Evaluation of Hydroalcoholic Extract of*Excoecaria agallocha* Bark in the Management of Non-Alcoholic Fatty Liver Disease

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CERTIFICATE

This is to certify that Arun Baidya has carried out the research on the project entitled **"Evaluation of Hydroalcoholic Extract of *Excoecaria Agallocha* Bark in the Management of Non-Alcoholic Fatty Liver Disease"** under my supervision, in the division of Pharmacology and Toxicology—Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032.

He has incorporated his findings into this thesis, which has the same title as what he submitted in partial fulfilment of the requirement for the award of Degree of Master of Pharmaceutical Technology degree from **Jadavpur University**. I am satisfied that he has carried out his thesis with proper care and confidence to my entire satisfaction.

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I declare that **" Evaluation of Hydroalcoholic Extract of *Excoecaria Agallocha* Bark in the Management of Non-Alcoholic Fatty Liver Disease "** is my own work, that it has not been submitted for any other university's degree or test, and that all of the sources I have utilised or quoted have been mentioned and recognised with thorough references.

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ABSTRACT/PREFACE

The present study entitled "**Evaluation of Hydroalcoholic Extract of *Excoecaria Agallocha* Bark in the Management of Non-Alcoholic Fatty Liver Disease**" covers original research work conducted by the author for the award of Master of Pharmacy in the Department of Pharmaceutical Technology, Jadavpur University, Kolkata.

Non-alcoholic fatty liver disease (NAFLD) is a chronic lipid and carbohydrate metabolic disease that is frequently linked with endoplasmic reticulum stress, dysregulated energy balance, and cellular and molecular inflammation. The use of anti-hyperlipidemia, anti-hyperglycemia, or anti-inflammatory medications that are already on the market to treat NAFLD has not produced the desired results. Consequently, natural products are receiving more attention as a potential treatment option for NALFD. It appears that some natural products have the ability to improve NAFLD symptoms by restoring energy balance, lipid and carbohydrate metabolism, and other metabolic processes.Oxidative stress has an important role in the pathophysiology and progression of NAFLD. There is accumulating evidence suggests that the phenolic and flavonoid-rich *Excoecaria agallocha* L extract possesses hypoglycemic and antioxidant qualities. The purpose of this study was to ascertain if *E. agallocha* extract protects against NAFLD caused by a high-fat diet. After a 16-hour fast, a single intraperitoneal injection of 40 mg/kg of streptozotocin (STZ) dissolved in 0.01M citrate buffer (pH 4.5) was administered to create the NAFLD rat model. *Excoecaria agallocha* L. extract (200 and 400 mg/kg/day) was given orally to a subgroup of STZ-induced NAFLD model rats for 30 days, whereas rats in other diabetic control groups were given simply isotonic saline in the same amount of *E. agallocha* extract.To investigate the effects of E. agallocha extract on rats, different parameters such as oxidative parameters, antidiabetic and histopathology were measured, in addition to other biochemical tests. The administration of *E. agallocha* extract resulted in dose-dependent reductions in higher blood sugar, SGOT, SGPT, TC, TG, LDL, and VLDL levels. Various oxidative stress parameters were also significantly altered in a NAFLD rat model and treated by *E.agallocha* extract. Histopathological studies on the liver confirmed the possible protective effect of the E. agallocha extract. The findings point to the potential utility of *E. agallocha*. extract as a novel therapeutically beneficial hepatoprotective agent, particularly in NAFLD.

Keywords: Non-Alcoholic Fatty Liver Disease, Metabolic disorder, Oxidative stress, *Excoecaria agallocha*, Phytochemical analysis.

CONTENTS

INTRODUCTION

1.1 Epidemiology

Chronic liver disease known as non-alcoholic fatty liver disease (NAFLD) is typified by a significant fat accumulation in the liver of people who consume little or no alcohol. In wealthy countries, it has emerged as the most prevalent liver disease. The prevalence rate increased from 8.2% to 10.9% over that time, and the total number of NAFLD patients increased dramatically from 391.2 million in 1990 to 882.1 million in 2017(Ge et al., 2020). Unhealthy eating habits and insufficient physical exercise are contributing to the increased prevalence of fibrosis,non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (HCC) variants of NAFLD(Sayiner et al., 2016). NAFLD is a widespread disorder due to a lack of approved treatments, inadequate disease progression prediction markers, and insufficient screening of high-risk asymptomatic individuals. Although the main course of treatment consists on lifestyle modifications, alternative therapeutic approaches are also being explored. By learning more about the mechanisms behind disease development, therapeutic intervention, and diagnostic biomarkers, it is feasible to mitigate the effects of sickness. Combination medications are being used more often. This study aims to identify the pathological features, immunological mechanisms, and current therapeutic strategies for NAFLD(Petagine et al., 2023). It suggests that the global profile of NAFLD patients is males with type 2 diabetes and obesity who have an average age of 51.7 years. The incidence of NAFLD, diabetes, and metabolic illnesses appears to be increasing linearly; this is especially the case for those who have central obesity, metabolic syndrome, and diabetes. Among the metabolic comorbidities are hypertension (37%) and metabolic syndrome (40%) (LH et al. 2019). NAFLD can coexist with type-2 diabetic mellitus (T2DM) in up to 70% of cases, and T2DM patients have a doubled risk of dying from any cause. Additionally, Patients with excessive obesity had a 90% greater incidence of NAFLD(Alexander et al., 2018). As a result, the prevalence of NAFLD represents a substantial worldwide health burden that has to be addressed by clinicians.

1.2. Fatty liver disease:

Similar to the obesity epidemic, fatty liver disease is a common cause of elevated liver tests. The primary technique for determining the presence or absence of fatty liver disease is liver biopsy examination. Adult livers may contain up to 5% fat, and the size of the triglyceride (TGs) droplets indicates where the fat is being deposited. In fatty liver disease, which is frequently characterised by large droplet steatosis or mixed large and micro droplet steatosis, macrovisccular droplets typically do not cover the whole hepatocyte(Brunt, 2007). triglycerides (TGs) are the preferred nutrient for storage because of their higher caloric density and insoluble nature in water. They deposit in adipocytes in higher organisms, although under normal circumstances, they accumulate in other cell types. For example, during their long seasonal flights, migrating birds store enormous amounts of TGs in their liver, which is then used to prepare the delicious delicacy fois gras. People who store excess calories in their livers run the risk of developing unhealthy liver illnesses and grave clinical consequences. One of the most important roles of TGs is to buffer changes in energy supply and demand(J. Cohen, 2011).
Two significant causes of chronic liver disease globally are non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD). Both may result in cirrhosis and are distinguished by histological lesions, some of which include steatosis (Yeh & Brunt, 2014). 50% of deaths associated with cirrhosis are caused by alcohol, making it a major cause of end-stage liver disease of ALD. 20% of individuals with liver disease now use alcohol, making alcohol the most prevalent cause of cirrhosis in India (34.3%). Steatohepatitis, cirrhosis with consequences, and

fatty liver are all included in ALD. Based on its reversible nature with abstinence and asymptomatic character, steatosis—which affects 90% of heavy drinkers—is deemed harmless.

Nevertheless, in comparison to abstinent controls, it signifies metabolic stress that lowers long-term survival and raises the chance of severe liver disease (Mitra et al., 2020). Adults are more likely to be diagnosed with NAFLD, which is defined by triglyceride buildup that exceeds 5% of liver weight. From simple steatosis to NASH, which may advance to cirrhosis and hepatocellular cancer, it encompasses a variety of illnesses. Being caught early is critical since diagnosis is only achievable in the absence of substantial alcohol use.

De Ritis identified the ratio of aspartate transaminase (AST) to alanine transaminase (ALT) in serum, which is a sign of long-term problems in chronic viral diseases such as NAFLD, alcoholism, and hepatitis approximately half a century ago. Long-term consequences, such as fibrosis and cirrhosis, are predicted by an increased AST/ALT ratio (Botros & Sikaris, 2013).

1.3. NAFLD:

Lipid accumulation in hepatocytes may alter liver function as a result of genetics and other stresses. NASH might develop from steatosis that causes inflammation if these circumstances persist. NASH may ultimately result in liver failure, cirrhosis of the liver, and advanced fibrosis. In developed nations, the main causes of NAFLD, a common liver disease, include sedentary lifestyles, Western diets, obesity, diabetes, insulin resistance, and AT dysfunction. Possible consequences include NASH, hepatocellular carcinoma, fibrosis, and cirrhosis. NAFLD is partially caused by non-liver factors such internal dysbiosis and adipose tissue inflammation as well as hepatic lipotoxicity. Higher calorie consumption is causing metabolic inflammation in NAFLD and other obesity-related diseases more and more (Kořínková et al., 2020). Patients with NASH often show no symptoms; high serum transaminases are the first indication of the condition during regular testing. Some people, nevertheless, could feel nausea, fatigue, and lose their appetite. More severe symptoms such as edema, jaundice, and portal hypertension may occur in individuals if the illness advances to cirrhosis.

Fat accumulation in liver cells is a hallmark of the initial stage of NAFLD, also known as simple steatosis. Another term for this illness is NAFLD. It is generally asymptomatic and can be detected using imaging tests or blood tests. The main treatments for simple steatosis are lifestyle changes like losing weight and being more active. Simple steatosis is usually harmless and avoidable. Metabolic syndrome, dyslipidemia, obesity, and type 2 diabetes are risk factors. The risk of cardiovascular disease may rise with simple steatosis, even though it often does not result in liver damage (Friedman et al., 2018).

The liver fat buildup, inflammation, and hepatocyte destruction that define NASH, a stage of NAFLD, are present. Stenosis, cirrhosis, and even hepatocellular cancer may develop as a result of it. Although the exact nature of the progression process is unknown, elements such as lipotoxicity, oxidative stress, and dysbiosis of the gut microbiota are probably involved. Usually, a liver biopsy is needed for the diagnosis, and weight reduction is the mainstay of lifestyle changes for care. The number of pharmacological treatments is restricted; however, several innovative remedies are now being studied(Younossi et al., 2019).

An excessive accumulation of extracellular matrix proteins, mostly collagen, brought on by persistent liver injury characterizes fibrosis, a critical stage of nonalcoholic fatty liver disease. Driven by activated hepatic stellate cells, this process eventually results in scarring of liver tissue. The degree of fibrosis is a critical determinant in predicting long-term outcomes; severe fibrosis increases the possibility of morbidity and mortality associated with liver disease. Although non-invasive techniques like elastography and serum biomarkers have enhanced the monitoring process, Liver biopsy is the most dependable tool for precise staging. The most effective strategy for fibrosis regression is weight reduction, and management focuses on treating metabolic risk factors (Friedman et al., 2018).

The most severe stage of NAFLD, known as cirrhosis, is marked by a large amount of fibrosis and a deformation of the typical architecture of the liver. At this stage, there is a greater chance of hepatic decompensation, portal hypertension, hepatocellular cancer, and reduced liver function. Clinical characteristics, imaging tests, and non-invasive indicators are often used in the diagnosis process. A liver biopsy may be required for conclusive staging. The goals of management are to treat problems, stop more damage, and address metabolic risk factors. Liver transplantation may be considered in more advanced instances (Chalasani et al., 2018).

1.4.Pathogenesis of NAFLD

- **Lipid Accumulation**

The liver is essential for the import of free fatty acids (FFAs) and for the synthesis, export, storage, and metabolism of fats. NAFLD may arise as a result of problems with any one of these functions. (Berlanga et al., 2014). The liver absorbs free fatty acids primarily through the portal vein from dietary fatty acids and lipolysis in peripheral adipose tissues, particularly visceral and subcutaneous. The intricate control of free fatty acid storage and release within adipose tissues involves a sophisticated regulation by a variety of cytokines and hormones. These factors elicit distinct responses from adipose tissues, indicating a nuanced sensitivity to the complex interplay of regulatory signals. Liver FFAs are closely linked to visceral adipose tissues because they exhibit more sensitive lipolytic activities during fasting and exercise than subcutaneous adipose tissues. Thus, central obesity- which is typified by a large amount of visceral adipose tissue, contributes significantly to the pathophysiology of NAFLD (Ono et al., 2010).

The livers of patients with NAFLD accumulate fat, mostly as triglycerides. Triglycerides are created by esterifying free fatty acids and glycerol. (Buzzetti et al., 2016). In the hepatocytes, TGs can be stored as lipid droplets or released into the bloodstream as very low-density lipoprotein. To convert TGs into very low-density lipoproteins through esterification, Apolipoprotein B is necessary (Berlanga et al., 2014). Hepatic fat accumulation arises when there's an imbalance, with increased fatty acid uptake and de novo synthesis surpassing the rates of oxidation (Musso et al., 2009). In individuals grappling with type 2 diabetes mellitus or obesity, the onset of insulin resistance triggers uncontrolled lipolysis within the adipose tissue. This process culminates in an overwhelming influx of non-esterified fatty acids (NEFAs) into the liver, where the hepatocytes absorb them with the help of fatty acid transport protein FATP5, FATP2, and another protein transporter like caveolin-1, FA-building protein. CD36, also known as FA translocate, promotes NEFA uptake and intracellular trafficking in a variety of cells, such as macrophages, adipocytes, myocytes, hepatocytes, and enterocytes. The transcription factor forkhead box protein O1, insulin, and muscle contraction all promote FFA uptake by inducing

CD3 translocation. Pregnane X receptor (PXR), Liver X receptor (LXR), and peroxisome-activated receptor gamma (PPAR-γ) all share a common target in CD36, which has been demonstrated to increase in a murine model of hepatic steatosis. The precise involvement of CD36 in human diseases remains unclear; there is a notable association in morbidly obese individuals with NAFLD. In this context, the mRNA levels of CD36 have demonstrated a correlation with both liver fat content and apoptosis(Arab et al., 2018).

There are notable differences in the hepatic triglyceride sources between NAFLD patients and the general population. In particular, free fatty acid (FFA) accounts for only 60% of hepatic TG; the remaining 25% is ascribed to hepatic DNL, and the other 15% is acquired from dietary fats. (Méndez-Sánchez et al., 2007). DNL is a metabolic process primarily occurring in the liver and adipose tissue. This intricate series of metabolic events transpires within the cytoplasm, specifically the mitochondria, where surplus carbohydrates undergo conversion into fatty acids that are subsequently esterified into triglycerides. The initiation of DNL involves the transformation of acetyl-CoA into malonyl CoA facilitated by acetyl-CoA carboxylase, involving multiple cycles and enzymatic reactions. This malonyl CoA serves as a crucial substrate in the synthesis of 16-carbon palmitic acyl CoA, with the help of fatty acid synthase enzyme. Fatty acid synthase assumes a pivotal role in the intricate process of synthesizing fatty acid. Based on the state of metabolism, FAs are converted to TGs and either quickly metabolized or stored.(Bullón-Vela et al., 2018). Transcriptional factors like sterol regulatory element binding protein-1(SREBP-1), carbohydrate response element binding protein (ChREBP), peroxisome proliferator-activated receptor (PPAR-γ), and can all be activated to increase hepatic DNL—different types of SREBP isoform exit: SREBP1c and SREBP2. SREBP2 regulates the cholesterol metabolism. When cellular cholesterol levels are reduced, SREBP2 becomes activated. It undergoes translocation from ER to the nucleus, and it exerts its influence on the transcription of specific genes associated with cholesterol synthesis, secretion, uptake, and transport. This regulatory process aims to enhance the availability of intracellular cholesterol(Musso et al., 2013). Insulin stimulates SREBP1c through LXR, which then increases fatty acid synthase transcription. ChREBP stimulates a liver type of pyruvate kinase transcription, which raises the synthesis of citrase, a substrate for the synthesis of fatty acid(Shimada et al., 2014).

Dietary factors have a major impact on how NAFLD develops. Western diets' high-fat content has been connected to dyslipidemia, insulin resistance, and the start of metabolic and cardiovascular illness.(Zhou & Fan, 2019). Fructose, unlike glucose, possesses the ability to influence hepatic lipid metabolism through direct activation of ChREBP and SREBP1c, along with a reduction in mitochondrial beta-oxidation. This dual mechanism ultimately promotes the development of NAFLD(Pierantonelli & Svegliati-Baroni, 2019).

- **Insulin Resistance**:

When insulin attaches to its receptor in healthy humans, a series of processes take place that led to the phosphorylation of number of substrates, including insulin receptor substrates (IRS)-1, 2,

3, and 4. These phosphorylated substrates are essential for the body's transmission and amplification of the insulin signal(Dowman et al., 2010). IRS proteins experience serine phosphorylation, a process that dampens insulin signalling by reducing the level of insulin-induced tyrosine phosphorylation (Malaguarnera et al., 2009). Various enzymes, including c-Jun-N-terminal kinase (JNK-1), kappa kinase beta (IKK-b), and protein kinase (PKC), participate in stimulating the serine phosphorylation of the substrate for the insulin receptor, leading to a reduction in glucose uptake, decreased activation of glycogen synthase, and inhibition of the phosphorylation of forkhead box protein O (FOXO) are mechanisms that collectively lead to the stimulation of hepatic gluconeogenesis(Malaguarnera et al., 2009; Polyzos et al., 2009). The inhibition of IRS1/2 signaling can be attributed to primary factors such as FFA, ROS, ER stress, and adipocyte-mediated alterations. The influences of IKK-b, JNK-1, and PKC play a pivotal role in this context. These elements collectively contribute to the hindrance of IRS1/2 signaling pathways (Marušić et al., 2021). Research findings have indicated that inflammatory cytokines, TNF-∝ hinders the effect of insulin-induced tyrosine phosphorylation of IRS1 by augmentation in the phosphorylation of Ser307(Rui et al., 2001). These outcomes are facilitated through the activation of the IKKβ-NF-κB pathway (Yuan et al., 2001). Elevated levels of TNF-∝ within adipose tissue could potentially contribute to the advancement of NAFLD by fostering systemic insulin resistance and triggering inflammation in diverse tissues(Fontana et al., 2007). The heightened secretion of IL-6 in adipose tissue, driven by JNK1 activity, led to an upregulation of hepatic SOCS3 expression. Consequently, this upregulated SOCS3 prompted the degradation of critical molecules associated with insulin signalling in the liver, ultimately resulting in the development of insulin resistance in the liver (Sabio et al., 2008). Elevated levels of IL-6 in the serum have been documented as notably higher in individuals with NAFLD(Coulon et al., 2012). Adipocytes also release distinct adipokines, including specialized leptin and adiponectin (Sun & Karin, 2012). Leptin significantly influences the dephosphorylation of IRS1(B. Cohen et al., 1996). Patients with NAFLD exhibit elevated circulating leptin levels compared to control, and there is a positive correlation between the severity of NAFLD and the levels of circulating leptin(Sakurai et al., 2021). Conversely, adiponectin plays a crucial role in improving NAFLD. This effect is mediated through AdipoR1 and AdipoR2. Within the liver, AdipoR1 facilitates the activation of AMP-activated kinase, while AdipoR2 is responsible for triggering the activation of PPAR alpha. Both of these pathways contribute to heightened insulin sensitivity(Yamauchi et al., 2007). The livers of individuals with obesity exhibit elevated levels of hepatic DAG. DAG is a lipid intermediate involved in TG synthesis, and in a state of normal physiological conditions, heightened TG levels coincide with a parallel increase in DAG. Activation of PKC by DAG leads to the inhibition of insulin receptor kinase activity(Pafili & Roden, 2021).

1.5. Indirect relation between diabetics and NAFLD

NAFLD has a complicated and contradictory connection with antidiabetic drugs. An increased risk of acquiring or aggravating NAFLD has been linked to some antidiabetic medications despite the fact that diabetes is still a substantial risk factor for NAFLD. There is a reciprocal link between diabetes and NAFLD, with one illness raising the chance of the other. Diabetes is a risk factor for the advancement of NASH and cirrhosis, two more severe types of NAFLD. Up to 70% of people with type 2 diabetes mellitus (T2DM) have NAFLD (Targher et al., 2021).

