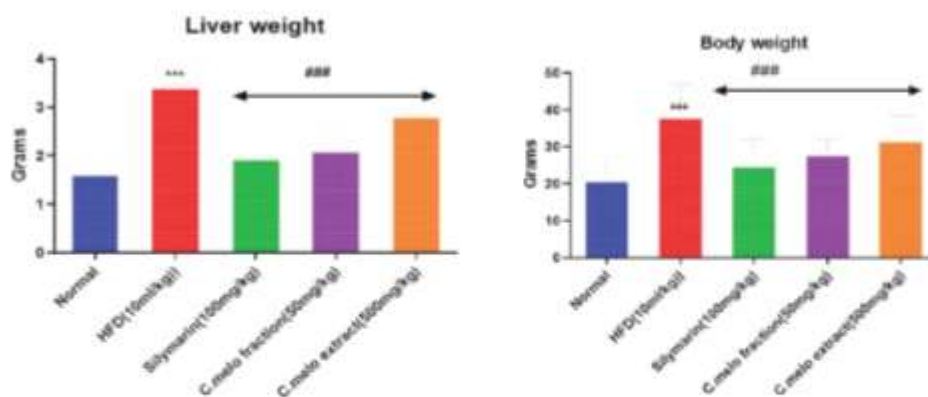


Figure 4: Effect of *Cucumis melo* extract and fraction on body weight and liver weight. *** $p < 0.001$ compare to normal, ### $p < 0.001$ when compared to disease control.

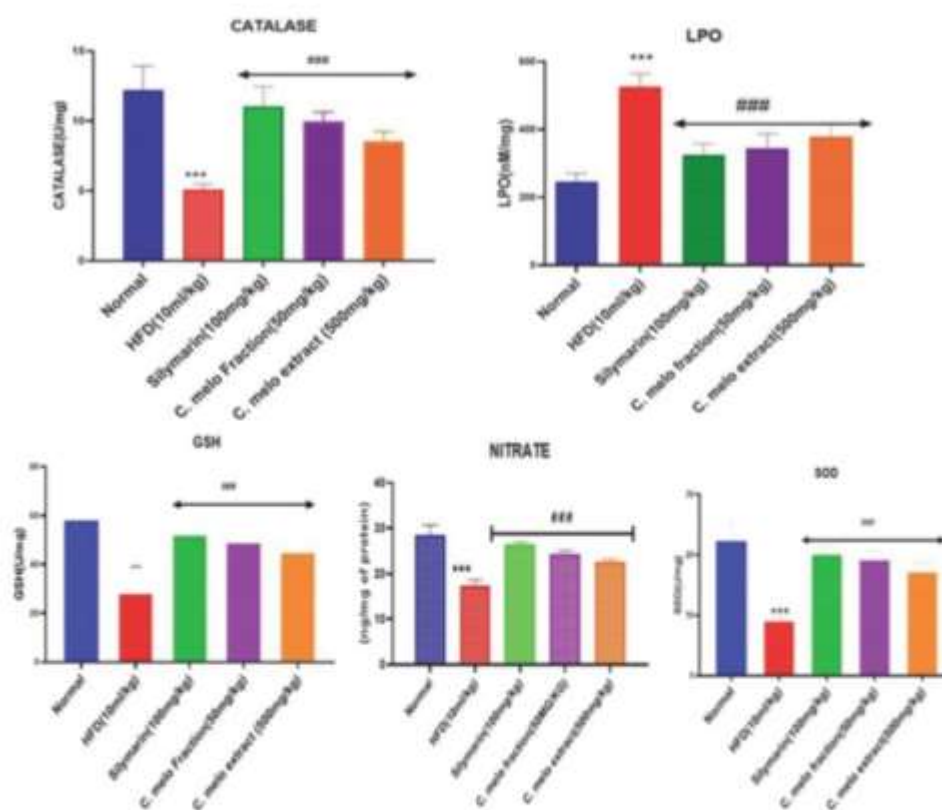


Figure 5: Effect of *Cucumis melo* extract and fraction on catalase, GSH, Nitrate and LPO. *** $p < 0.001$ compare to normal, ## $p < 0.01$, ### $p < 0.001$ when compared to disease control.