Insulin, sulfonylureas, and thiazolidinediones (TZDs) are among the antidiabetic drugs that have been linked to the onset or progression of NAFLD. Increased lipogenesis, weight gain, modifications to lipid and glucose metabolism, and altered insulin sensitivity are all potential causes of NAFLD. That being said, not all antidiabetic drugs are harmful to the liver. Metformin, SGLT2 inhibitors, and GLP-1 receptor agonists are among the more recent medication types that have shown possible advantages (Talamantes et al., 2023). The idea that antidiabetic drugs may primarily cause NAFLD emphasizes how crucial customized treatment is to the care of diabetic patients. Healthcare professionals need to carefully weigh the benefits and dangers of glycemic management for the liver. Regularly monitoring liver function and fat content in diabetic patients may be part of this. Patients at high risk for NAFLD may also benefit from using newer antidiabetic medications that may improve liver health. Lifestyle interventions, such as diet and exercise, can also improve liver health and diabetes control. Medication selection should be customized based on the unique characteristics of each patient, including body weight, liver function, and NAFLD risk factors (Kim & Lee, 2020). The physiological resemblances, genetic homogeneity, fast disease progression, well-characterized model, hormonal responses, histological similarities, features of the metabolic syndrome, responsiveness to interventions, availability, cost-effectiveness, ethical considerations, and versatility in study designs of Wistar rats make them an ideal model for studying metabolic disorders such as NAFLD. In addition to having NAFLD, they may also acquire other characteristics of metabolic syndrome and have well-characterized hormonal responses, as shown in scientific literature. Larger-scale research may benefit from the affordability and accessibility of Wistar rats. They do not, however, quite mimic every facet of human NAFLD, especially when it comes to the molecular pathways involved and the way the illness progresses over time(Ghezzi et al., 2012). The study that follows focuses on several variables that should be examined as a result of diabetes, including tissue antioxidants, serum variables, blood glucose level, blood cholesterol level, and histopathology of the affected organs.

1.6.NASH:

The developing stage of NAFLD known as NASH is marked by variable degrees of fibrosis and necroinflammation. NASH and subacute hepatic failure are associated(Basaranoglu & Neuschwander-Tetri, 2006). One way to explain the switch from NAFLD to NASH is with the "two-hit hypothesis." The accumulation of fatty acids in the liver, which is closely associated with insulin resistance, is the first attack in the early stage. This build-up not only increases the vulnerability of the liver cell but also sets the stage for further shocks or injuries such as proinflammatory cytokines, oxidative stress, mitochondrial dysfunction, and lipid peroxidation(Giorgio et al., 2013). The first hit hypothesis mentioned above is the pathophysiology of NAFLD.

Genetic and epigenetic factors are believed to play a role jointly in mitochondrial dysfunction in NASH(Caligiuri et al., 2016). The hub of free fatty acid β-oxidation predominantly takes place in the mitochondria of hepatocytes. Within the mitochondria, long-chain fatty acids undergo oxidation and subsequent transportation. This metabolic pathway is linked with the reduction of

oxidized NAD+ and FAD to their respective reduced forms, namely NADH and FADH2(Fromenty et al., 2004). NADH and FADH2 undergo reoxidation to NAD+ and FAD, transferring electrons to the mitochondrial respiratory chain. This process generates reactive oxygen species (ROS), contributing to oxidative stress if protective mechanisms are insufficient(Basaranoglu et al., 2013). Elevated FFA flux exhausts mitochondria, necessitating alternative sites for FFA processing, such as endoplasmic reticulum and peroxisomes. Mitochondrial exhaustion triggers oxidative stress via partially reduced oxygen molecules. Mitochondrial CYP2E1 attempts to oxidize surplus FFAs, intensifying ROS production. Excess FFAs are additionally oxidized in peroxisomes, where FADH2 and NADH electrons contribute to increased ROS formation. This process induces mitochondrial dysfunction, manifested by reduced proteins, DNA, and ATP (Noureddin & Sanyal, 2018). Impaired mitochondrial respiratory chain (MRC) function has the potential to initiate the generation of reactive oxygen species (ROS). When there is a disruption in the flow of electrons within the respiratory chain, the intermediates involved in the respiratory process may divert electrons towards molecular oxygen, resulting in the formation of superoxide anions and hydrogen peroxide(Wei et al., 2008).

Lipid peroxidation of polyunsaturated fatty acids (PUFAs) is initiated by an imbalance caused by increased quantities of ROS and decreased antioxidant levels. Cytotoxic aldehydes such as 4-hydroxy-2,3-transnonenal (4-HNE) and malondialdehyde (MDA) are produced during the process of lipid peroxidation. The collaboration between ROS and aldehydes serves to intensify oxidative stress, concurrently promoting elevated levels of inflammatory cytokines. These events play a pivotal role in instigating hepatic inflammation, ultimately contributing to the emergence of various hepatic lesions associated with NASH(Basaranoglu & Neuschwander-Tetri, 2008). ROS activation triggers the nuclear factor-κB, leading to the initiation of transforming growth factor-α (TNF-α) synthesis. This activation also results in elevated expression levels of various cytokines, such as TGF-β, interleukin-6 (IL-6), IL-8, TNF-α, and Fas ligand(Rolo et al., 2012).

Additionally, the activation of death receptors is linked to Fas-ligand, which has been demonstrated to be correlated with heightened necrosis through the activation of caspases and the induction of cell death via Fas-ligand. The impact of TNF-α on NASH is intensified by an irregular cytokine profile and heightened TNF-α receptor expression within the liver. This results in increased lipid peroxidation of mitochondrial membranes, thereby exacerbating their functionality and further triggering oxidative stress. TNF-α induces inflammation by activating NADPH oxidase, which triggers TNF receptor-1 expression(Ucar et al., 2013). NF-κB has been proposed as a pivotal contributor to the escalation of inflammation in the liver(dela Peña et al., 2005).

1.7. Treatment:

Treatment for NAFLD mostly consists of lifestyle modifications and management of associated metabolic diseases. There are currently no FDA-approved medications, especially for NAFLD, even though a few techniques have shown promise.

- **Lifestyle Modifications:**

- **Weight loss:** A 7-10% reduction in body weight can significantly improve liver histology in NASH patients.

- **Diet:** Mediterranean diet and low-carbohydrate diets have shown benefits.
- **Exercise:** Regular physical activity, both aerobic and resistance training, can reduce liver fat (Vilar-Gomez et al., 2015).

- **Management of Metabolic Comorbidities:**

- Treating diabetes, hypertension, and dyslipidemia can improve NAFLD outcomes.
- Metformin, while not directly improving liver histology, may reduce the risk of hepatocellular carcinoma(Mantovani et al., 2018).

- **Pharmacological Interventions:**

- Vitamin E: Shows some benefits in non-diabetic NASH patients.
- Pioglitazone May improve liver histology in patients with and without diabetes.
- GLP-1 receptor agonists: Emerging evidence supports their use in NASH (Chalasani et al., 2018).

- **Emerging Therapies:** Several drugs are in various stages of clinical trials, targeting different pathways:

- FXR agonists (e.g., obeticholic acid)
- PPAR agonists
- CCR2/CCR5 antagonists
- FGF21 analogs(Friedman et al., 2018)

- **Liver Transplantation:** This is for patients with end-stage liver disease or hepatocellular carcinoma due to NAFLD(Younossi et al., 2016).

Phytochemicals obtained from plants are one of the most important natural sources of inspiration for medication development. These chemicals, produced for specific purposes, can act as a natural template for the development of novel medications. A minimum of 12,000 such compounds have been identified, accounting for less than 10% of the total. The chemical diversity of these chemicals may provide the core scaffolds for future drugs(Lai & Roy, 2004). A renewed surge of interest in the use of phytochemicals and their potential as a foundation for drug discovery has occurred throughout the past 25 years(Newman & Cragg, 2007). Approximately 60% of all pharmaceuticals used in clinical settings worldwide are made up of natural materials or their derivatives, with medicinal plants accounting for 25% of all medications (Gurib-Fakim,2011).
More than 28 percent of newly launched chemical entities in the market have their origins in natural items. According to research on plants, there are between 250,000 and 350,000 plant species that have been discovered, of which roughly 35,000 are used medicinally worldwide (Kong et al., 2003). According to the World Health Organization (WHO), 85% of traditional medicines produced from plants are used by 65% of the world's population and 80% of

developing nations. This indicates the importance of plant-based medical systems(Cragg & Newman, 2013).

People in rural regions can receive primary healthcare services via the usage of medicinal plants. According to a 2004 survey, between 40 and 50 percent of individuals in wealthy nations use traditional medicines, whereas 80% of people in third-world countries rely on them for their primary healthcare requirements. These traditional medicines are primarily plant-based treatments (Oyebode et al., 2016).

LITERATURE REVIEW

2. LITERATURE REVIEW

Guo et al., 2022 Nonalcoholic fatty liver disease (NAFLD) is a widespread chronic liver ailment affecting around 25% of the world's population. It is linked to lipid deposition, oxidative stress, ER stress, and lipotoxicity. Hepatic steatosis is one of its distinguishing features. Natural products include active substances that can reduce protein levels and treat NAFLD, perhaps leading to the creation of new pharmaceuticals. These compounds might open up new options for research and drug development.

Alongi, D.D. et al 2001 Plants have a diverse spectrum of secondary metabolites, which can have a variety of biological actions and so serve as valuable sources of medical or pharmaceutical goods. Plants have been utilised for medicinal reasons throughout history, and many contemporary medications are derived from them. Morphine, the first pharmaceutically active chemical isolated from the plant Papaversomniferum, was discovered in 1805. The discovery of morphine ushered in an era in which plant-derived medications were refined, analysed, and supplied in exact quantities. Recently, there has been an increase in interest in alternative medicines and the therapeutic use of natural goods, particularly plant-derived medications, with plants accounting for around 25% of all prescription drugs worldwide.

Kaliamurthi & Selvaraj, 2016A milky mangrove found along India's coastline is called *Excoecaria agallocha*. There are about fifty volatile chemicals, fifteen terpenoids, and twenty polyphenols in the leaf, stem, latex, and root extracts. An ethanolic leaf extract yielded a large variety of chemicals. High concentrations of polyphenols and terpenoids found in *E. agallocha* have been linked to antibacterial, anticancer, and anti-diabetic properties as well as endocrine, epidemic, and endemic disease control.

Li et al., 2009; Mondal et al., 2016 The compounds flavonoids, diterpenoids, phenolic acids, triterpenoids, tannins, and have been discovered from *E. agallocha*. The majority of the diterpenoids in *E. agallocha* are present in its roots, bark, wood, leaves, and stems. There are several types of diterpenoids, including labdane, beyerane, artisane, isopimarane, daphnane, tigliane, and kaurene.

Yin et al., 2008 Agallochaols,agallochaexcoerins,agallochins, excoeagallochaols, and excoecarins are among the most often isolated diterpenoids. *E. agallocha* leaves and stems include triterpenoids, phenolic acids, flavonoids, sterols, and tannins, according to reports. Triterpenoids are classified into oleanane, cycloartane,taraxerane, friedelane, and lupane kinds.

Thirumurugan et al., 2009 Investigated the potential anti-diabetic effects of *E. agallocha* leaves in rats given an alloxan injection. The findings demonstrated a significant hypoglycemic impact of the ethanolic leaf extract at 500 mg/kg in both normal and alloxan-induced diabetic mice.

Sofia & Teresa, 2016 Research has indicated that when evaluated using reputable assays, the bark **(Subhan et al., 2008)** and leaf (**Laith et al., 2016**) Antioxidant properties of *E. agallocha*

extracts are notable. based on the scavenging of free radicals by 2,2-diphenyl-1-picrylhydrazyl (DPPH) in diverse plant components extracted using different solvents.

Babuselvam et al., 2012; Y. Li et al., 2010 It has been demonstrated that diterpenoids, such as agallochaols, that are extracted from *E. agallocha* twigs and stems have anti-inflammatory qualities. By blocking NF-kB or AP-1 activation, they successfully inhibited TNF-α and IL-6 produced by lipopolysaccharide (LPS) in murine macrophage RAW 264.7 cells. *E. agallocha's* analgesic and anti-inflammatory qualities were assessed in a different investigation. As a result, the overarching goal of this study is to explore the scientific basis for therapeutic usage and then assess the biological potential of these plants.

The plant's components are high in polyphenols and flavonoids, which may explain why it possesses antioxidant properties. A common way that NAFLD spreads is by the overproduction of free radicals. The generation of reactive oxygen species in the mitochondria brought on by hyperglycemia may be a crucial step in the development of diabetic complications.

Fish poisoning has been done using latex in Southeast Asian nations. Its wood and bark are used in traditional medicine in Thailand to treat flatulence**(Karalai et al., 1994)**. In South China's coastal districts, the herb has historically been used to cure ulcers, rheumatism, leprosy, and paralysis **(Y. Li et al., 2012)**.

According to recent research, *E. agallocha* extract (500 µg/disc) has antimicrobial action**(Rajeswari & Bhaskara-Rao, 2015)** and silver nanoparticles from *E. agallocha* fruit extract **(Nagababu et al., 2017)**.

AIM, OBJECTIVE AND RATIONALE

3.1. AIM

To evaluate the Pharmacological activity of *Excoecaria agallocha* (AGL) plant extract for the management of non-alcoholic fatty liver disease.

3.2. OBJECTIVE

1. To evaluate the hepatoprotective effect of AGL extract against the NAFLD model.
2. To evaluate the therapeutic effect of AGL on the liver by measuring such biomarkers, e.g., Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Low-density lipoprotein (LDL), High-density lipoprotein (HDL), Triglyceride (TG), Total cholesterol (TC).
3. In vitro evaluation of hypoglycemic activity
4. To determine the antioxidant potential of AGL.
5. To observe the histopathological changes in experimental rats

3.3.RATIONALE

- Very few drugs are available for management of NAFLD
- Natural product may act as lead molecule for the development of drugs with improved safety profile
- Scientific literature suggests that *Excoecaria agallocha* exhibits antioxidant, anti-inflammatory and hypoglycemic activity.

PLANT PROFILE

4.1. Scientific name:*Excoecaria agallocha L*

Fig 1: *Excoecaria agallocha*Plant

4.2. Taxonomical distribution

- Kingdom: Plantae
- Phylum: Tracheophyta
- Class: Magnoliopsida
- Order: Malpighiales
- Family: Euphorbiaceae
- Genus: Excoecaria
- Species: E. agallocha

4.3. Synonyms

Excoecaria affinis Endl., Excoecaria camettia Willd.

4.4. Common names:

a. Sanskrit- Agaru, gangwa, gaourai
b. Hindi- Gangiva, tejbala.
c. Bengali- Gewa,
d. English- Blind-your-eye mangrove, Milky mangrove, River poison tree
e. Malayalam- Komatti, Kannampotti, Kammetti,

4.5. Geographical Distribution

E. agallocha L. (AGL) thrives in mangrove forests in Australia, Asia, and the Southern Pacific. Information is provided in the **Table 1.**

Table 1 : Geographical distribution of *Excoecaria agallocha* L.

Sl no	Specific Region	Countries and subarea
1.	Asia-temperate	China - Guangxi,Guangdong, Hainan; Taiwan; Japan - Ryukyu Islands;
2.	Asia-tropical Indian Subcontinent	India - Andhra Pradesh,Andaman and Nicobar, Goa, Karnataka, Kerala, Maharashtra, Orissa, Pondicherry, Tamil Nadu, West Bengal; Bangladesh; Sri Lanka
3.	South Eastern Asia	Myanmar; Cambodia;Indonesia; Papua New Guinea; Thailand; Malaysia;Vietnam; Philippines
4.	Australia	Australia, New South Wales,Queensland, Northern Territory, Western Australia
5.	Pacific South	Fiji; Niue;New Caledonia; Tonga

4.6.Botanical Description

E. agallocha L. is a mangrove plant that belongs to the Euphorbiaceae family. This little tree can grow up to 15 meters tall. The **Table 2** provides a detailed description of the botanical features.

Table 2:Botanical features of *Excoecaria agallocha* L.

Sl no	Plant part	Morphological description
i.	Flowers	Flowers: 2-3 mm in diameter, yellow, fragrant, unisexual, with three stamens and no filaments; male flowers on catkin spikes The female flowers are in an axillary raceme, having a pedicellate shape, pale green colour, three lobes on the calyx, three cells on the ovary, and a trifid style.
ii.	Fruits and roots	Fruits: 3 lobed, 8 mm in diameter. around 4 mm length seeds. About 1 mm length radicle
iii.	Stem	Bark exudate rapid and copious, sometimes deciduous
iv.	Leaves	The leaves are alternating, ovate-elliptic or orbicular, with a short acuminate apex, narrowing base, and entire or sinuate-crenate margins. They measure 3-8 cm×1.5-3 cm and are glabrous and petiolate.
v.	Branches	Lenticellate, glabrous
vi.	Roots	Superterranean bands create elbow-shaped pegs rather than pneumatophores as a result of horizontal roots spreading and mingling with one another.

4.7.Ethnobotanical Information

It is commonly recognized that the plant has significant economic, ecological, and medical uses.In addition to its traditional uses as an emetic and purgative, it is also used to heal ulcers, wounds, and stings from dangerous marine organisms. The bark oil of E. agallocha is also used to treat paralysis, leprosy, and rheumatism.

Additionally, it has been traditionally used to treat dermatitis, hematuria, and conjunctivitis. This plant secretes latex, which has been used as a purgative, abortifacient, and to cure rheumatism, paralysis, ulcers, and leprosy. The inhabitants of India, Malaysia, and New Caledonia utilise the plant's leaves and latex as fish poison. Leprosy is cured in Sri Lanka with the smoke from burning wood, and swellings of the feet and handsare treated with the root mashed with ginger. A milky sap or latex that the plant exudes can result in skin blisters and discomfort, as well as momentary blindness if it gets in the eye. This further indicates the plant's deadly properties. Due of its deadly properties, latex is employed as both a dart and fish poison. A reddishsoft material called "Tejbala," which is extracted from the lower trunk of *E. agallocha*, is said to have been employed as a purported "aphrodisiac tonic" in Indian "materia medica". Burmese people utilise

the leaves as an epilepsy treatment. In the Solomon Islands, coconut milk is combined with the plant's latex to create a potent emetic and purgative. The oil extracted from the trees is used by the Malays to cure skin infections and irritation. The plant's roots are used as a component in embrocations and as a remedy for swellings and toothaches.

4.8.Chemical constituents

E. agallocha has a variety of chemical elements, the most common of which are alkaloids, diterpenoids, flavonoids, terpenoids, and tannins.Some of their names are given in Table 3.

Table 3: Compound presence in E. agallocha

Sl no	**Types of compounds**	**Compound names**	**Presence in *Excoecaria agallocha***
1	Flavonoid	Quercetin	Present in leaves; known for its antioxidant properties.
		Kaempferol	Found in leaves; contributes to antioxidant and anti-inflammatory effects.
2	Phenolic Compound	Tannic Acid	Found in bark; contributes to astringency and defensive properties.
		Syringic Acid	Detected in bark; known for antioxidant properties.
		Gallic Acid	Found in bark; contributes to antioxidant and antimicrobial properties.
3	Terpenoid	Lupeol	Found in leaves and bark; has potential anti-inflammatory and antimicrobial effects.
		Betulin	
		Taraxerol	
4	Steroid	β-Sitosterol	Present in leaves; has potential anti-inflammatory and cholesterol-lowering effects.
		β–sitostenone	
5	Unique to Excoecaria	Excoecarins	Specific to the genus; may have defensive and toxicological effects
		Excoagallochaol A-D	
		Agallochaol	

Collection, identification, and Phytochemical screening

5.1. Collection of the plant materials

The whole plant was collected in the month of November, 2023 from the Coastal region of sundarban, Kakdwip, South 24 parganas, 743347, West Bengal. The plant material was identified by taxonomist, R. K. Gupta, Scientist-E & Head of office, Central National Herbarium, Botanical Survey of India (BSI), Howrah-711103 and it has been identified as *Excoecaria agallocha L* belonging to the family Euphorbiaceae. A voucher specimen (JU/AB-01) of the authenticated *Excoecaria agallocha L* has been deposited on dated 21/12/2023 in the herbarium of BSI.

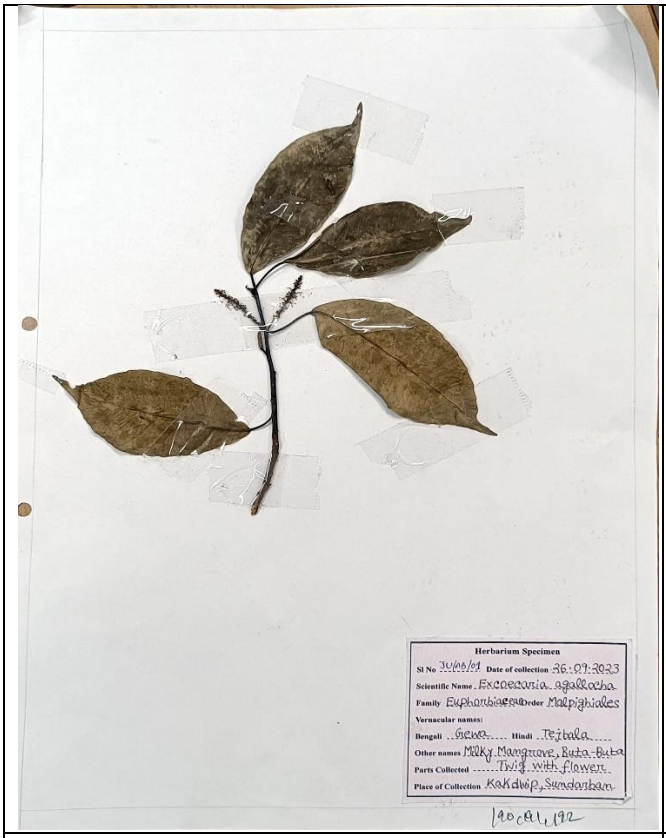


Fig 2: Herbarium sheet

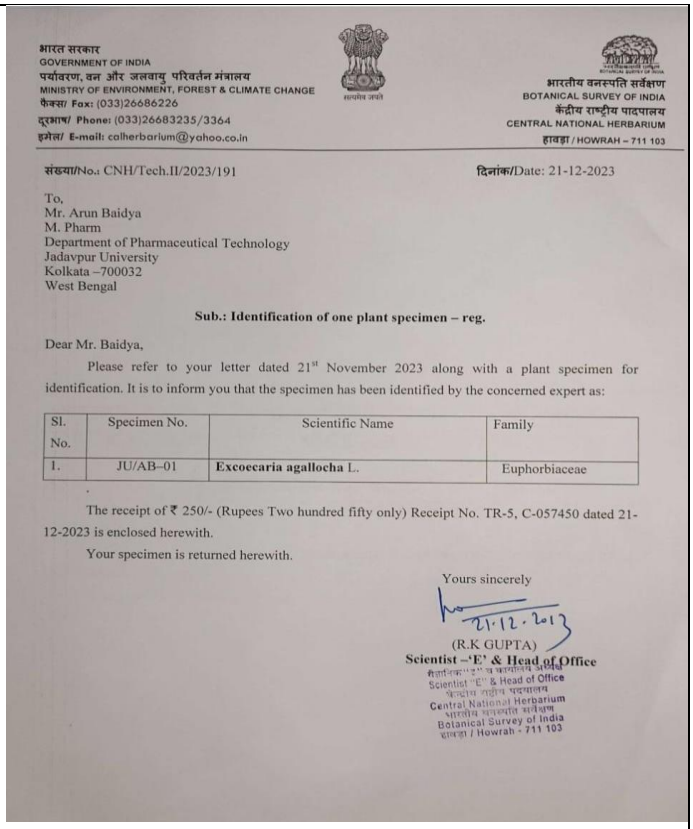


Fig 3: Authenticated certificate from BSI

5.2. Processing of *E. agalloch*

For the purpose of removing soil and other impurities, tap water was used to wash the bark portion of *Excoecaria agallocha* (AGL). After the bark was properly dried in a shaded area at room temperature (25ºC±2), the plant components were ground into a fine powder for use in other experiments.

5.3. Extraction procedure

In a Soxhlet apparatus, the AGL bark was extracted using Ethanolic extract, hydro-alcoholic (ethanol : water - 50:50). The extract was filtered, and then the previous step was carried out twice more. In a rotating evaporator operating at low pressure, the solvent was totally eliminated.

After lyophilization, a concentrated extract was produced, which was then kept for later use in vacuum desiccators at 20°C. The yield value was about 13.76% w/w.

5.4. Soxhlet extraction:

One hot continuous extraction method is Soxhlet extraction, in which a dried crude drug is subjected to constant extraction with the same solvent. The Soxhlet extractor primarily consists of three parts:

Fig 4: Soxhlet Extraction

1. At the top condenser. Here, the solvent vapor gets condensed and drips into the main chamber of the Soxhlet extractor.

2. In the middle is the Soxhlet apparatus's main chamber, which contains the Thimble. The main chamber is loaded with the Thimble, which is the powdered crude drug wrapped in a piece of filter paper.

3. At the bottom- a round-bottom flask. As it is more popularly known, it holds the solvent or menstruum. A sufficient amount of solvent necessary for extraction is loaded in this flask.

The solvent is boiled in the round-bottom flask with the help of a heating mantle. The temperature is set at a temperature around the solvent's boiling point. One side tube originating from the round-bottom flask opens at the top of the main chamber of the Soxhlet apparatus and carries the solvent vapors to the condenser. Another thin tube, the siphon tube, is connected to the main chamber. As the solvent level in the chamber reaches the top of the siphon tube, the entire solvent is transferred into the round-bottom flask. The color of the solvent in the side tube should be monitored continuously. As long as any tinge of color is visible in the siphon tube, the extraction should be continued. When the solvent in the side tube gets colorless, the liquid containing the phytoconstituents should be collected and evaporated in a vacuum. If any solid residue is visible, the extraction should be continued. Otherwise, the extraction procedure need not be continued. The importance of this technique is that the process can be done multiple times to achieve effective extraction.

After the extraction, a finished extract is obtained through several downstream processing methods. First, the solvent is removed using a suitable method, which is referred to as concentration. After the concentration procedure, the plant extract is subjected to phytochemical analysis. The preliminary qualitative phytochemical analysis detects the presence of secondary metabolites. The quantitative estimations of the total amount of a particular kind of secondary metabolite, such as the TPC and TFC, are also under the scope of the present study.

5.5. Determination of physicochemical parameters:

5.5.1 Moisture content determination:

1 gm of powder was put into a flat petri dish, which was then heated to 105°C in a hot air oven to eliminate any moisture from the powdered plant. The samples were dried and then weighed in a desiccator at room temperature. Using the previously weighed powdered medication as a reference, the % moisture content was calculated. [Ahmed et al, 2013].

$$\text{Total moisture content (\%)} = \frac{\text{Weight of dried sample}}{\text{Weight of sample taken}} \times 100$$

5.5.2 Total ash content:

2 gram of dried powder plant material was weighed in a silica crucible. The powder was heated to 450°C in a muffle furnace for two to three hours, or until it was carbon free. It was then allowed to cool in a desiccator. To ensure thorough detecting ash value, the powder was weighed after that. For thirty minutes, it was heated in the furnace once more. It was then chilled and weighed. The ash's weight and the powdered material's weight were compared.(Ahmed & Hasan, 2015).

$$\text{Percentage of ash} = \frac{\text{Weight of ash}}{\text{Weight of sample taken}} \times 100$$

5.5.3 Determination of water-soluble ash:

The ash obtained was digested with water.25ml of solvent was used and 20-30 min time was taken for boiling in a water bath. The contentment in the crucible was filtered by using Whatman filter paper and the filter paper was free from ash. The filter paper with residue was removed carefully without any loss, folded and put in the same crucible, dried in hot air oven and was ignited in muffle furnace at the temperature of 450°C for 1h. Then that was cooled by using a desiccator and final weight was taken. The percentage of the water-soluble ash was calculated with the reference of the previously weighed powdered drug (Ahmed & Hasan, 2015).

$$\text{Percentage of soluble ash} = \frac{\text{Weight of soluble ash}}{\text{Total weight of ash}} \times 100$$

5.5.4 Determination of acid insoluble ash value:

The previously weighed total ash was boiled with 25 ml of 2N HCl for 15 mins and then the solution was filtered through an ash less filter paper. After filtration, the filter paper with ash was placed in a silica crucible and ignited at 450ºC in a muffle furnace until the ash becomes carbon free. Next the crucible was cooled in room temperature and the weight of the acid insoluble ash was calculated with reference to the weight of the powdered drug previously taken(Ahmed & Hasan, 2015).

$$\text{Percentage of acid insoluble ash} = \frac{\text{Weight of acid insoluble ash}}{\text{Total weight of ash}} \times 100$$

5.5.5 Determination of extractive value:

The dry powdered plant material of *Excoecaria agallocha* was extracted with Hexene, chloroform, methanolic, and hydroalcoholic 1 g of the coarsely powdered plant material was weighed in a weighing bottle and transferred into a dry conical flask. Then the flask was filled with different solvents (15 ml) separately. The flasks were corked and kept aside for 24 h at room temperature, shaking frequently. The mixtures were filtered through Whatman No. 1 filter paper into a measuring cylinder. After the filtration, it was then transferred into a weighed Petry plates. The resulting extracts were dried out by allowing the filter to completely evaporate the solvent. The following formula was used to compute and record the extraction value in %. The Hexene, chloroform, methanolic, and hydroalcoholic extract of *Excoecaria agallocha*(Khandelwal, 2006).

$$\text{Extractive value (\%)} = \frac{\text{Weight of dried extract}}{\text{Weight of plant material}} \times 100$$

Preliminary phytochemical screenings of *E. agallocha*

The preliminary phytochemical screening of the hydroalcoholic extract of *Excoecaria agallocha* was carried out by various standard methods (Khandelwal, 2006) which are mentioned below.

5.6. Chemical tests

5.6.1. Tests for alkaloids:(Souza, 2010)

- **Wagner's test:**2milliliters of extract solution were treated with 0.1 milliliters of Wagner's reagent and two milliliters of mild hydrochloric acid. A reddish-brown coloration indicated a positive response to alkaloids
- **Hager's test:**Few drops of Hager's reagent has been added to 1 ml of the sample solution. When a yellow-colored precipitate forms, alkaloids are present.
- **Mayer's test:** In 1 ml of sample solution with a few drops of potassium mercuric iodide, or Mayer's reagent. When a yellow-colored precipitate forms, alkaloids are present.
- **Dragendroff test:**0.1 ml of dilute hydrochloric acid and 0.1 ml of Dragendroff's reagent were added to a test tube containing a 2m1 extract solution. The production of orange-brown color precipitate indicates the presence of alkaloids.

5.6.2. Tests for glycoside:

- **Kellar kiliani test:**In a test tube, add a small sample,ferric chloride solution, glacial acetic acid, and concentrated sulfuric acid. Observe the interface for colour changes and observe the reaction (Evans, 2009).
- **Bontrager's test:** 1millilitre of extract solution was treated with a few millilitres of mild sulphuric acid before being filtered and chloroform extracted. The layer was treated with 1 ml. of ammonia. The formation of a crimson colour indicates the presence of anthraquinone glycosides.

5.6.3. Test for saponin:

- **Frothing test:** dried powdered plant sample was added to distilled water and shaken vigorously and If froth appears, it conforms the presence of Saponins.

5.6.4. Test for flavonoid:

- **Shinnoda test:** magnesium powder and few drops of concentrated HCl solution was added to 2ml of sample solution. Pink color obtains because of the presence of flavonoid.

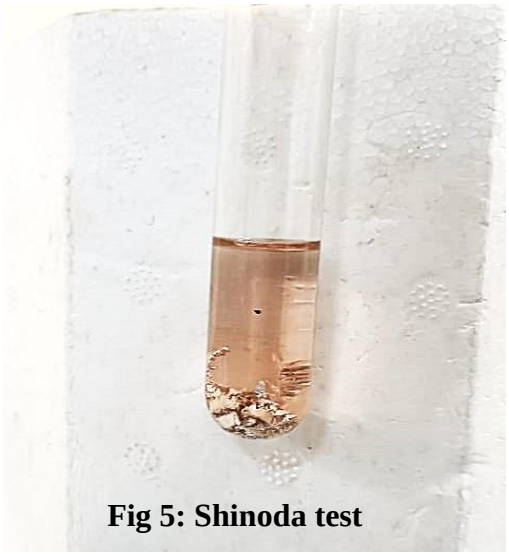


Fig 5: Shinoda test

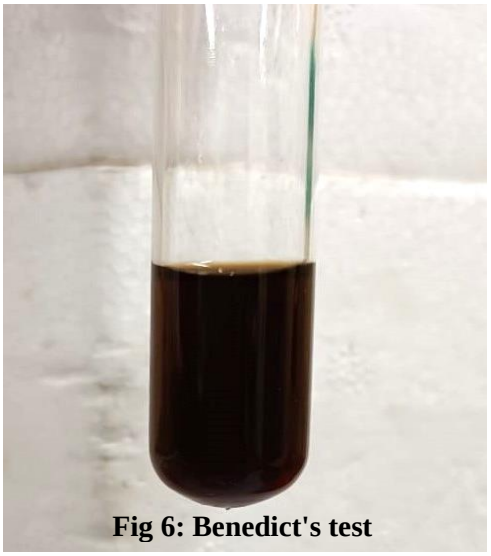


Fig 6: Benedict's test

5.6.5. Tests for tannin:(Segelman et al., 1969)

- **Ferric chloride test:** 5% ferric chloride solution in few drops was added into 2ml of sample. If it becomes blue, tannin can be present.
- **Lead acetate test:** few drops of lead acetate solution was added to 2-3ml of the sample solution and formation of white precipitate obtains due to the presence of tannin.
- **Gelatin test:** in the 2 ml of sample solution gelatin solution was added. Formation of white precipitate obtains due to the presence of tannin.

5.6.6. Test for steroid:

- **Liebermann burchard test:**2 milliliters of the test extract, 1 milliliter of CHCl3, 1 milliliter of acetic anhydride, and 1 drop of concentrated H2SO4 has been added. If the solution becomes red, then blue, and lastly bluish green in color, it indicates the existence of steroidal nucleus, whereas the creation of purple or red color indicates the presence of triterpenoid.
- **Sulkowski test:** 1-2 mg of powdered drug was dissolved in 1ml of CHCL3 solution and 1ml of concentrated H2SO4 was added to that. The presence of sterols is indicated by the colours bluish red, cherry red, and purple in the chloroform layer, whilst the creation of a reddish-brown colour at the interface shows the existence of a triterpenoidal nucleus.(Bosila and El-Sharabasy, 2009)

5.6.7. Test for carbohydrate:

- **Molisch's test:** 1ml of Molisch reagent was added to 2ml of test solution. Then 1ml of concentrated sulphuric acid solution was also added. In the junction of two liquids, violet ring appears for the presence of carbohydrate.
- **Fehling's test:** to the test sample, first 1ml of Fehling solution A and the 1ml of Fehling's solution B were added. Then the whole mixture was boiled. Red precipitate appears for the presence of carbohydrate.(Nielsen, S.S. et al. 2010)
- **Benedict's test:**In order to detect the production of a reddish-brown precipitate, which would indicate the presence of carbohydrates, the test solution was combined with a few drops of Benedict's reagent, an alkali solution containing a cupric citrate complex, and heated in a water bath.(Bhandary et al., 2012)
- **Barfoed's test:** 2ml of Barfoed's reagent was mixed with 1ml of test solution. Then the mixture was boiled. Brick red precipitate appears for the presence of monosaccharide.

5.6.8. Test for phenolic compounds

- **FeCl3 test:** The test solution received 3-4 drops of FeCl3. The creation of a bluish-black colour indicates the presence of phenol (Prakash et al., 2015).
- **Lead acetate test:** In the test tube, The extract was mixed with some drops of 10% lead acetate solution in the test tube. The production of a white precipitate shows the presence of phenols. (Babbar et al., 2011).

5.6.9. Test forAnthraquinones

- A test tube contains extract, chloroform, and 10% sulfuric acid. Mix thoroughly and let stand for 10-15 minutes. A positive result is indicated by a colour change in the lower chloroform layer, typically pink, red, or orange, indicating the presence of anthraquinones (Harborne & Dey, 1989).

5.6.10. Test for Protein test

- **Millon's Test:**Add Millon's reagent to 2-3 mL of protein solution, mix well, heat, and observe colour change (Plummer, D.T. et al. 1987).

- **Xanthoproteic test:**In a test tube, mix 2-3 mL of protein solution with concentrated nitric acid (HNO3), heat, and cool. Add excess ammonium hydroxide solution and observe any color change (Sadasivam, 1996).

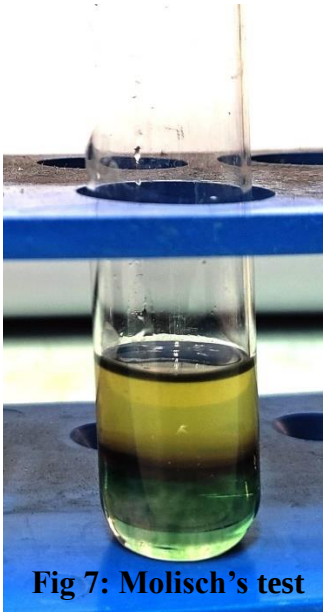


Fig 7: Molisch's test

Fig 8: Xanthoproteic test

5.7. Preparation and characterization of tincture

A total of 21 amber-colored bottles with 10 ml capacity were used. To these, 10 ml of alcohol with varied strengths—40, 50, 60, 70, 80, and 90 per cent (V/V) and absolute alcohol were added. Each of the twenty-one bottles contained 1g of *Excoecaria agallocha*bark powder. Each batch was allowed to macerate for a specific number of days: 7, 14, and 21. The materials were filtered through a sintered glass crucible (G4) while under reduced pressure after the required amount of maceration time. Physical characteristics were investigated, including color, pH, specific gravity, and total solid content (Barman S et al. 2024).

Figure 9: **Tinctures of different alcohol concentrations after maceration for differing periods**

Materials and Methods

6.1.In vitro Antioxidantstudy of Plant extract

Chemicals Used

Standard Ascorbic acid(C6H8O), Gallic acid (C7H6O5), Quercetin (C15H10O7), dibasic sodium phosphate, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), Sodium phosphate dibasic dihydrate (Na2HPO4.2H2O), Potassium dihydrogen phosphate (KH2PO4), Hydrogen peroxide (H2O2) (35% in water), α-glucosidase, 3, 5, di-nitro salicylic acid (DNS) and all other chemicals of analytical grade were obtained commercially.

6.1.1. DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging assay

Material and methods:

Preparation of 1M DPPH solution:

3.4 mg DPPH was dissolved in 100 ml methanol to produce 1M DPPH solution.

Preparation of dilutions of the plant extracts:

*E. agallocha*hydroalcoholic (AGL-HA) extract1mg was dissolved in 1ml methanol by the vortex. The solution was filtered through a 0.45µ filter (Millipore). From there, the dilution was prepared with the help of methanol. The IC50 value (50% inhibitory concentration) was calculated by nonlinear regression using GraphPad Prism version 10.3.0 software. IC50 was determined through nonlinear regression of the percentage relative activity f(concentration) plots.

Using the following formula, the DPPH scavenging capacity was determined:

Scavenging % = ((Absorbance control-Absorbance sample)/(Absorbance control))×100

Methodology:

Using a DPPH free radical scavenging experiment created in our lab, the antioxidant capacity of AGL-HA extract was evaluated. This 96-well microplate included an aliquot of 50 µL sample solution at various concentrations (50-100 µg/mL) and 950 µL of 1M DPPH methanolic solution. The reaction mixture was allowed to sit at 37°C in the dark for one hour. The extracts' capacity to scavenge free radicals was demonstrated by the removal of the original purple tint. The absorbance of the reaction mixture at 517 nm was measured using a UV-visible spectrophotometer.(Bhatti MZ et al. 2015). The positive control in this experiment was ascorbic acid. The above formula was used to obtain the inhibition percentage.

6.1.2.NO scavenging assay

A Nitric Oxide (NO) scavenging test is a typical method used to assess the capacity of compounds to neutralize nitric oxide radicals, which are reactive species involved in several biological processes and illnesses.

Material and methods:

Preparation of 50mM Phosphate buffer (pH 7.4)

0.8899g Na2HPO4.2H2O, 0.68045g KH2PO4 were dissolved in 100ml water and maintain pH 7.4.

Preparation of Nitrous Oxide Solution:

Dissolve sodium nitroprusside (Na2[Fe(CN)5NO) in PBS to prepare a working solution. This solution releases nitric oxide in a controlled manner.

Preparation of 1mg/ml extract solution:

AGL-HA extract 1mg was dissolved in 1ml methanol by the vortex. The solution was filtered through a 0.45µ filter (Millipore).

Griess Reagent Preparation:

Prepare the Griess reagent by mixing equal volumes of sulfanilamide and naphthylethylenediamine dihydrochloride in an acidic solution (typically phosphoric acid). This reagent reacts with nitrite to form a colored azo dye.