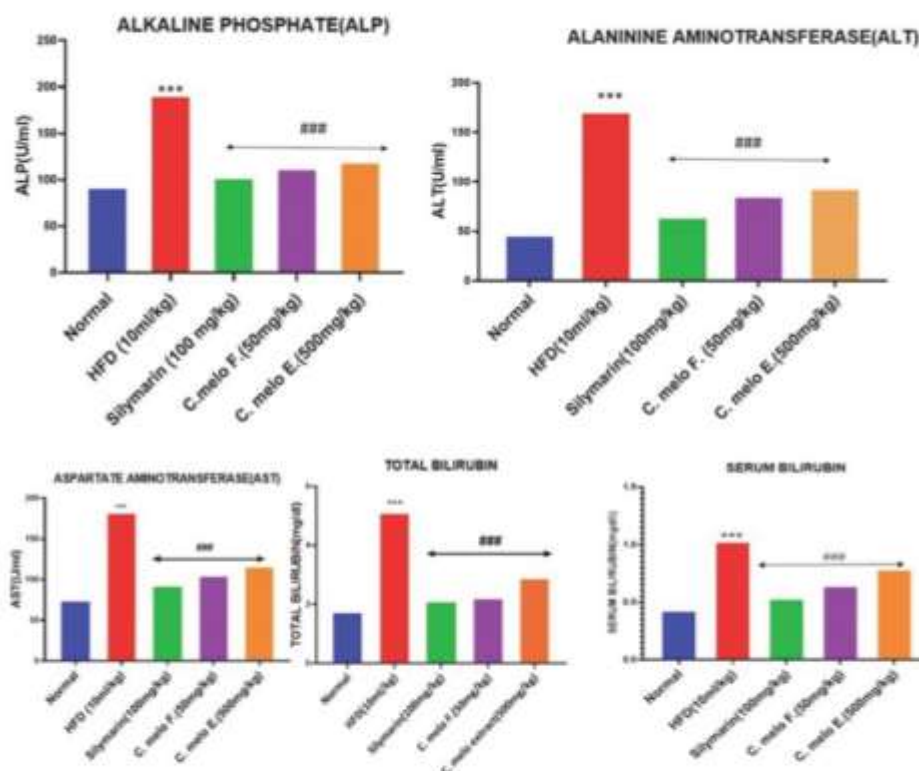


Figure 6: Effect of *Cucumis melo* extract and fraction on AST, ALT, ALP, Serum bilirubin and Total bilirubin. *** $p < 0.001$ compare to normal, ## $p < 0.01$, ### $p < 0.001$ when compared to disease control.

with a BE of -11.1kcal/mol via forming 2 hydrogen bonds and 23 non-hydrophobic interactions (Figure 11 and Table 1).

DISCUSSION

The present study was designed to explore the hepatoprotective and antioxidant effect of *C. melo* in HFD-induced hepatotoxicity in albino mice. In HFD animals, elevated hepatic fatty acid metabolism results in cholesterol and triglyceride accumulation as well as elevated liver inflammation and liver damage.²⁹ Firstly, both *C. melo* extract and fraction showed significant cell viability compared to HFD-treated groups in HepG2 cells. Secondly, the extract and a fraction were screened for its hepatoprotection in albino mice. In animal study, the significantly increased body weight ($p < 0.001$) and liver weight ($p < 0.001$) could be due to the accumulation of fat which was reversed in the treatment groups. This indicates the potential role of phytosterols in the regulation of lipid metabolism. In the present study, HFD-treated animals showed abnormal levels of antioxidant markers TP, GSH, LPO, SOD, nitrate and catalase, levels and were reversed in the

C. melo extract and fraction groups. Similarly, lipid markers like HDL, LDL, VLDL, TG and TC were also abnormal in the HFD-treated animal and were found to be towards normal in *C. melo* extract and fraction groups. Serum ALP, AST, ALT and bilirubin are considered as a specific marker for hepatic damage and were significantly elevated in the HFD-treated animals and were found to be towards normal in *C. melo* extract and fraction groups. Histology of the liver showed remarkable histological changes after treatment. In HFD-treated groups, cirrhosis or liver injury was confirmed by clogged blood vessels, disintegrating tissue, fatty degeneration and excessive inflammation. Whereas, Silymarin, *C. melo* extract and fraction anomalies HFD diet mediated histological pattern of the liver.