Methodology:

Napthyl ethylene diamine dihydrochloride (0.1% w/v) was often used in place of 1-napthylamine (5%), the Griess Illosvory reagent. After mixing 0.5 ml of saline phosphate buffer, 2 ml of sodium nitroprusside (10 mM) and 0.5 ml of hydroalcoholic (AGL) extracts (1000 µg/ml) or standard (ascorbic acid) solution, the reaction mixture was incubated for 150 minutes at 25°C. To finish the diazotisation process, 0.5 mL of the reaction mixture was mixed with 1 mL of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and incubated for an additional five minutes. Naphthalene diamine dihydrochloride (1 millilitre) was then added, mixed, and allowed to stand at 25°C for 30 minutes. The nitrate concentration was measured at 546 nm.
(Patel A et al. 2010). The positive control was ascorbic acid. The following formula determined the scavenging activity of the extract:

NO scavenging activity % = ((Absorbance control-Absorbance sample)/(Absorbance control))×100

6.1.3. Determination of Total Phenolic Content (TPC):

Principle:

The reduction of the Folin–Ciocalteu reagent (FCR) in the presence of phenolics is the basis of the widely used Folin–Ciocalteu (F–C) test for phenolic analysis. Molybdenum-tungsten blue is produced as a consequence, and its concentration increases linearly with the concentration of phenolics in the reaction media. It is detected spectrophotometrically at 760 nm. By lowering precipitate formation and raising the assay's reactivity, lithium sulfate is added to the reagent, which improves the FCR. The simple and popular F-C test may determine the total phenolics in fruits and vegetables. According to the technique, a test tube should be filled with 0.5 ml of deionized water, 0.125 ml of diluted fruit extracts, and 0.125 ml of FCR and left to react for six minutes—next, 1.25 millilitres of a sodium carbonate solution (7%) (Malta & Liu, 2014).

Material and Methods:

Bioactive substances that provide health advantages are called phytochemicals, especially phenolics in plants. These substances have various biological effects, including antioxidant activity, and may be found in edible and inedible plant components. Their redox properties allow them to scavenge, which allows them to serve as singlet oxygen quenchers, reducing agents, and hydrogen donors. In addition, natural antioxidants have antiviral, antibacterial, anti-inflammatory, antiallergic, antithrombotic, and vasodilatory properties. Researchers and food makers are becoming more interested in the potential of these phytochemicals for maintaining health and protecting against cancer and coronary heart disease (Babbar et al., 2011).

Preparation of Folin cio-calteau phenol reagent :

Dilute the Folin-Ciocalteu concentrate with deionized water. A common dilution ratio is 1:10. The solution is mixed thoroughly. Adjust the pH to around 10-11.

Preparation of standard gallic acid dilutions:

Gallic acid with a 95% purity level was used to create standard dilutions. A methanol solution containing 1 mg/ml was produced, and then six dilutions of 1000, 500, 250, 125, 62.5, and 31.25 µg/ml were serially diluted.

Preparation of % $NaHCO_3$ solution:

Measure 7.5 grams of sodium bicarbonate.Add the sodium bicarbonate to 250 mL of deionized water. Stir until completely dissolved. This forms a 0.2 M solution.

Preparation of 1mg/ml extract solution:

AgL-HA extract 1mg was dissolved in 1ml methanol by the vortex. The solution was filtered through a 0.45µ filter (Millipore).

Methodology:

Folin-Ciocalteu reagent (10 times diluted with de-ionized water) and 0.1 mL aliquot of various extracts (4 mg/mL) were mixed in a 96-well plate. Stir the liquid and let it sit at room temperature for five minutes before adding 0.75 mL of 6% sodium carbonate. After a 90-minute procedure, the absorbance at 725 nm was measured. The typical calibration curve (0–25 µg/mL) was created using gallic acid. The whole phenolics were expressed in milligrams of gallic acid

equivalent per gramme of dry weight. Negative control was provided by substituting 0.1 mL of DMSO for the extract.(Bhatti et al., 2015)

6.2.4. Determination of Total flavonoid content(TFC):

Principle:

Plant extracts were tested for TFC using the aluminium chloride colourimetry technique. This approach uses Al (III) as a complexing agent, forming Al(III)-flavonoid chelates. Depending on the pH level and the circumstances of the experiment, flavonoids have a considerable propensity to bind metal ions, usually at a 1:1 ratio (Shraim et al., 2021)

Preparation of NaNO2 solution:

Measure 0.1 grams of sodium nitrite. Add the sodium nitrite to 100 mL of deionized water. Stir until completely dissolved. Ensure the solution is homogeneous.

Preparation of AlCl3 solution:

Measure 0.5 grams of aluminum chloride. Add the aluminum chloride to 50 mL of deionized water. Stir until completely dissolved. This results in a 10% (w/v) solution of $AlCl_3$. nsure the solution is well-mixed to achieve uniformity.

Preparation of NaOH solutions:

For prepare a 1 M solution, weigh 40 grams of NaOH pellets.Slowly add the NaOH pellets to 1 liter of deionized water while stirring continuously.

Preparation of 1mg/ml extract solution:

AGL-HA extract 1mg was dissolved in 1ml methanol by the vortex. The solution was filtered through a 0.45µ filter (Millipore).

Methodology:

1. 5 mL of methanol and 0.5 mL of various extracts (1 mg/mL) were mixed together on a 96-well plate. 2.8 mL of distilled water, 0.1 mL of potassium acetate (1 M), and 0.1 mL of 10% aluminium chloride were then added. The mixture was allowed to come to room temperature for thirty minutes. An absorbance measurement was made of the reaction mixture at 415 nm. Using rutin as the standard, the calibration curve (0–8 µg/mL) was drawn. Milligrams of rutin equivalent per gramme of dry weight was used to represent the flavonoid content. (Bhatti et al. 2015).

6.2. Evaluation of in vitro antidiabetic potential

6.2.1. α amylase test

Principle:

α-amylase enzymes catalyze the hydrolysis of α-1,4-glycosidic linkages in starch and similar polysaccharides. One necessary biochemical approach to evaluate the activity of these enzymes is the alpha-amylase in vitro test. Monitoring when substrate vanishes or when products emerge under carefully regulated circumstances quantifies this process. Typical substrates include specific synthetic substrates, starch, amylose, and amylopectin. The assay's sensitivity and specificity may be impacted by the substrate used. The Dinitrosalicylic Acid (DNS) Method, the Iodine-Starch Reaction, the Chromatometric Substrate Method, and the Turbidimetric Method are examples of colourimetric techniques. Through the conversion of insoluble starch into soluble products, these techniques assess the decrease in turbidity, the reduction in sugars generated by the enzyme, and the intensity of the blue colour(Souza, 2010).

Preparation of 100mM phosphate buffer pH 6.8:

1.7798g Na2HPO4.2H2O, 1.3609g KH2PO4 were dissolved in 100ml water and maintain pH 6.8.

Preparation of α-amylase solution:

To preparing a 1 mg/mL solution and need 50 mL, weigh 50 mg of alpha-amylase.Add the weighed alpha-amylase to a beaker containing 50 mL of phosphate buffer. Stir until completely dissolved.

Preparation of DNS solution:

Add 1 gram of DNS and 30 grams of sodium potassium tartrate to 100 mL of 1 M sodium hydroxide solution in a beaker.Stir until the DNS and sodium potassium tartrate are completely dissolved.Transfer the solution to a 1-liter volumetric flask and add deionized water to make up the volume to 1 liter. Mix thoroughly.

Preparation of 1mg/ml extract solution:

AGL-HA extract 1mg was dissolved in 1ml methanol by the vortex. The solution was filtered through a 0.45µ filter (Millipore).

Methodology:

The conventional procedure was followed with a bit of modification to determine the extract's and fractions' alpha-amylase inhibitory activity. A reaction mixture consisting of 50 µl phosphate buffer (100 mM, pH = 6.8), 10µl α-amylase (2 U/ml), and 20 µl of extract and fractions at different concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml) was preincubated for 20 minutes at 37°C in a 96-well plate. After adding 20µl of 1% soluble starch (100 mM phosphate buffer, pH 6.8) as a substrate, the mixture was further incubated for 30 minutes at 37°C. Next, 100µl of DNS colour reagent was added, and the mixture was boiled for 10 minutes—the mixture's absorbance after mixing. Using a Multiplate Reader, the measurement was made at 540 nm. The

standard was acarbose at a range of concentrations (1 mg/ml). The findings were presented as a percentage of inhibition (Telagari M et al. 2015). The formula was used to compute the % inhibition, which was used to express the results-

Inhibitory activity (%) = (1-AS/AC) ×100

Where,

AS = absorbance in the presence of test substance

AC = absorbance of control

6.2.2. α glucosidase:

Principle:

α-glucosidase in vitro assays are an essential biochemical method for determining the activity of α-glucosidase enzymes, and they are especially significant in the fields of food science, medication development, and diabetes research. The release of glucose or a chromogenic/fluorogenic group from a particular substrate under regulated circumstances is measured to quantify the activity. p-Nitrophenyl-α-D-glucopyranoside (pNPG), 4-Methylumbelliferyl-α-D-glucopyranoside, maltose, and other naturally occurring oligosaccharides are examples of common substrates. Among the measurement techniques are the glucose oxidase-peroxidase system for natural substrates, spectrophotometric measurement of p-nitrophenol using pNPG, and fluorometric quantification of 4-methylumbelliferone using 4-methylumbelliferyl-α-D-glucopyranoside (Daou et al., 2022).

Preparation of 100mM phosphate buffer pH 6.8:

1.7798g Na2HPO4.2H2O, 1.3609g KH2PO4 were dissolved in 100ml water and maintain pH 6.8.

Preparation of glucosidase solution:

For preparing a 1 mg/mL solution weigh 50 mg of alpha-glucosidase enzyme.Add the weighed enzyme to 50 mL of phosphate buffer. Stir until completely dissolved.

Preparation of 0.1M Na2CO3 solution:

Weigh out 10.6 grams of sodium carbonate. Add the weighed sodium carbonate to a beaker containing approximately 800 mL of deionized water. Stir until completely dissolved. Transfer the solution to a 1-liter volumetric flask. Add deionized water to bring the total volume up to 1 liter. Mix thoroughly.

Preparation of 1mg/ml extract solution:

AGL-HA extract 1mg was dissolved in 1ml methanol by the vortex. The solution was filtered through a 0.45µ filter (Millipore).

Methodology:

The standard protocol was utilized with minor adjustments to ascertain the extract and fractions' α-glucosidase inhibitory activity. A 96-well plate was preincubated for 15 minutes at 37°C with a reaction mixture that included 50 µl phosphate buffer (100 mM, pH = 6. 8), 10 µl-glucosidase (1 U/ml), and 20 µl extract and fractions at various concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml). Following the addition of 20 µl P-NPG (5 mM) as a substrate, the mixture was incubated at 37°C for a further 20 minutes. 50 µl Na2CO3 (0.1 M) was added to stop the reaction. The absorbance of the p-nitrophenol that was released at 405 nm was measured using the Multiplate Reader. Various acarbose dosages (0.1–0.5 mg/ml) were utilised as a benchmark. It was (Telagari & Hullatti, 2015).The formula was used to compute the % inhibition, which was used to express the results.

-

Inhibitory activity (%) = $(1-A_S/A_C) \times 100$
Where,
A_S = The absorbance in the presence of the test substance
A_C= Theabsorbance of control

6.3. In vivo Animal Study:

Material and Methods:

6.3.1. Animals

The optimal study employed 7-8 weeks of Wistar albino rats of either sex that weighed 140-160 g. Rats were maintained in a conventional laboratory setting with 12-hour light/dark cycles, a temperature of 25-28 °C, and a relative humidity of 55-60%. They were fed a high-fat diet, which contained 30% fat, 20-30% protein, 35% carbohydrate, and 25% fructose, and they were given access to water as needed. Prior to the trial, the animals spent a week becoming used to the lab environment.

6.3.2. Drugs and chemicals:

We bought streptozotocin from HI Media Laboratories Pvt Ltd. India, and we got the standard drug metformin from USV Private Limited in Himachal Pradesh. The reagent kits used were from Span Diagnostics Ltd. India. The suppliers of trichloroacetic acid (TCA), thiobarbituric acid (TBA), phenazonium methosulfate (PMS), 5,5'-dithio bis-2-nitro benzoic acid (DTNB), nicotinamide adenine dinucleotide (NADH), and reduced glutathione (GSH) were SISCO Research Laboratory, Mumbai, India. The glacial acetic acid and potassium dichromate were from Ranbaxy, Mumbai.

6.3.3. Induction of diabetes in rats:

A single intraperitoneal injection of 40 mg/kg streptozotocin, prepared in 0.1 mol/L citrate buffer at pH 4.4, was given to rats. Diabetic animals were employed in tandem with age-matched control rats that were given citrate buffer. Blood samples were taken two days following the STZ injection, and the Accu-check Counter TS diagnostic kit technique (Span Diagnostic, Surat, India) was used to determine the plasma glucose levels. In the current investigation, rats having plasma glucose levels more than 250 mg/dl after four weeks were chosen.

6.3.4. Treatment schedule:

The rats were continued with high-fat diets throughout the study. The animals were divided into Five groups (n = 6) and received the treatment for 28 days.

Experimental groups and dose schedule:

- Group I (NC): Normal control group (Rats on normal diet).
- Group II (DC): Disease control group + Streptozotocin (STZ) (Rats fed with HFD daily for 28 days).
- Group III (Low): HFD rats + STZ +*Excoecaria agallocha* extract (AGL-HA) low dose (200 mg/kg body weight) orally daily for two weeks.
- Group IV (High): HFD rats + STZ +AGL-HA high dose (400 mg/kg body weight) orally daily for two weeks.
- Group V (STD): Standard control group (HFD rats + STZ + Metformin 250 mg/kg body weight) orally daily for two weeks.

6.3.5. Estimation of fasting blood glucose (FBG) level

Using a one-touch glucometer, the FBG level was tested on the 0th, 7th, 14th, 21st prime, and 28th day. All rats in each group had their blood drawn by heart puncture for the purpose of estimating the serum lipid profile (LDL, VLDL, TC, TG) and serum biochemical parameters (SGOT or AST, SGPT or ALT, De-ritis ratio) after the 21st hour of the last dosage and 18 hours of fasting. The animals were then killed in order to obtain liver tissue for the purpose of measuring various endogenous antioxidant parameters.

6.3.6. Body weight:

On days 0 through 28, the body weight of the rats in each group was measured. To ensure accuracy, the weight was measured using a conventional digital weight balance.

6.3.7. Serum Liver Function Test

Numerous serum biochemical parameters, such as serum glutamic pyruvic transaminase (SGPT) or alanine transaminase (ALT), and serum glutamic oxaloacetic transaminase (SGOT) or aspartate aminotransferase (AST), were measured in the collected blood. Commercially accessible kits from Span Diagnostics Ltd., India were used for all of the analyses.

6.3.8. Serum lipid profiles

Using commercial kits from Span Diagnostics Ltd. India, serum lipid profiles such as total cholesterol, HDL cholesterol, triglycerides, and LDL cholesterol were measured in STZ-induced diabetic rats.

6.3.9. Sacrifice and collection of livers

The deaths occurred during the conclusion period for each group. The rats were fasted for eight hours before being humanely killed with overdose ofketamine and xylazine at the end of the intervention period. Their livers were removed and cleaned with regular saline. Each liver had a piece of around 5 mm removed, which was then preserved in 10% neutral buffered formalin for histology. Five milliliters of sodium phosphate buffer (pH 7.5; 50 mM) containing 10% Triton X100 was used to homogenize 0.5 grams of each heart that was removed. After homogenization, the tissue samples were centrifuged for 10 minutes at 4°C at 15,000 rpm. After being decanted into Eppendorf tubes with labels, the supernatants were kept at -20◦C until further analysis could be performed.

6.3.10. Histopathological Studies

Hematoxylin and eosin staining, oil-o-red, and standard paraffin tissue processing were applied to liver tissues that had been preserved in 10% normal saline. A Digital Bright-field Microscope (OMAX, USA) was used to view the slides, and digital photomicrographs were taken.

6.3.11. Estimation of Liver Antioxidant Stress

Catalase activity, lipid peroxidation, and reduced glutathione (GSH) levels were among the oxidative stress indicators measured in tissue supernatants.

6.3.12. Reduced glutathione (GSH) level

The GSH level was ascertained using Ellman's method. To summaries, 100 µL of the tissue supernatant was deproteinized with 300 µL of 10% TCA, and the resulting combination was centrifuged at 3500 rpm for five minutes. After that, a 96-well plate was pipetted with 200 µL of the deproteinized samples and 50 µL of Ellman's solution, and it was incubated for five minutes. At 415 nm, the absorbance was measured. Utilizing a GSH standard curve, the GSH level was determined. (Gl, 1959)

6.3.13. Catalase activity

The technique that was previously developed was used to determine the catalase activity. In summary, 340 µL of 50 mM sodium phosphate buffer (pH 7.0) was combined with 100 µL of the supernatants. After that, the mixture was mixed with 150 µL of 2 M H_2O_2. For three minutes, absorbance was measured at 240 nm at one-minute intervals (Aebi, 1984).

6.3.14. Lipid peroxidation levels

The thiobarbituric acid reactive substances (TBARS) were measured and expressed as malondialdehyde (MDA) levels using the previously mentioned procedure. In short, 100 µL of the supernatant was mixed with 100 µL of 8.1% SDS solution, 375 µL of 20% acetic acid, and 1 mL of 0.25% thiobarbituric acid (TBA) in a reaction mixture. After one hour of boiling, 200 µL of the heated liquid was pipetted onto a 96-well plate, and the absorbance was measured at 532 nm. An MDA standard curve was used to extrapolate MDA values (Chowdhury & Soulsby, 2002).

6.3.15. Statistical Analysis

Each and every result is displayed as mean ±SEM. Graph Pad Prism 10.3.0 (Graph Pad software, USA) was used to analyze the data for statistical significance using one-way analysis of variance (ANOVA) and post hoc Dunnett's test. P values were deemed statistically significant when they were <0.05.

RESULTS

7.1. Physicochemical evaluation

The physicochemical evaluation of plants involves assessing both physical and chemical properties to understand their composition, quality, and potential applications. The below results evaluate the potential physiochemical parameters of Excoecaria agallocha.

Table 3: Physicochemical evaluation of *Excoecaria agallocha*

Standardization parameters	**Value (%w/w)**
Moisture content	43.73 ± 1.26
Ash value	
Total ash value	16.00± 1.98
Acid insoluble ash value	2.75± 0.27
Water soluble ash value	13.35± 0.86

Values are mean ± SD, (n= 3)

Extractive value (%w/w):-	
Solvent	**Extractive value (gm/Kg)**
Ethyl Acetate	51.39
Hydroalcoholic	146.50

7.2. Table 4: Preliminary phytochemical screening of *Excoecaria agallocha* hydroalcoholic extract

S.NO		PLANT EXTRACT	Hydro-alcoholic Extract	methanolic Extract
1.	Alkaloids	Mayer's reagent	Positive	Positive
		Hager's reagent	Positive	Positive
		Wagner's reagent	Positive	Positive

		Dragendorff's reagent	Positive	Positive
2.	Phenol phenolic compounds	$Fecl_3$	Positive	Positive
		Lead acetate test	Positive	Negative
3.	Saponin	Frothing test	Positive	Positive
4.	Carbohydrate	Molisch test	Positive	Positive
		Fehling's solution	Positive	Positive
		Benedict's test	Positive	Positive
		Barfoed's test	Positive	Positive
5.	Protein and amino acids	Millon's test	Negative	Negative
		Xantho Protein test	Negative	Negative
6.	Glycosides	Bontrager's test	Positive	Positive
		Kellar Kiliani test	Positive	Positive
7.	Flavonoids	Lead acetate solution test	Positive	Positive
		Shinoda test	Positive	Positive
8.	Phytosterols	Liebermann-Burchard test	Positive	Negative
9.	Tannins	Ferric chloride test	Positive	Positive
		Lead acetate test	Positive	Positive
10.	Anthraquinones		Positive	Negative

7.3. Observation of absorbance in Total Phenolic estimation

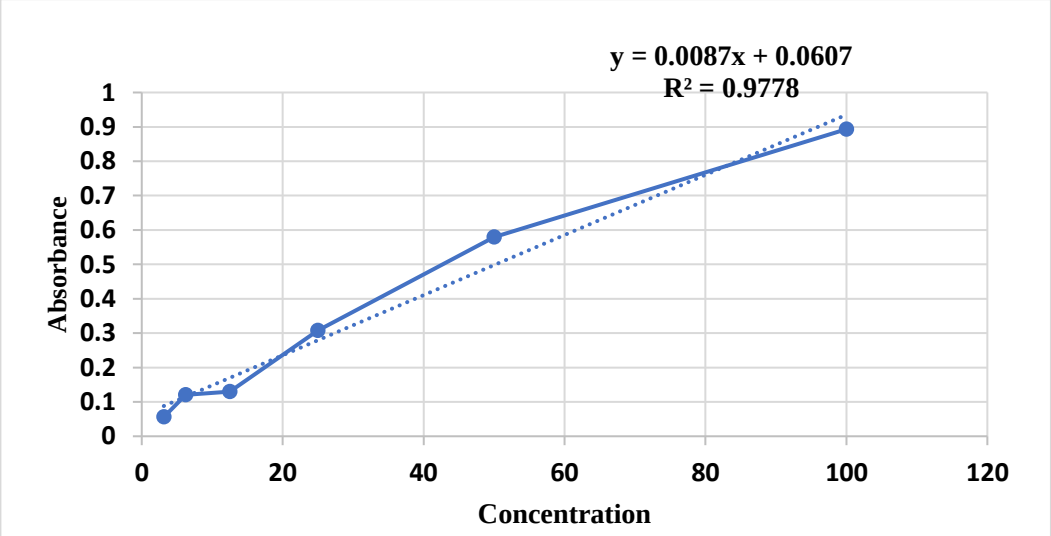
The absorbance of the Standard and test solution was represented in **table5** The total phenolic content in terms of mg Gallic Acid Equivalent (GAE) of Hydroalcoholic extract was found to be 131.18 mg/g. These results suggest that the higher presence of phenolic components was responsible for the levels of antioxidant activity. The standard curve for gallic acid is shown in the **figure 10**.

Table 5: Observation table for TPC

Sl No.		Concentration(µg/ml)	Absorbance
1	STANDARD (Gallic acid)	3.125	0.055
2		6.25	0.012
3		12.5	0.012
4		25	0.030
5		50	0.057
		100	0.893
1	SAMPLE (Hydroalcoholic extract)	1000	1.202

Values are mean ± SD, (n= 3)

Figure 10: Standard curve of Gallic acid



7.4. Observation of absorbance in total flavonoid estimation

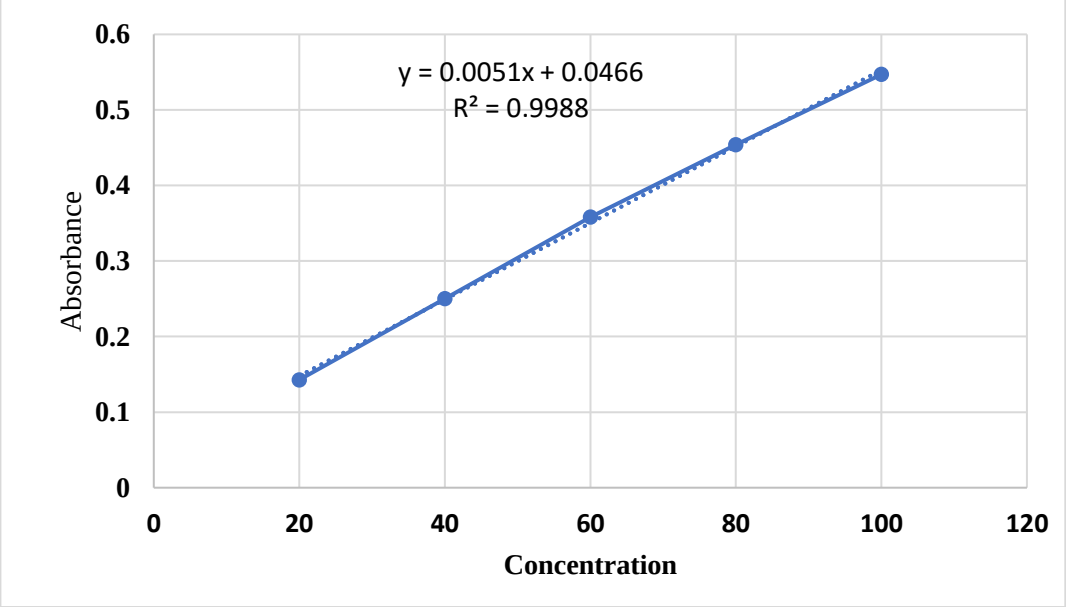
The content of the flavonoid compound in the Hydroalcoholic extract of *Excoecaria agallocha* barkwas measured by aluminium chloride reagent in terms of quercetin equivalent. It was found

to be 46.20mg/g. The absorbance of the Standard and test solution was represented in **table 6**. It is well known that flavonoids have significant antioxidant activity and have a positive impact on human nutrition and health. The ethyl acetate extract contains a substantial quantity of flavonoids, which may contribute considerably to the plant's antioxidant activity. The Standard curve of Quercetin is shown in **Figure 11**.

Table 6:

Sl No.		**Concentration(µg/ml)**	**Absorbance**
1	STANDARD(QUERCETIN)	1000	0.145
2		500	0.085
3		250	0.048
4		125	0.025
5		62.5	0.019
1	SAMPLE (Hydroalcoholic extract)	1000	0.281667

Figure 11: Standard curve of Quercetin



7.5. Characterization of tincture

The different characteristics of the prepared tincture are shown in table 7, 8, and 9. The pH of the prepared tincture to varying strengths of alcohol is represented in table 7. The study shows that greater alcohol concentration in the tincture increases the acidity of the tincture, while storage for a more extended period also slightly increases acidic properties. The solid content of the tincture also decreases with time, as seen from table 8, while greater alcohol concentration also imparts diminished solid content. Finally, as seen in table9, the specific gravity of the prepared tinctures does not vary substantially with time.

Table 7: pH of the tincture

Alcohol Strength	**pH**		
	7 Days	**14 Days**	**21 Days**
40%	6.03	5.65	5.33
50%	5.90	5.79	5.64
60%	5.31	5.66	5.49
70%	5.73	5.82	5.50
80%	5.70	5.60	5.45
90%	5.35	5.56	5.69
Absolute(100%)	5.35	5.23	5.09

Table 8:Total Solid Content of Tincture

Alcohol Strength	7 Days	14 Days	21 Days
40%	19.76	17.23	14
50%	17.02	15.34	12.53
60%	16.90	15.39	12.30
70%	16.97	14.83	10.38
80%	15.69	13.75	10.41
90%	12.34	13.88	10.16
Absolute(100%)	9.92	7.14	5.50

Table 9: Specific Gravity of Tincture

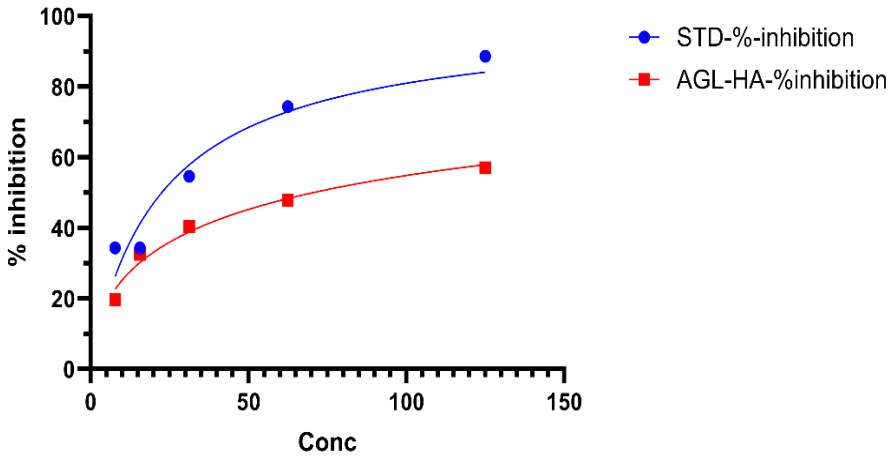
Alcohol Strength	7 Days	14 Days	21 Days
40%	0.972	0.943	0.954
50%	0.932	0.914	0.964
60%	0.900	0.912	0.994
70%	0.778	0.893	0.991
80%	0.887	0.895	0.901

90%	0.844	0.888	0.921
Absolute(100%)	0.779	0.799	0.802

7.6. Percentage Inhibition of DPPH radicals:

The decrease in DPPH absorbance at 517 nm caused by antioxidants was utilised to determine its capability for reduction. When DPPH free radicals were present in large concentrations, the extract demonstrated its highest capacity to donate hydrogen. The extract has an IC50 value of 70.52µg/ml, indicating antioxidant activity. On the other hand, the DPPH radical showed an IC50 value of 22.56 µg/ml for the well-known antioxidant ascorbic acid. When compared to ascorbic acid, E. agallocha exhibited considerable scavenging effects with increasing concentration. The results of the DPPH scavenging activity are shown below in Figure 11, representing the DPPH radical scavenging assay of standard and test sample.

Figure 11: DPPH radical scavenging assay

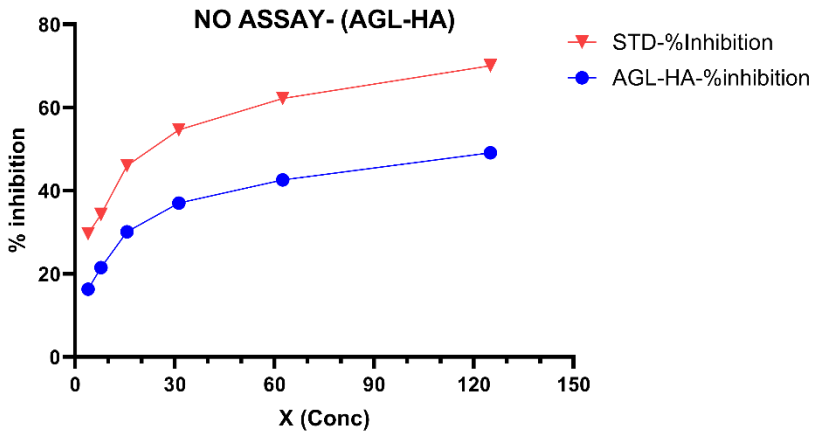


7.7. PercentageInhibition of Nitric oxide radicals

In an aerobic environment, nitric oxide is an extremely unstable species. Using NO2, N2O4, and N3O4 as intermediates, it combines with O2 to yield the stable products nitrates and nitrite. With the Griess reagent, an estimate is obtained. Nitrous acid concentration falls in the presence of test

chemical, a scavenger. The degree of reduction corresponds to the degree of scavenging.The IC50 values of AGL-HA extract and ascorbic acid were 120.6 µg/ml and 23.06 µg/ml, respectively. The results showed that the test sample was adequate for scavenging Nitric oxide in a dose-dependent manner. The outcome is comparable to that of the ascorbic acid reference standard. Figure 12

Figure 12: Inhibition graph of Nitric Oxide radical scavenging ASSAY

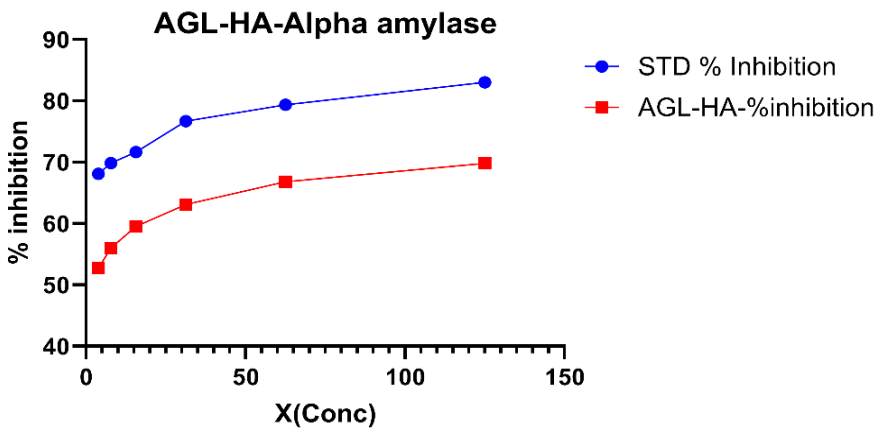


7.8. Determination of *in-vitro* Antidiabetic Activity:

7.8.1. α-amylase inhibition assay:

α-amylase is one of the enzymes that hydrolyses the α-1,4 glycosidic linkages in starch to produce glucose and maltose. α-amylase hydrolyses complex polysaccharides into oligosaccharides and disaccharides and then hydrolyses by α-glucosidase to monosaccharides. The in vitro antidiabetic activity was evaluated by using α amylase inhibitory assay. The hydroalcoholic extract of *E. agallocha* produces some inhibitory effects on this enzyme. Acarbose was used as a standard, which shows marked inhibitory effect of the enzyme (my thesis ref). The IC50 of Acarbose and hydroalcoholic extract of *E. agallocha* was calculated and found to be 0.1985 µg/ml and 2.440 µg/ml, respectively. Figure 13 represents the graph of α-amylase inhibition assay of Acarbose and test sample.

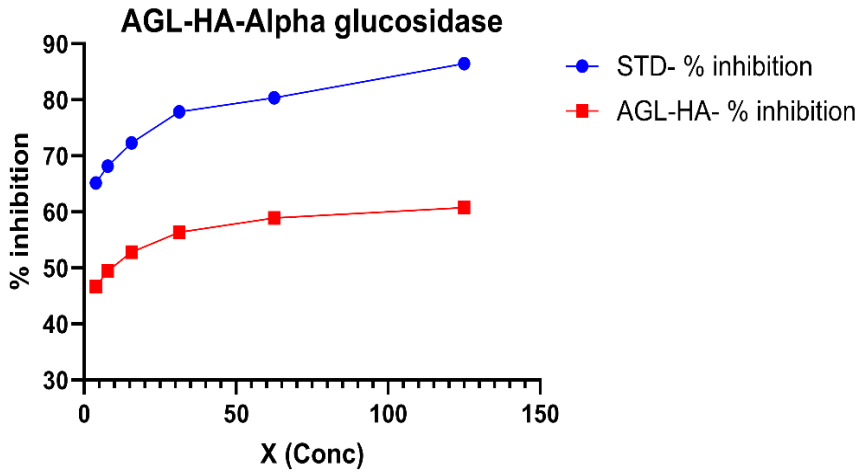
Figure 13: Graph of α-amylase inhibition assay of Acarbose and AGL-HA sample



7.8.2. α-glucosidase inhibition assay

The α-glucosidase assay is used to evaluate the inhibition of the enzyme α-glucosidase, which plays a key role in carbohydrate digestion. Inhibitors of α-glucosidase can help manage postprandial blood glucose levels, making this assay valuable in diabetes research and drug development.The hydroalcoholic extract of *E. agallocha* produces some inhibitory effects on this enzyme. Acarbose was used as a standard, which shows marked inhibitory effect of the enzyme. The IC50 of Acarbose and hydroalcoholic extract of *E. agallocha* was calculated and found to be 0.6816 µg/ml and 8.246 µg/ml, respectively. Figure: 14 represents the graph of α-amylase inhibition assay of Acarbose and test sample.

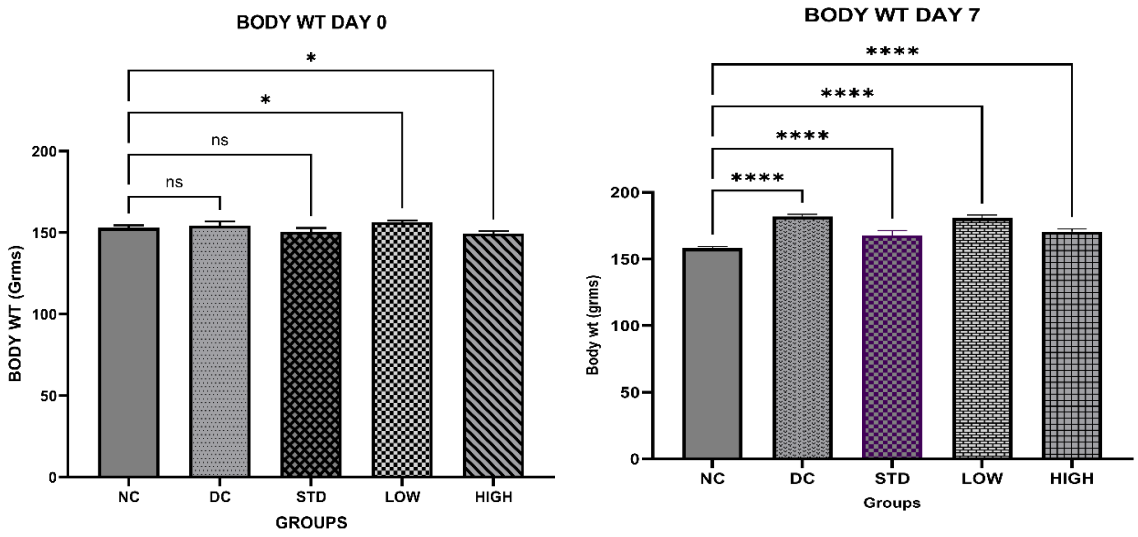
Figure 14: Graph of α-glucosidase inhibition assay of Acarbose and AGL-HA sample

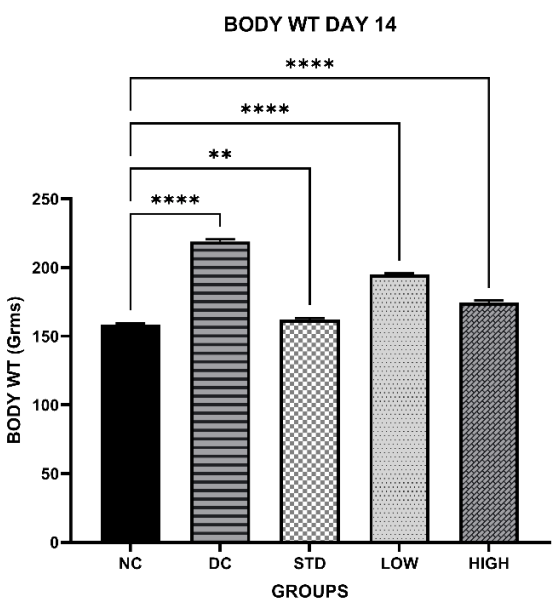


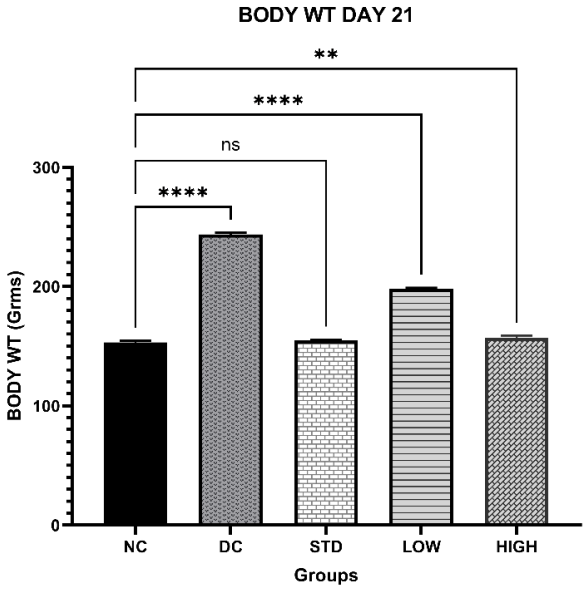
7.9. Effect of AGL-HA extracton body weight

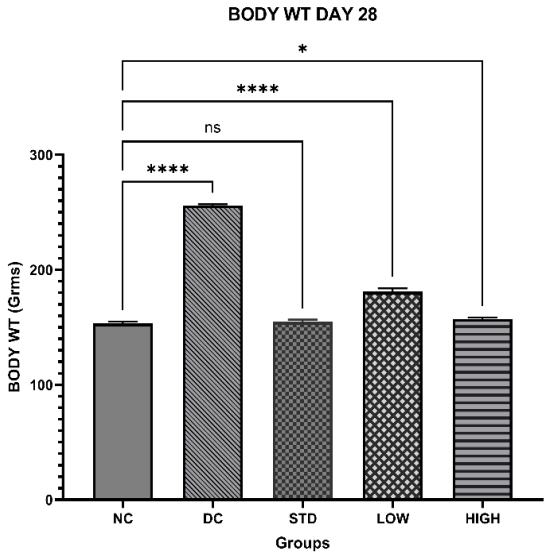
The final body weights were significantly ($p < 0.05$) decreased in the diabetic control group as compared to the normal control group. Administration of AGL at the doses of 200 and 400 mg/kg significantly ($p<0.05$) improved the body weight when compared to the diabetic control group.