Moreover, the current work traced the 160 reported phytochemicals of *C. melo* to propose a probable mechanism against the HFD-induced hepatotoxicity. Out of 160 phytochemicals, 16 were predicted to show a positive druglikeness and were further predicted to target 27 protein molecules and were involved in 65 pathways, in which 7 pathways

were identified to be associated with liver cirrhosis. Among 27 predicted targets FXR, HMGCR was identified as a major modulated and therapeutic target involved in the pathogenesis of hepatotoxicity by modulating all 7 pathways within the network. FXR indirectly reduces lipid-induced hepatic inflammation by decreasing intracellular FA levels.³¹ It is highly expressed in the liver and intestine which further standalone in activating

varieties of inflammatory markers causing hepatocellular or cholestatic conditions.³¹ Several tetracyclic triterpenes from fruits and vegetables have been reported to inhibit the production of FXR by blocking the inflammatory cellular signaling pathways primarily MAPK, PI3-Akt and NF-kB.³² *C. melo* phytochemicals exhibit potent antioxidant, anti-inflammatory and anti-colitis activity by reducing the FXR expression in bile formation,

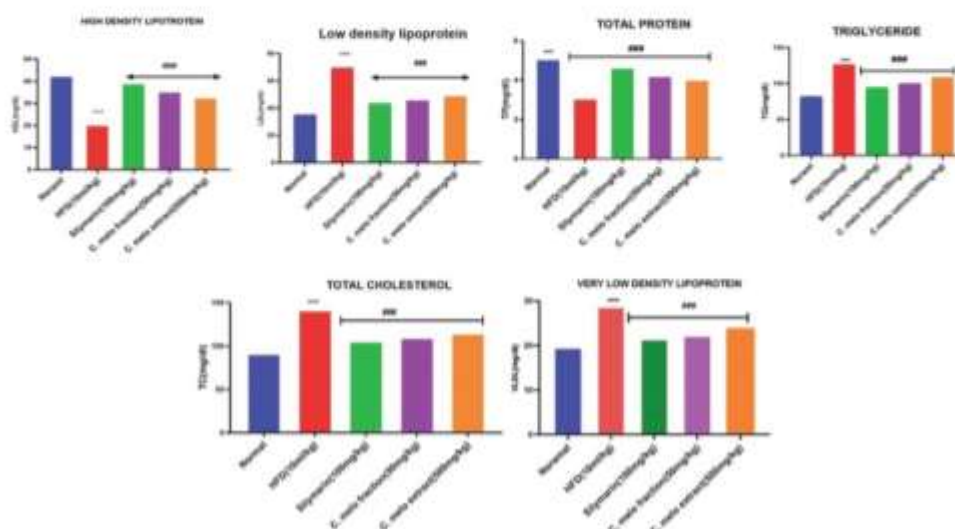


Figure 7: Effect of *Cucumis melo* extract and fraction on HDL, LDL, VLDL, TC, TG, TP. *** $p < 0.001$ compare to normal, ## $p < 0.001$ when compared to disease control.