Fig 15: Significant difference in body weight level at different days









7.10.Effect of AGL-HA extract on fasting blood glucose level

Rats given STZ to induce diabetes had considerably ($p < 0.05$) higher FBG levels than the healthy control group. When FRHE was administered to diabetic rats at dosages of 200 and 400 mg/kg, the FBG level was dramatically ($p < 0.05$) lowered towards normal in comparison to the diabetic control group.

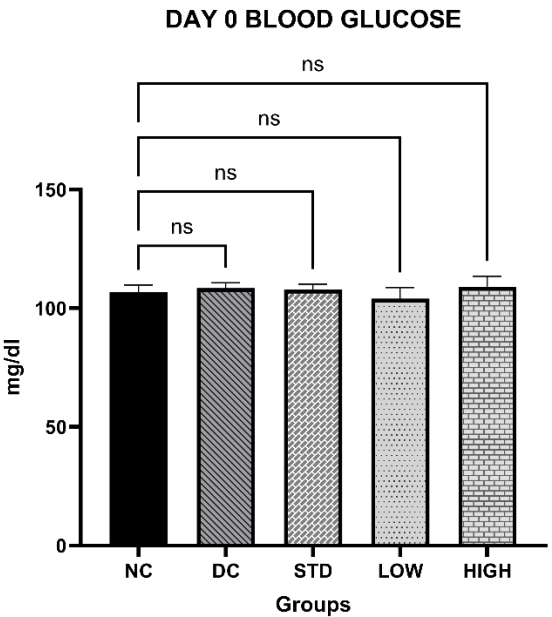
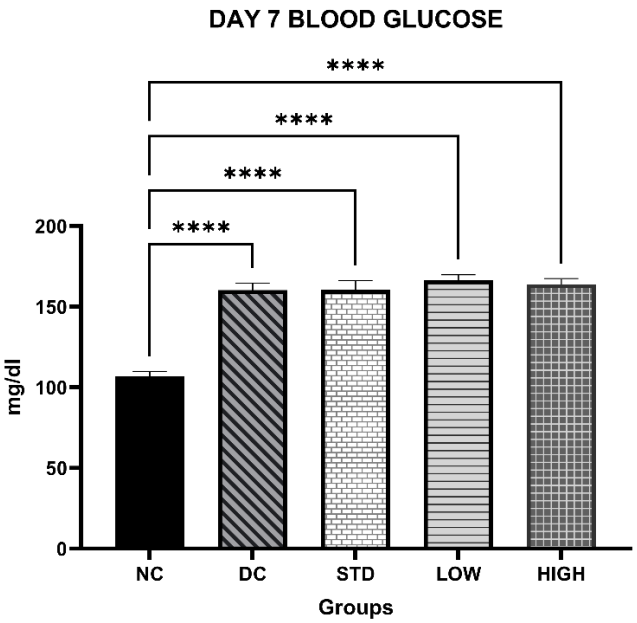
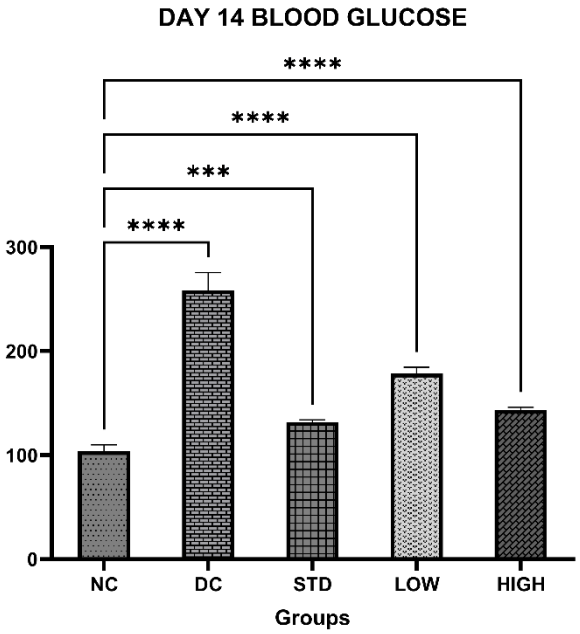
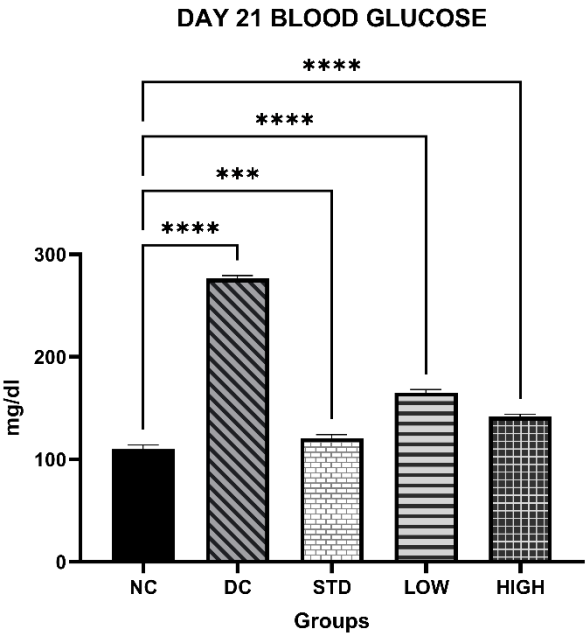
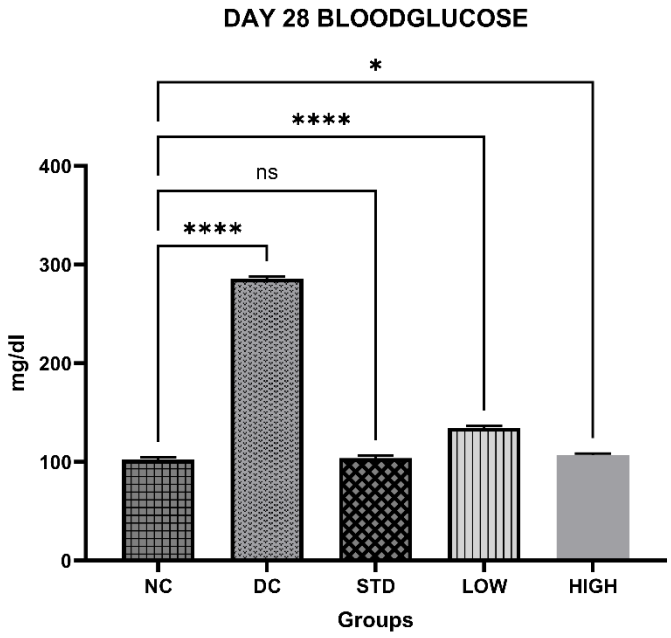


Fig 16: Significant difference in blood glucose level at different days


DAY 7 BLOOD GLUCOSE
****
****
****
****
200
150
100
50
0
mg/dl
NC
DC
STD
LOW
HIGH
Groups


DAY 14 BLOOD GLUCOSE
****
****
***
****
300
200
100
0
NC
DC
STD
LOW
HIGH
Groups


DAY 21 BLOOD GLUCOSE
****
****
***
****
300
200
100
0
mg/dl
NC
DC
STD
LOW
HIGH
Groups

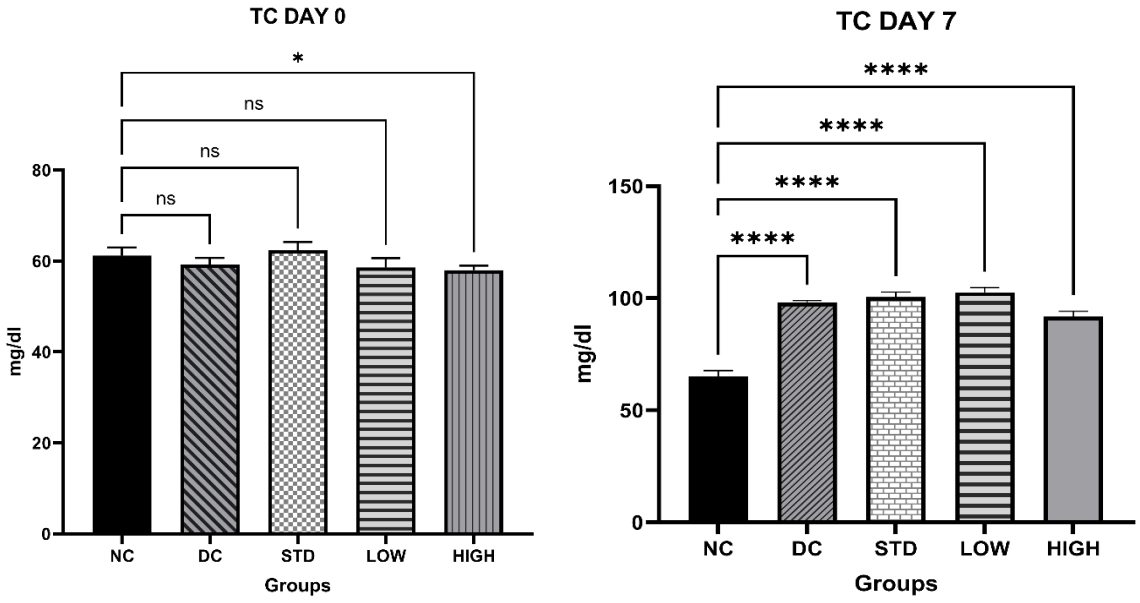


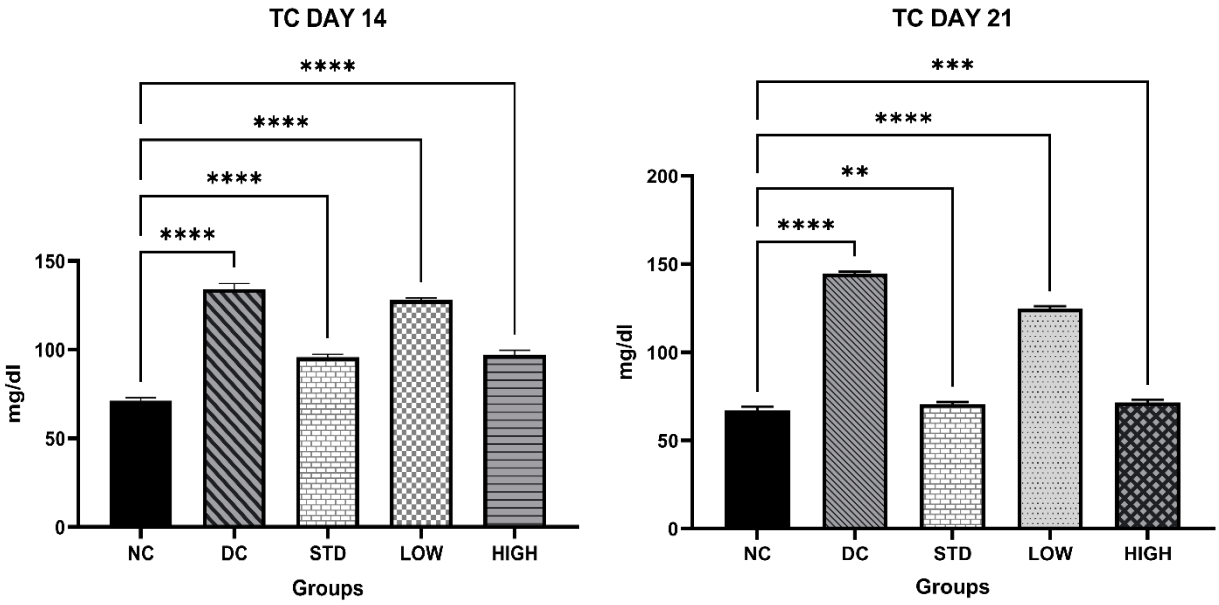
7.11. Estimation of serum lipid profiles:

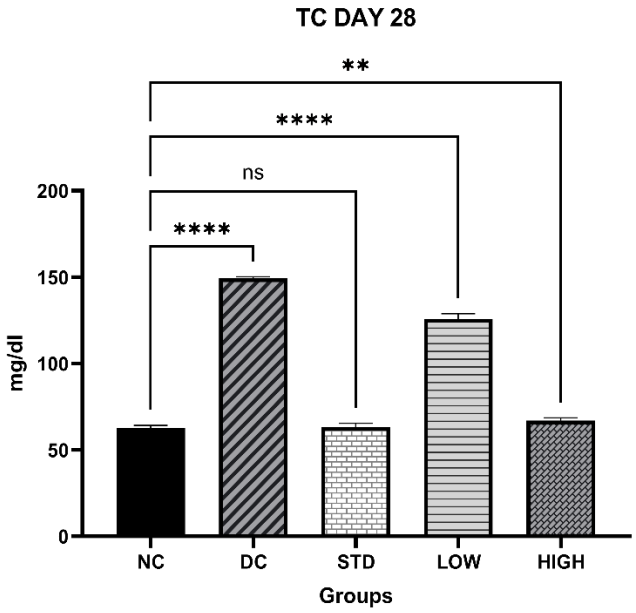
In STZ-induced diabetic mice, serum lipid profiles, including total cholesterol, triglycerides, and HDL cholesterol, were considerably ($p < 0.05$) raised. Level dropped considerably ($p < 0.05$) in comparison to the usual control group. When compared to the diabetic control group, treatment with AGL at the dosages of 200 and 400 mg/kg considerably ($p <0.05$) decreased the total TC, TG, LDL, and VLDL and significantly (p - 0.05) improved the HDL level.

7.11.1. Effect of AGL-HA extract on TC

Fig17: Significant difference in TC level at different days







7.11.2. Effect of AGL-HA extract on TG

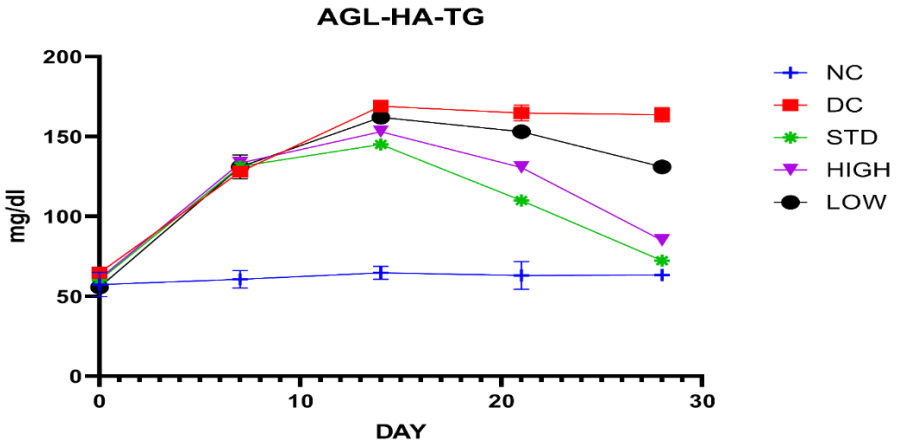
Table 10: TG Data mean and SD

AGL-arun	NC	DC	STD	HIGH	LOW
DAY 0	57.33±7.57	64.67±1.52	60±2	61±1.73	55.67±2.08
DAY 7	60.67±5.50	128±1.73	131.33±2.51	133.33±0.57	131±7.54
DAY 14	64.67±4.04	169±1	145±2.62	153±2.64	162±3
DAY 21	63±8.66	164.67±5.03	122±2.64	130.67±3.05	153±3

DAY 28	63.33±1.52	163.67±4.5	72.33±2.08	85.67±2	131±2.64

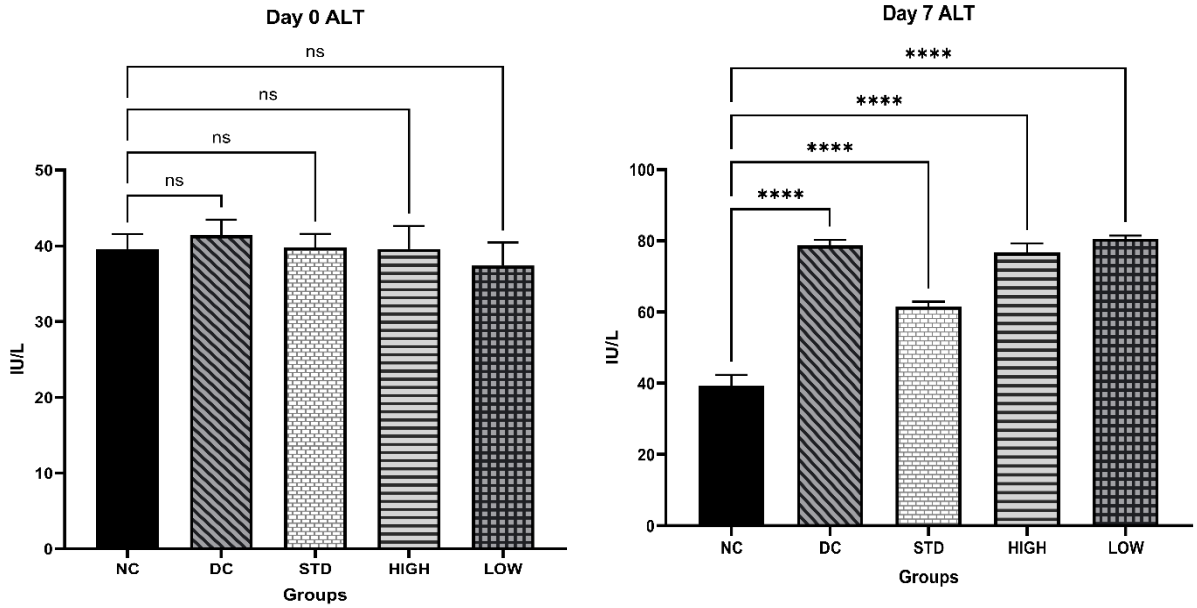
Values are mean ± SD, (n= 5)

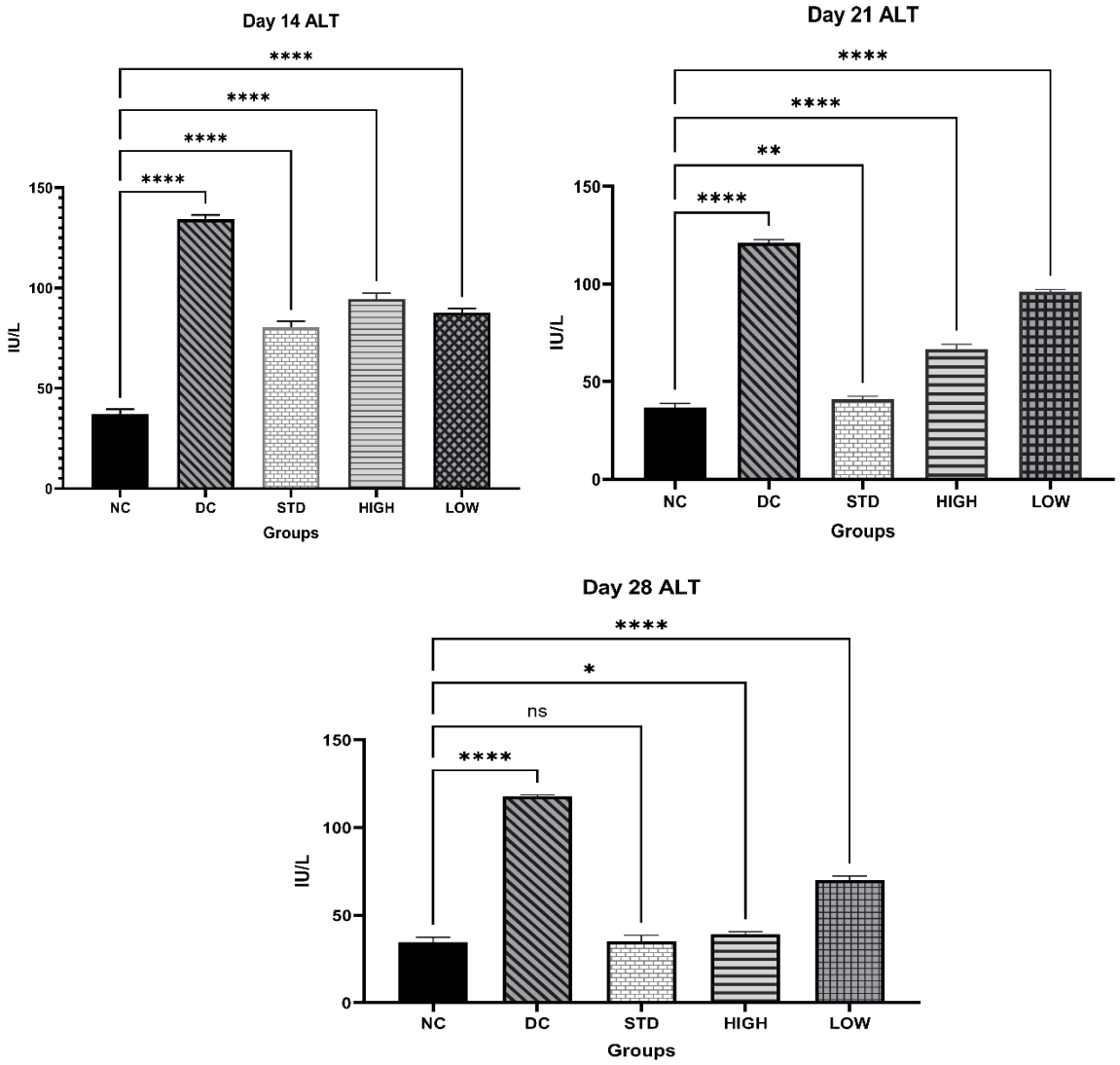
Fig 18: Line diagram of TG data



7.11.3. Effect of AGL-HA extract on ALT

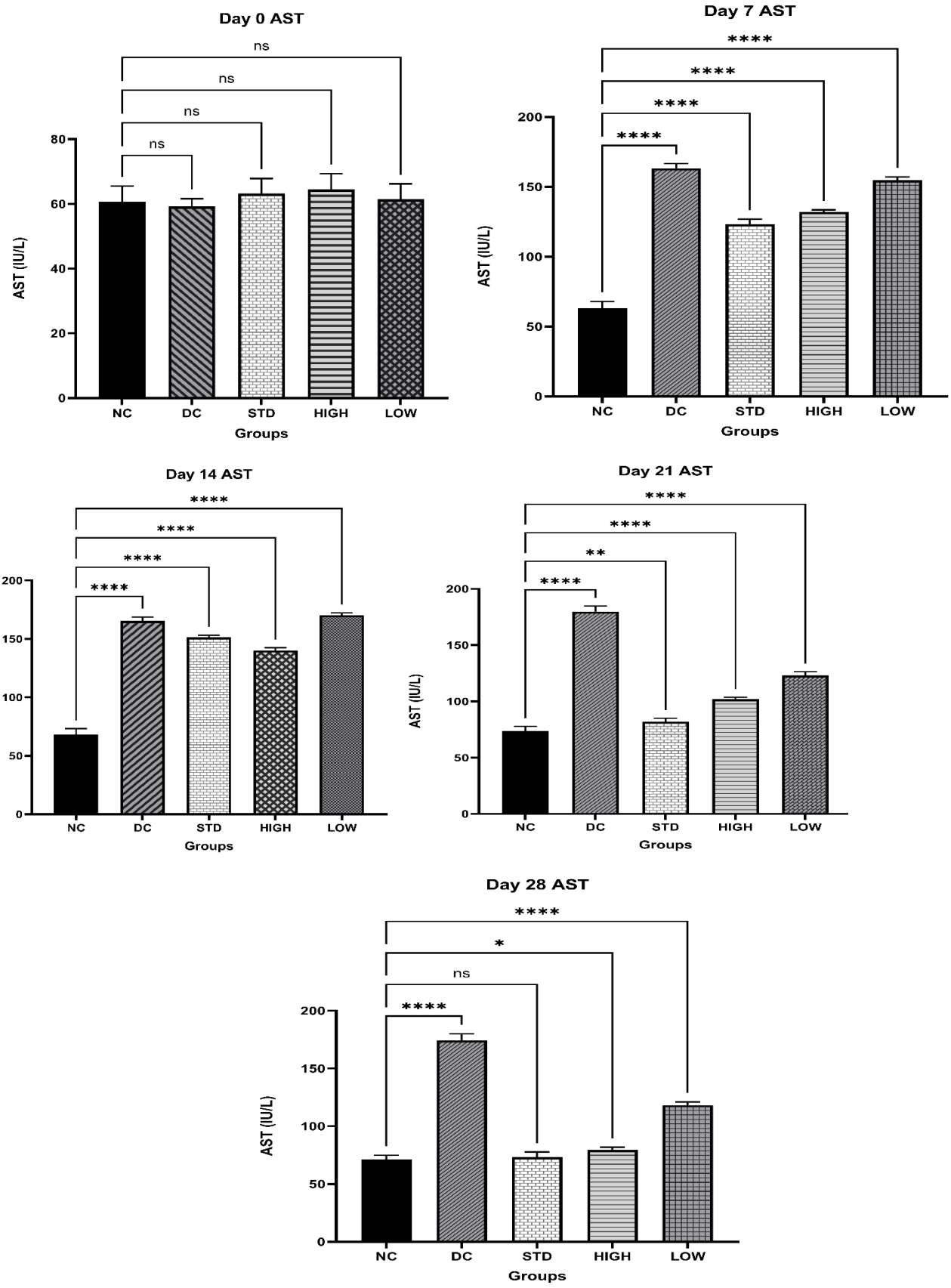
Fig 19: Significant difference in ALT level at different days





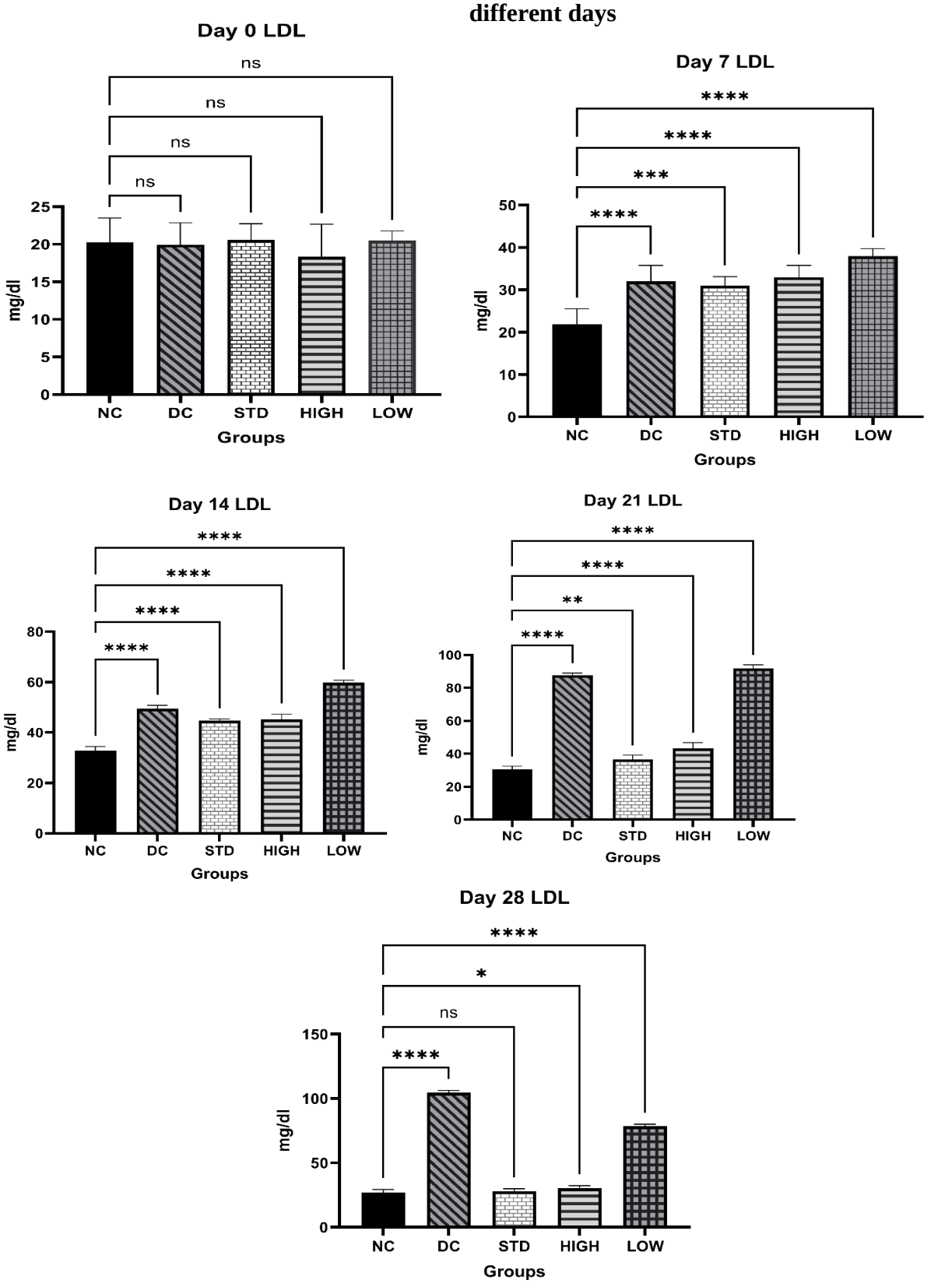
7.11.4. Effect of AGL-HA extract on AST

Fig 20: Significant difference in AST level at different days



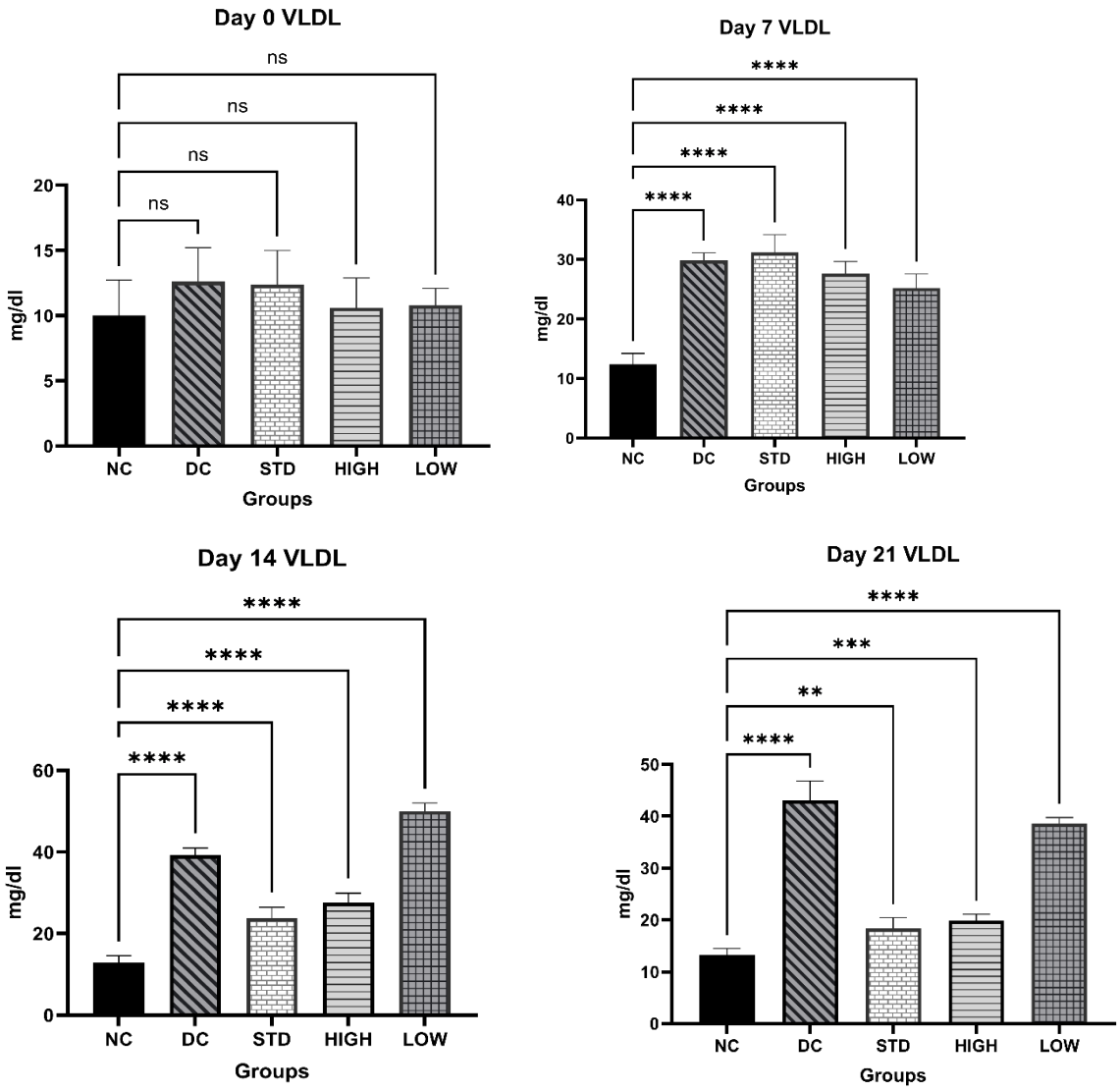
7.11.5. Effect of AGL-HA extract on LDL

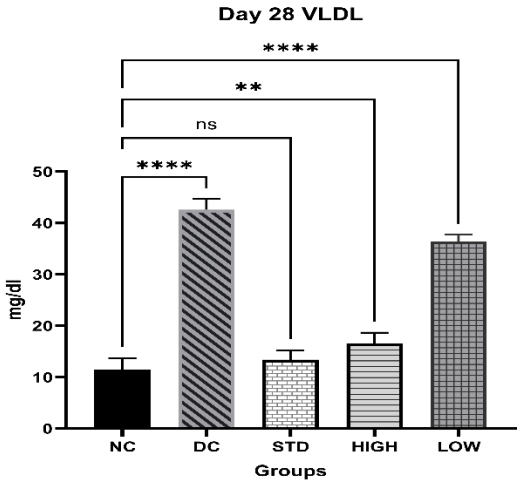
Fig 21: Significant difference in LDL level at different days



7.11.6.Effect of AGL-HA extract on VLDL

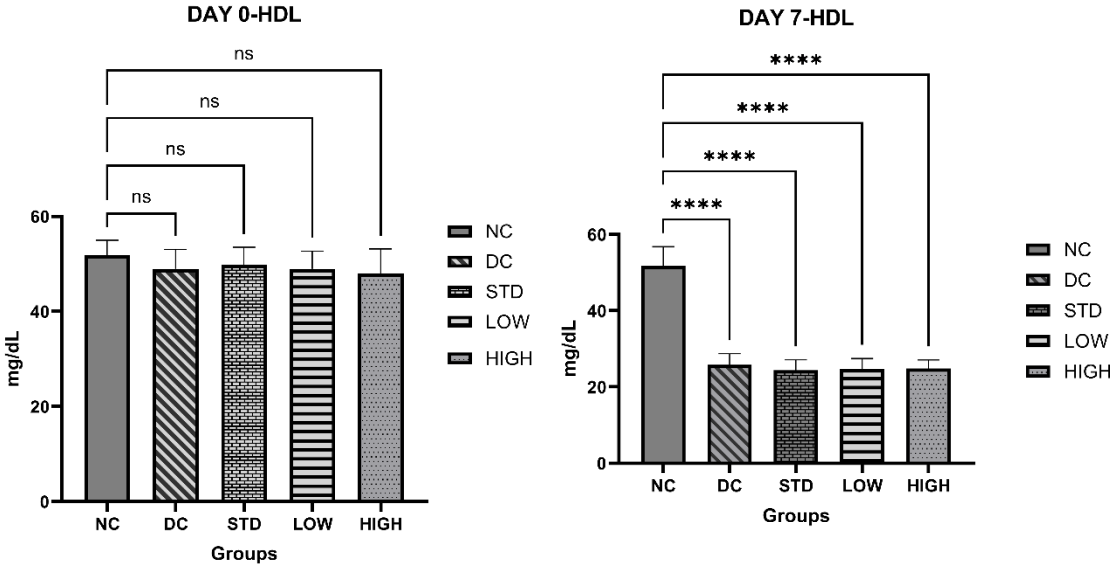
Fig 22: Significant difference in VLDL level at different days

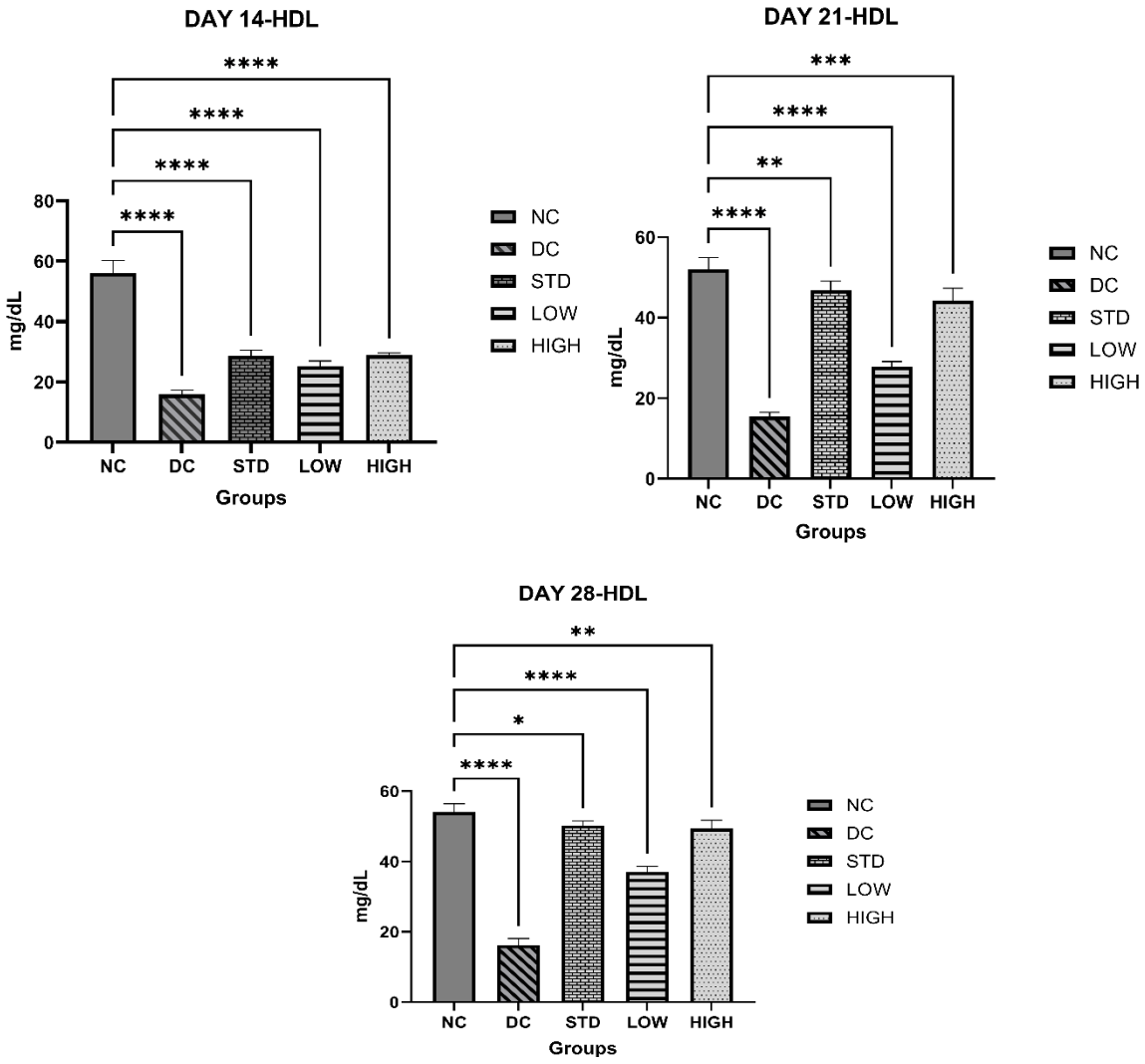




7.11.7. Effect of AGL-HA extract on HDL

Fig 23: Significant difference in HDL level at different days





7.12. Table 11: Liver Weight after Sacrifice:

Si.No	Group Name	Liver weight
1.	NC	6.7 ± 1.31
2.	DC	18.68 ± 2.56
3.	Low	13.53 ± 1.91
4.	High	10.15 ± 1.02
5.	STD	8.11 ± 2.73