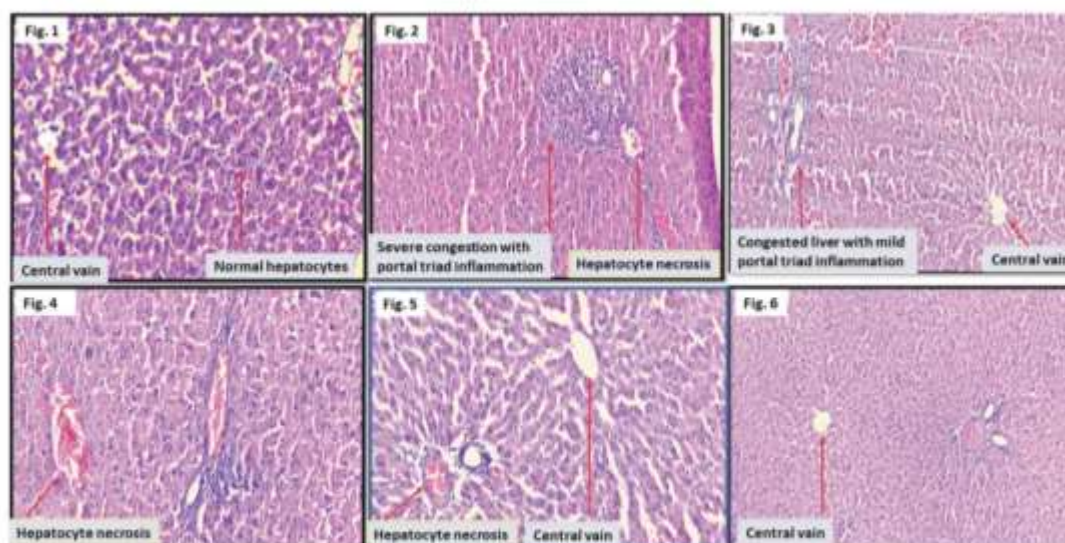


Figure 8: Histopathological changes on HFD induced hepatotoxicity. I: Normal control, II: HFD, III: Silymarin+HFD, IV: *C. melo* extract (250 mg/kg+HFD), V: *C. melo* extract (500 mg/kg+HFD), VI: *C. melo* fraction (50 mg/kg+HFD).

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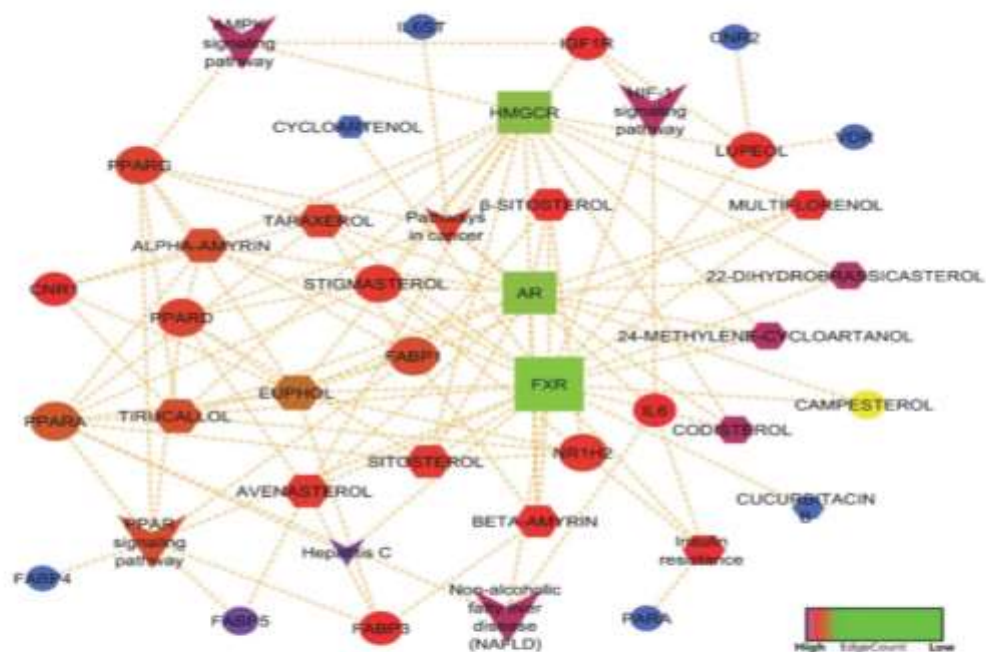


Figure 9: Network representation of Cucumis melo Linn phytochemicals with their probable protein molecules involved in Liver cirrhosis.

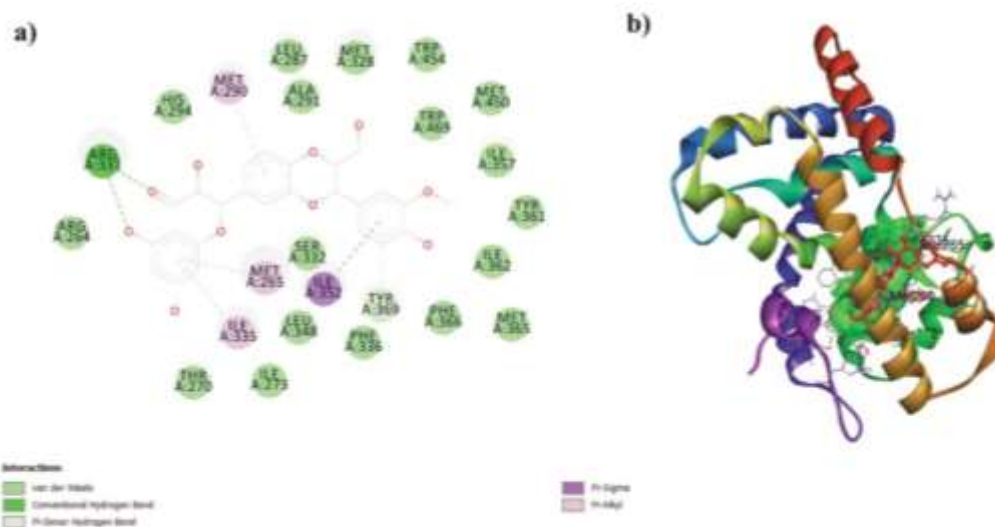
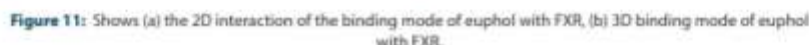


Figure 10: Shows (a) the 2D interaction of the binding mode of silymarin with FXR, (b) 3D binding mode of silymarin with FXR.



Hence, the present investigation on hepato-protective activity of *C. melo* could be due to the modulation of PPAR, Insulin resistance, pathways in cancer, AMPK, non-alcoholic fatty liver disease and HIF-1 signaling pathways.

The current investigation employed *in vivo* experimental evaluation to explore the HFD-induced hepatotoxicity of *C. melo* extract and fraction followed by computational approaches. Evaluation of the extract and fraction demonstrated an increase in antioxidant enzyme activity and reduced biochemical levels. Histopathology analysis revealed improved clogged vessels, disintegrating tissue, fatty degeneration and excessive inflammation. Further, we depicted the interaction of the phytochemicals from *C. melo* with proteins involved in the etiology of hepatotoxicity. Gene enrichment analysis identified PPAR signalling pathway as a likely major pathway targeted by the phytochemicals to counter hepatotoxic activity. Key constituent like euphol suggested their potential role in inhibiting the FXR target. Further, the identified phytochemicals and enriched fractions need to be validated for clinical trials and their interaction studies with the standard anti-hepatotoxicity drugs to develop and formulate the adjunct therapies for effective management. On the other hand, it provides the critical suggestive possible mechanism of action that may be taken advantage of developing the *C. melo* based research plans.

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SUPPLEMENTARY DATA AVAILABILITY

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

CONTRIBUTIONS

SRU: Conceptualized the research, Experimentation and wrote the original draft. NAK: Conceptualized the research, revised the draft and supervision. FB, AR and DP: Assisted SRU in experimentation and writing manuscript. VSP: Revision and drafting. HRD: Conceptualized the research and revision. All authors have reviewed and approved the final version of the manuscript.

AVAILABILITY OF DATA AND MATERIALS

The datasets analyzed during the current study are available from Sanjay R. Ugare (First Author) and Nayeem A. Khatib (corresponding author) on request.

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Table S1: Phytocompounds of *C. melo* which were retrieved from PubChem possess Drug likeness score.