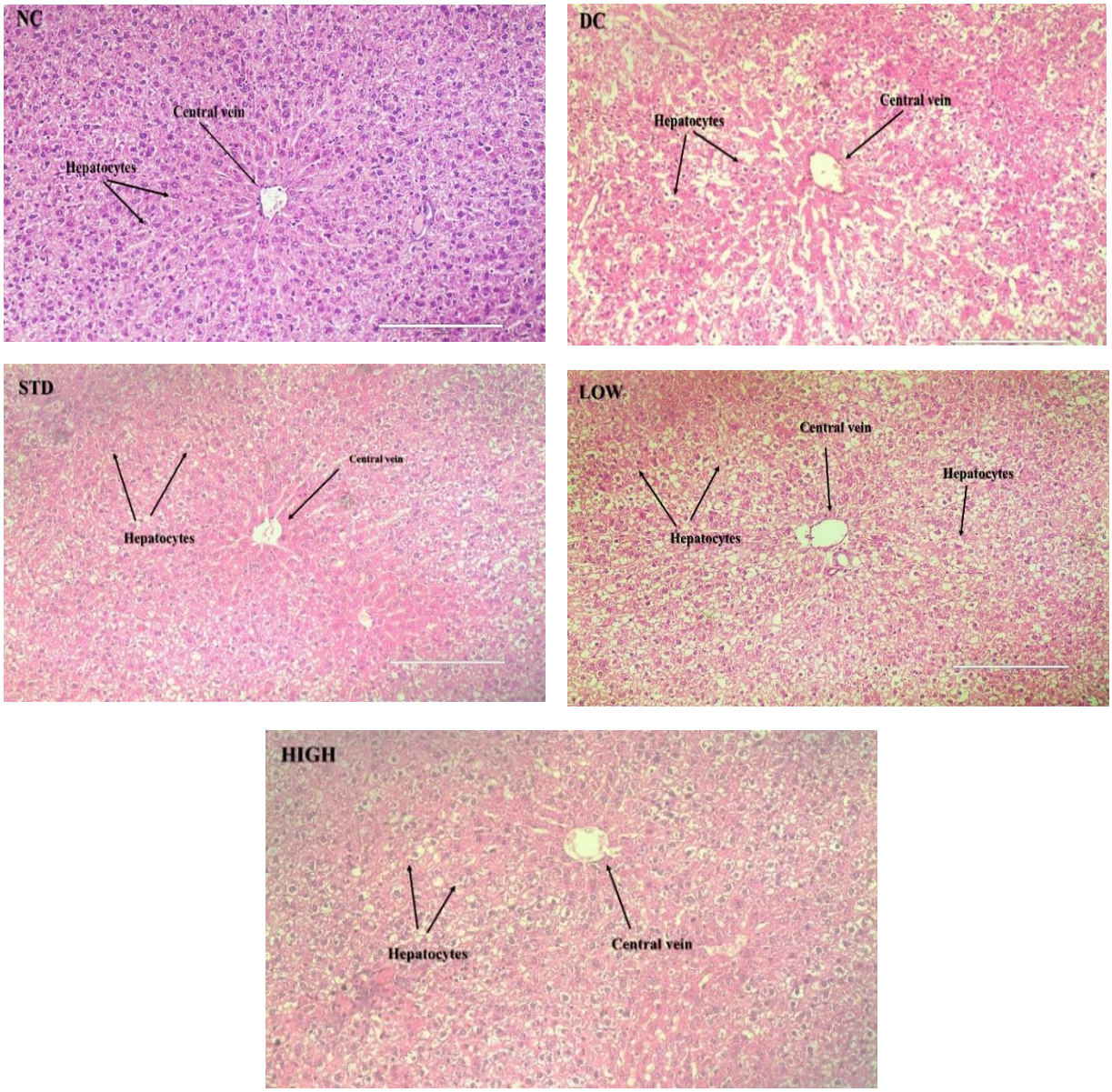
Values are mean ± SD, (n= 3)

7.13. Histopathological studies:

7.13.1. Hematoxylin and Eosin staining (H&E staining)

The histological study of the liver in Disease group rats revealed significant deterioration of the liver tissue. Treatment with a High dose of AGL-HA extract (400 mg/kg) enhanced the cellular structure, with visible central veins surrounded by hepatocytes and well-organised hepatic ducts, indicating that the plant extract has hepatoprotective properties.

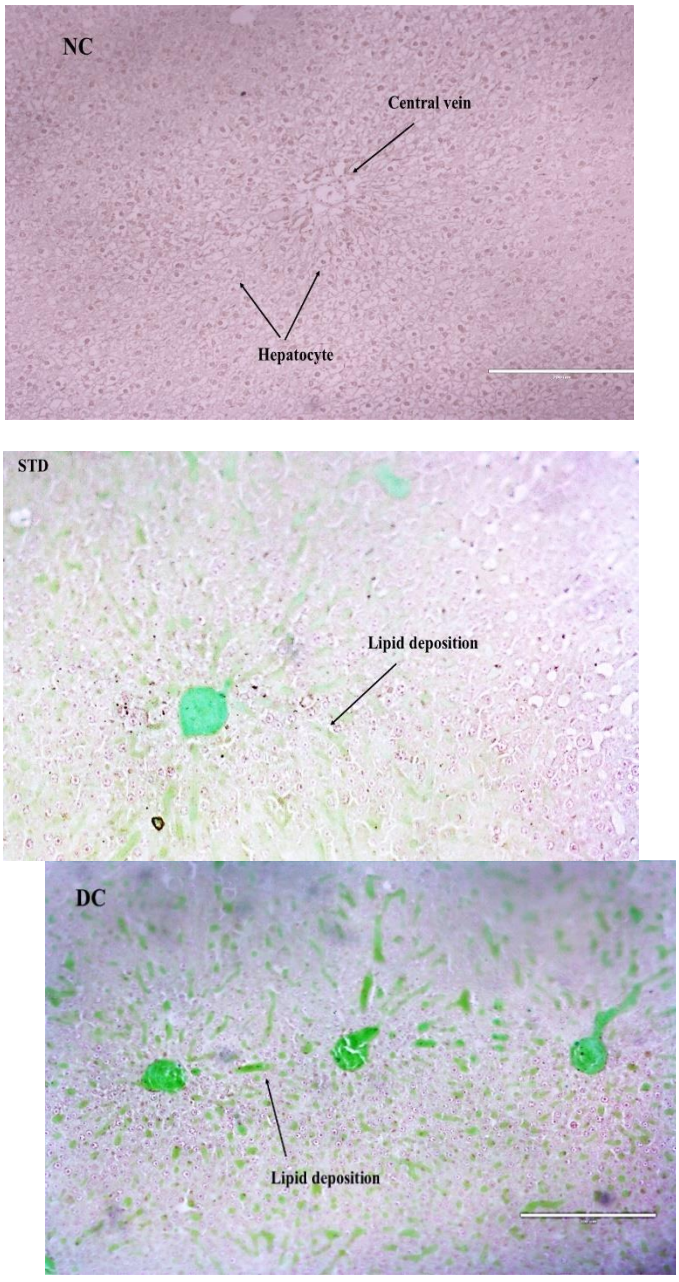
Fig 24: H&E histology of different liver tissue

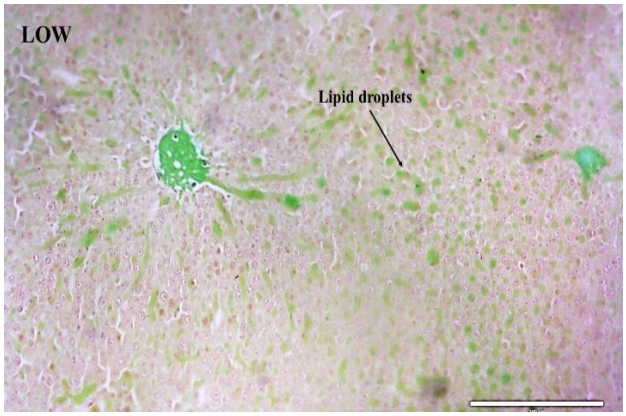


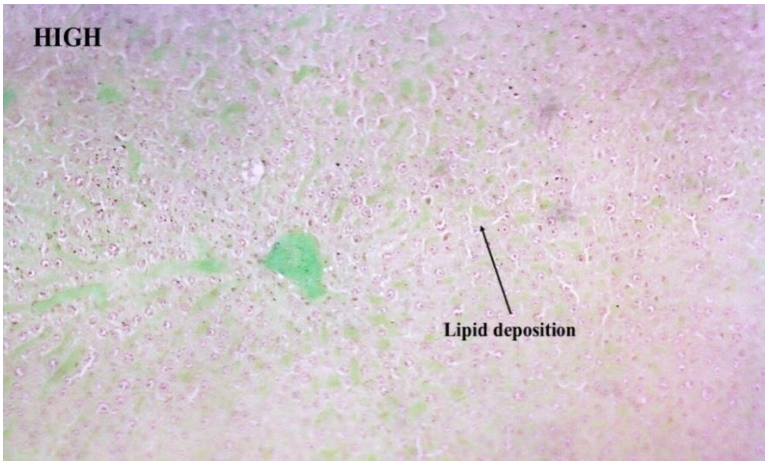
7.13.2. Oil O Red

Lipoproteins, cholesterol esters, and neutral lipids may all be stained using Oil Red O (ORO), a fat-soluble dye. Because of its cellular permeability, it has been widely utilised for tissue staining and intracellular lipid staining. Triglycerides, which are the main neutral fats, have an orange-red stain. The Oil O Red histological study reaveled the significant diposition of fat in liver tissue of Disease groups. Treatment with Low dose of AGL-HA (200 mg/KG) dose not significant reduction in lipid deposition. But the treatment with Standard drug and High dose of AGL-HA (400 mg/KG) significantly reduce lipid deposition level.

Fig 25: Oil O Red histology of different liver tissue







7.14. Estimation of liver antioxidation level:

The hepatic levels of GSH and catalase activity were significantly ($p < 0.05$) reduced upon induction of T2D, and the MDA level was exacerbated concurrently. Following treatment with AGL-HA Extract, these levels and activity were markedly restored, with rats receiving the high dosage (400 mg/kg bw) showing even better activity compared to those receiving metformin. Hepatic oxidative indicators were either hardly affected or not affected at all when the infusion was given to normal animals.

Fig 26: Significant difference in GSH level at 28th days:

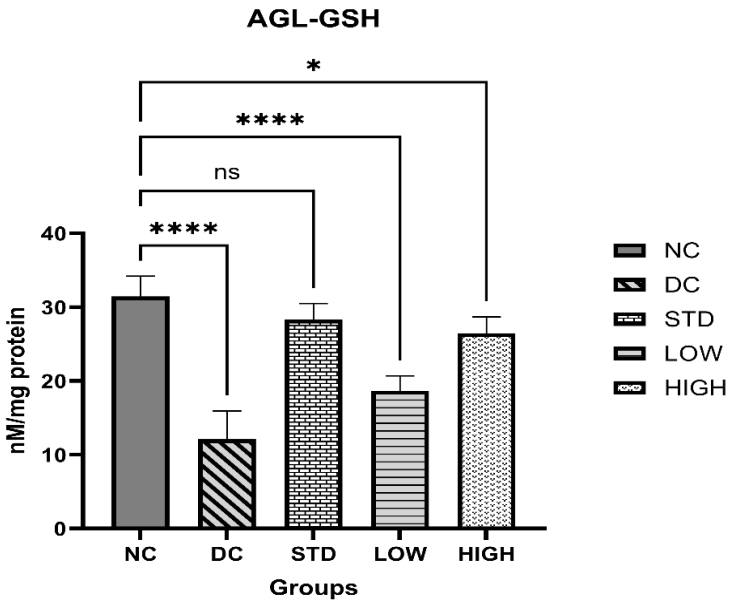


Fig 27: Significant difference in catalase activity at 28th days:

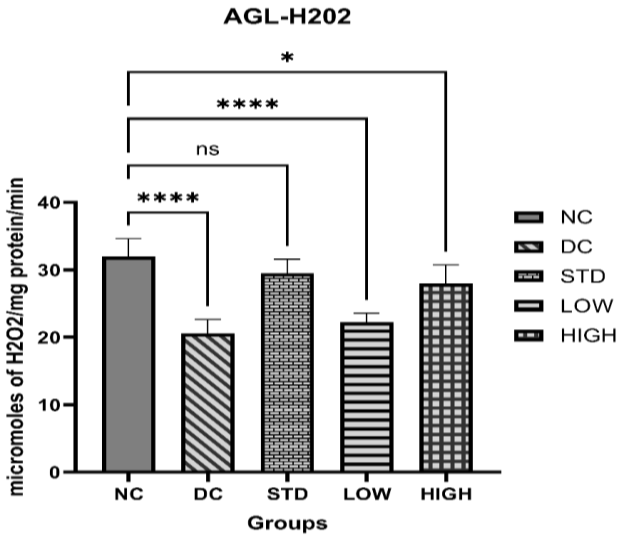


Fig 28: Effect of AGL on MDA levels at 28th day:

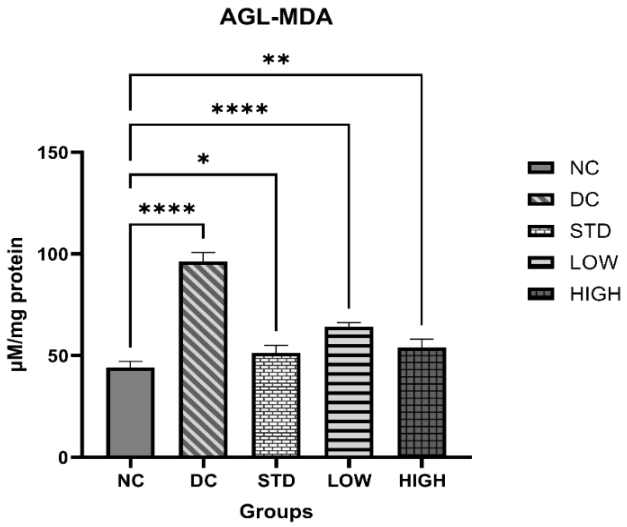
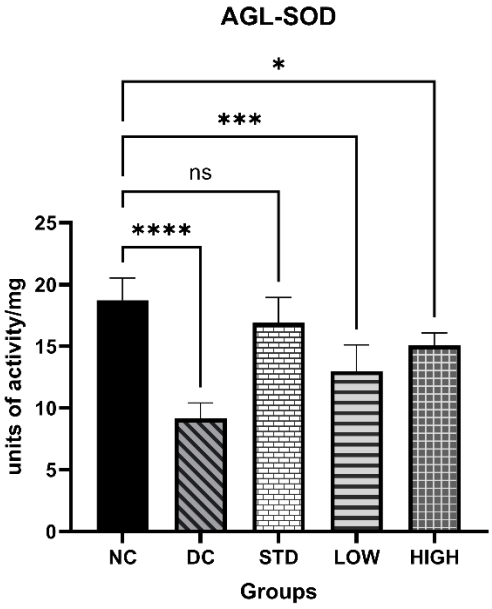


Fig 29: Effect of AGL on SOD levels at 28th day:



DISCUSSION

In ThisStudy we conduct various phytochemical analyses of *Excoecaria agallocha*, revealing the presence of various chemical constituents like carbohydrates, glycosides, flavonoids, saponins, steroids, alkaloids, gums, fixed oils, fats, tannins, phenols. We also examined the antioxidant properties of the mangrove plant *Excoecaria agallocha*,it showed a significant result in antioxidant tests like DPPH and the Radical Scavenging test. Before the In-Vivo Animal Study We conducted some In-Vitro antidiabetic Studies (Alpha-amylase, Alpha-glucosidase) and they showed positive results and encouraged us to conduct the In-vivo Animal study.

NAFLD is a serious condition that can result in morbidity and death due to micro and macrovascular consequences. Liver dysfunctions, such as liver failure, are the primary cause of death associated with diabetes. The pathophysiology of NAFLD is associated with hyperglycemia caused by insulin resistance and pancreatic β-cell dysfunction. In T2D rats, it has been demonstrated that E. agallocha infusion reduces hyperglycemia, enhances insulin production and utilization, and lessens pancreatic β-cell dysfunction. In this work, E. agallocha therapeutic efficacy and mechanism on NAFLD in T2D rats are examined.

In the In-Vivo study, we administereda High-fat diet and a single injection of streptozotocin (STZ) at a dose of 40 mg/kg, i.p. to Wistar rats resulted in the development of oxidative stress damage in hepatic tissues, Diabetes, and eventually Non-alcoholic fatty liver disease. Rats showeda significant increase in Body weight,Blood Glucose, SGOT (AST), SGPT (ALT), TG, TC, LDL, And VLDL concentrations in the DC (disease Control) groups rats when compared to the normal group (NC).Oral administration of hydroalcoholic extract of *Excoecaria agallocha* L. High (400mg/KG) and Low (200mg/KG) doses were administrated to show the hepatoprotective and anti-NAFLD activity effect of the plant. A standard drug (Metformin) was treated in the STD group to standardize and for the comparison of the effect of plant extract efficacy in the study.

The In-Vivo result shows that the therapy with AGL-HA High dose considerably normalized increased levels of Bood glucose, Body weight, and Biochemical parameters, however therapy at Low dose was not that dramatically normalizing.

The study examined changes in rat liver cell and structure after 28 days, confirmed by histopathological (H&E, Oil-O-Red) investigations. The livers of the normal control and standard groups showed normal hepatocytes with no inflammation or fat deposition. The disease-control group showed hepatocyte deterioration, inflammation, and lipid deposition. A lower dosage of AGL (200 mg/kg) protected the liver, with minor degenerative alterations in

hepatocytes. A higher dose (400 mg/kg) showed liver protection, with normal hepatocyte shape and no lipid droplet and inflammatory changes.

In this study, we also tested Rat liver weight after sacrificing of rat at day 28 and examined liver tissue antioxidant teste with the liver tissue homogenate. We discovered a substantial imbalance between the prooxidant and antioxidant states in disease control as compared to normal control rats, shown by a decrease in serum SOD, GSH, and higher MDA content activity. As per Caturano et al., 2023, Oxidative stress, caused by excess hyperglycemia-mediated free radical production, is linked to the pathogenesis and pathogenesis of nonalcoholic fatty liver disease (NAFLD). In untreated diabetic rats, depleted GSH and catalase activity and increased MDA levels indicate a weakened antioxidant enzyme defense system, leading to increased hepatic oxidative stress in T2D. Antioxidants have been reported to protect against NAFLD by scavenging free radicals and modulating the endogenous antioxidant defense system. In these studies,the administration of Plant extract at High doses significantly normalized the levels of these antioxidants. According to Sabu and Kuttan (2004), the decrease in liver antioxidant status may be due to the body's heightened defense against the generation of lipid peroxides.

As a result, it is reasonable to conclude that the hydroalcoholic extract of *Excoecaria Agallocha*bark is remarkably effective against high fat diet/STZ induced NAFLD in Wistar rat due to enhancement of the endogenous antioxidant mechanism, ability to lower lipids and serum glucose.

Conclusion

The research assesses that the hydroalcoholic extract of Excoecaria agallocha bark has been shown to improve NAFLD, a metabolic condition. By efficiently scavenging free radicals and lowering oxidative stress markers, the phytochemical analysis of AGL extract uncovered a rich composition of bioactive compounds, such as polyphenols, flavonoids, and terpenoids, which have significant antioxidant properties in vitro and are essential for reducing oxidative damage caused by NAFLD. In vivo experiments on Wistar rats revealed that the extract alters NAFLD metabolic pathways, lowering fat formation and increasing anti-inflammatory properties. In In-vivo investigations on NAFLD animal models revealed that the extract lowered liver fat accumulation, improved liver function tests, and slowed histopathological progression. It also improved insulin sensitivity and lipid profiles, which are frequently disturbed in NAFLD patients. In the in-vivo investigation, rats treated with the extract demonstrated improvements in their quality-of-life ratings, decreased hepatic fat, and raised liver enzyme levels compared to the Placebo group.Because of its safety and tolerability, the extract might be utilized as an addition to NAFLD therapy. However, Larger-scale, randomized controlled trials are required to prove its effectiveness and safetywith future studies focusing on optimal dosing regimens, long-term effects, and potential interactions.Further research is needed to understand the exact mechanisms of action of AGL extract. Additional investigation is required to precisely comprehend the modes of action of the plant extract, which may involve pathways such as metabolic regulation, antioxidant, and anti-inflammatory actions. This study highlights the significance of investigating traditional medicinal plants as innovative therapeutic agents and adds to the increasing body of data in favor of plant-based treatments for complicated metabolic diseases like NAFLD.

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