Sl. No.	Compounds	Lipinski Rules			Log P	Drug likeness score
		Molecular wt. (G/mol)	HBA	HBD		
	Acceptable Values	<500	<5	<5	<10	0 to 2
1.	Euphol	426.7	1	1	8.74	0.55
2.	Tirucallol	426.7			8.74	0.55
3.	Alpha-amyrin	426.7	1	1	7.77	0.10
4.	Taraxerol	426.7	1	1	8.11	-0.9
5.	Avenasterol	412.7	2	1	7.95	0.25
6.	Stigmasterol	412.7	1	1	7.74	0.62
7.	Sitosterol	414.7	1	1	8.45	0.78
8.	Lupeol	426.39	1	1	8.35	-0.22
9.	beta-Amyrin	426.7	1	1	7.95	-0.22
10.	beta-Sitosterol	414.39	1	1	8.45	0.78
11.	Multiflorenol	426.39	1	1	7.89	-0.27
12.	Codisterol	398.7	1	1	7.7	0.43
13.	22-dihydrobrassicasterol	400.7	1	1	7.87	0.59
14.	24-Methylenecycloartanol	440.7	1	1	8.49	-0.48
15.	Campesterol	400.37	1	1	7.87	0.59
16.	Cycloartenol	426.7	1	1	8.56	-0.27

Table S2: Toxicity screening of phytocompounds from *Cucumis melo*.

Sl. No.	<i>Cucumis melo</i>	Pa	Pi	Side Effect
	Euphol	-	-	-
	Tirucallol	-	-	-
	Alpha-amyrin	-	-	-
	Avenasterol	0.246	0.228	Nephrotoxicity
	Sitosterol	0.258	0.205	Nephrotoxicity
	Taraxerol	-	-	-
	beta Amyrin	-	-	-
	beta-Sitosterol	-	-	-
	Multiflorenol	-	-	-
	Codisterol	-	-	-
	22-dihydrobrassicasterol	-	-	-
	24-Methylenecycloartanol	-	-	-
	Campesterol	-	-	-
	Cycloartenol	-	-	-
	beta-Sitosterol	-	-	-
	Multiflorenol	-	-	-

Pa: Probable activity to cause toxicity.

Pi: Probable inactivity to cause nontoxicity.

Running title

Table S3: ADME profile of each phytochemicals from *Cucumis melo*.

Compounds	GI Absorption	BBB permeability	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	LogKp (skin permeation)
Euphol	Low	No	No	No	No	No	No	-2.58
Tirucallol	Low	No	No	No	No	No	No	-2.58
Alpha-amyrin	Low	No	No	No	No	No	No	-2.51
Taraxerol	Low	No	No	No	No	No	No	-2.3
Avenasterol	Low	No	No	No	No	No	No	-2.72
Stigmasterol	Low	No	No	No	Yes	No	No	-2.74
Sitosterol	Low	No	No	No	No	No	No	-2.2
Lupeol	Low	No	No	No	No	No	No	-1.9
beta-Amyrin	Low	No	No	No	No	No	No	-2.41
beta-Sitosterol	Low	No	No	No	No	No	No	-2.2
Multiflorenol	Low	No	No	No	No	No	No	-2.41
Codisterol	Low	No	No	No	Yes	No	No	-2.53
22-dihydrobrassicasterol	Low	No	No	No	No	No	No	-2.5
24-Methylenecycloartanol	Low	No	No	No	No	No	No	-1.67
Campesterol	Low	No	No	No	No	No	No	-2.5
Cycloartenol	Low	No	No	No	No	No	No	-1.96

Table S4: Liver cirrhosis pathway and gene enrichment analysis.

Pathway	Description	Count Fin Gene Set	False Discovery Rate	Genes
hsa03320	PPAR signaling pathway.	8	1.95E-13	FABP4, PPARG, FABP1, FABP5, PPARD, FABP3, PPARA, FXR.
hsa04931	Insulin resistance.	4	0.00012	NR1H2, PPARA, IL6, FXR.
hsa04152	AMPK signaling pathway.	3	0.0039	IGF1R, PPARG, HMGCR, FXR.
hsa05200	Pathways in cancer.	6	0.00014	IGF1R, PPARG, PPARD, AR, IL6ST, IL6.
hsa04932	Non-Alcoholic Fatty Liver Disease (NAFLD).	3	0.0058	PPARA, IL6, FXR.
hsa05160	Hepatitis C	2	0.042	PPARA, FXR.

Table S5: Network based gene set enrichment analysis highlights different proteins and pathway in pathogenesis of liver.

Sl. No.	Pathway	Proteins
1.	PPAR signalling pathway.	(Fatty acid binding protein adipocyte) FABP4.
2.	PPAR signalling pathway.	Fatty acid-binding protein, liver (FABP1).
3.	PPAR signalling pathway.	Fatty acid binding protein muscle (FABP3).
4.	PPAR signalling pathway.	Fatty acid binding protein skin (FABP5).
5.	PPAR signalling pathway.	Peroxisome proliferator-activated receptor alpha (PPARA).
6.	PPAR signalling pathway.	Peroxisome proliferator-activated receptor delta (PPARD).
7.	PPAR signalling pathway.	Peroxisome proliferator-activated receptor gamma (PPARG).
8.	PPAR signalling pathway.	Farnesoid X receptor (FXR).
9.	Pathways in cancer.	Peroxisome proliferator-activated receptor gamma (PPARG).
10.	Pathways in cancer.	Peroxisome proliferator-activated receptor delta (PPARD).
11.	Pathways in cancer.	Androgen receptor (AR).
12.	Pathways in cancer.	Interleukin-6 (IL6).
13.	Pathways in cancer.	Insulin like growth factor1 receptor (IGF1R)
14.	Pathways in cancer.	Interleukin 6 cytokine family signal transduced(IL6ST).
15.	Insulin resistance.	Nuclear Receptor Subfamily 1 Group H Member 2(NR1H2).
16.	Insulin resistance.	Peroxisome proliferator-activated receptor alpha (PPARA).
17.	Insulin resistance.	Interleukin-6 (IL6).
18.	Insulin resistance.	Farnesoid X receptor (FXR).
19.	Non-alcoholic fatty liver disease.	Peroxisome proliferator-activated receptor alpha (PPARA).
20.	Non-alcoholic fatty liver disease.	Interleukin-6 (IL6).
21.	Non-alcoholic fatty liver disease.	Nuclear Receptor Subfamily 1 Group H Member 3(NR1H3).
22.	AMPK signalling pathway.	Peroxisome proliferator-activated receptor gamma (PPARG).
23.	AMPK signalling pathway.	Insulin like growth factor1 receptor (IGF1R).
24.	AMPK signalling pathway.	HMG-CoA reductase (HMGCR).
25.	AMPK signalling pathway.	Farnesoid X receptor (FXR).
26.	Hepatitis C	Peroxisome proliferator-activated receptor alpha (PPARA).
27.	Hepatitis C	Farnesoid X receptor (FXR).
28.	HIF1 Signalling pathway	Insulin like growth factor1 receptor (IGF1R)
29.	HIF1 Signalling pathway	Interleukin-6 (IL6).

ANNEXURE – III - CERTIFICATES OF ORAL & POSTER PRESENTATION





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SOCIETY FOR ETHNOPHARMACOLOGY, INDIA**
 (Globalizing Local Knowledge and Localizing Global Technologies)
Theme : Redefining Ethnopharmacology for the Global Health and Wellbeing

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This is to certify that Prof./Dr./Mr./Ms. Sanjay R. Ugare

has Presented a PAPER in ORAL/ POSTER SESSION in the 9th International Congress of the
 Society for Ethnopharmacology, India (SFEC-2022) held at JSS College of Pharmacy,
 JSS Academy of Higher Education & Research, Mysuru, Karnataka, India from 22nd to 24th,
 April 2022.

Title of presentation: Screening of Cucurbita pepo against therapeutic targets of liver cirrhosis: An in silico and in-vitro study.


Dr. T.M. Pramod Kumar
 Organizing Chairman, SFEC - 2022


Dr. K. Mruthunjaya
 Organizing Secretary, SFEC - 2022


Mr. Birendra K. Sarkar
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