Thesis Report

on

Mechanistic insights into the Hepatoprotective Activity of *Terminalia arjuna*

by

Deepshikha Tiwari (MT23243)

Under the supervision of

Dr. Jaspreet Kaur Dhanjal

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Certificate

This is to certify that the thesis titled **"Mechanistic Insights into the Hepatoprotective Activity of *Terminalia arjuna*"** being submitted by Deepshikha Tiwari to the Indraprastha Institute of Information Technology, Delhi, for the award of the Master of Technology, is an original research work carried out by him under my supervision. In my opinion, the thesis has reached the standards fulfilling the requirements of the regulations relating to the degree.

The results contained in this thesis have not been submitted in part or in full to any other university or institute for the award of any degree/diploma.

Dr. Jaspreet Kaur Dhanjal

Assistant Professor

Department of Computational Biology

Indraprastha Institute of Information Technology,

Delhi

New Delhi,110020

Declaration

I submit this project entitled "**Mechanistic Insights into the Hepatoprotective Activity of *Terminalia arjuna***" to the Department of Computational Biology, Indraprastha Institute of Information Technology (IIIT), Delhi. I declare that this is my original work carried out under the guidance of Dr. Jaspreet Kaur Dhanjal, Assistant Professor, Department of Computational Biology at IIIT-Delhi.

Deepshikha Tiwari
MTech Student
Department of Computational Biology,
Indraprastha Institute of Information Technology, Delhi,
New Delhi 110020

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Abstract

Non-alcoholic fatty liver disease (NAFLD) affects roughly one quarter of the world's population and is tightly associated with obesity and metabolic syndrome; despite its high prevalence, no fully effective pharmacotherapies have yet been approved. *Terminalia arjuna*, a traditional medicinal plant of the Combretaceae family, has long been used for both cardioprotective and hepatoprotective applications. In this study, we employed a systems-biology framework, combining *in silico* transcriptomic profiling (RNA-seq) with gene-set and pathway enrichment analyses, to investigate three bioactive phytochemicals (Maslinic acid, ellagic acid, and plant-derived sterols) from *T. arjuna* influence liver gene-expression networks relevant to NAFLD.

Our data corroborate previous findings that Maslinic acid suppresses key lipogenic transcription factors (including SREBP-1c and members of the C/EBP family) while enhancing the expression of lipid-catabolic and oxidative-metabolism genes (such as PPAR-α and ATGL) through activation of the AMPK pathway, thereby reducing hepatic triglyceride levels and steatosis. Ellagic acid treatment signatures were marked by decreased oxidative stress and inflammatory markers (TNF-α, IL-6, ROS) alongside improved insulin sensitivity, whereas phytosterols exhibited the expected capacity to impede cholesterol uptake, diminish liver fat accumulation, and attenuate both oxidative injury and inflammatory signaling. Enrichment analyses further pinpointed modulation of several NAFLD-related pathways, including fatty-acid β-oxidation, cholesterol metabolism, cytokine-mediated inflammation, and antioxidant defences. Taken together, our results suggest that these *T. arjuna* derived phytochemicals may act synergistically to re-establish metabolic homeostasis in the fatty liver, promoting lipid clearance and antioxidant responses while repressing *de novo* lipogenesis and inflammation.

Overall, this work showcases the value of integrating network-level transcriptomic analysis with ethnopharmacological knowledge, offering a versatile computational strategy for uncovering multi-target mechanisms of hepatoprotection and guiding the development of plant-based NAFLD therapeutics.

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Chapter 1. Introduction

Medicinal plants have long been recognised as a cornerstone of human healthcare, providing a rich source of therapeutic agents across cultures and centuries. Nearly half of all small-molecule drugs approved between 1940 and 2014 were either directly derived from or inspired by natural products[1], highlighting their enduring value to modern medicine. Over the past two decades, advances in analytical chemistry, genomic exploration, and microbial cultivation have reinvigorated natural product research[2]. These developments have accelerated the identification of novel bioactive molecules targeting infectious, oncological, and metabolic diseases, while bioengineering of biosynthetic pathways has further expanded the diversity of phytochemicals available for drug screening[2].

Despite their long history of use, the extent to which herbal remedies are incorporated into conventional healthcare systems varies widely across regions. A global survey found that 39.7% of herbal medicine users rely primarily on recommendations from friends or family, whereas 17.7% and 23.3% regard pharmacists and physicians, respectively, as their main sources of information[3]. This trend reflects both a growing acceptance of plant-based therapeutics among healthcare providers and a continued reliance on informal knowledge channels. As a result, many pharmacies now stock standardised herbal preparations alongside conventional medicines[3]. However, clinical guidance on herb–drug interactions remains limited, and interdisciplinary collaboration between botanists, pharmacologists, and clinicians is still in its developmental stages[4].

Alongside integration challenges, safety remains a pressing concern. Many herbal formulations reach the market without undergoing rigorous pre-market evaluation, with estimates suggesting that up to 80% lack comprehensive toxicity testing[4]. This gap contributes to underreported adverse effects, including hepatotoxicity and nephrotoxicity. For example, a systematic review of Chinese herbal products identified aristolochic acid–containing preparations as a cause of severe liver injury[5]. Such findings underscore the necessity for stringent safety assessments and have prompted regulatory bodies to enforce stronger quality control through good manufacturing practices, extract marker standardisation, and post-marketing pharmacovigilance[4].

Quality assurance in herbal medicines is complicated by inherent variability in plant materials and processing methods. Factors such as harvest season, geographic origin, and extraction

techniques can substantially influence phytochemical content, resulting in inconsistent therapeutic efficacy. Modern quality control strategies, such as DNA barcoding for species authentication and HPLC-MS fingerprinting for quantitative phytochemical profiling, are now being employed to address these challenges[6]. These approaches not only ensure accurate dosing of bioactive compounds but also improve reproducibility in clinical trials and facilitate mechanistic research.

Together, these technological, regulatory, and analytical advancements have transformed herbal medicines from traditional, experience-based remedies into scientifically evaluated therapeutic agents. As research methods continue to evolve, the integration of phytochemical analysis, standardisation protocols, and robust safety evaluation is paving the way for the broader acceptance of plant-derived compounds in evidence-based medicine.

Within this landscape, *Terminalia arjuna* stands out as a medicinal plant of both historical and contemporary relevance. Native to the Indian subcontinent, the bark of *T. arjuna* (Combretaceae), commonly known as Arjuna, has been an integral part of Ayurvedic medicine for centuries[7]. Traditionally, decoctions of the bark were used to treat cardiovascular conditions such as dyslipidaemia, hypertension, heart failure, and angina[7]. In addition to its cardioprotective reputation, *T. arjuna* has been credited with hepatoprotective effects[8]. Modern pharmacological studies support these traditional claims, demonstrating that crude bark extracts exhibit anti-ischaemic, antioxidant, hypolipidaemic, and anti-atherogenic properties in animal models[7]. These diverse effects are attributed to its rich phytochemical profile, including triterpenoids, flavonoids, glycosides, and phytosterols, which act synergistically to produce physiological benefits[7]. Among these, the oleanane-type triterpenoid arjunolic acid—particularly abundant in the heartwood—has been identified as a key bioactive constituent[8–9]. Notably, clinical reports to date have not associated *T. arjuna* use with significant adverse effects[7,10].

Given its hepatoprotective potential, *T. arjuna* is an attractive candidate for investigation in non-alcoholic fatty liver disease (NAFLD), a chronic liver disorder of growing global concern. NAFLD is defined by excessive lipid accumulation in hepatocytes in the absence of significant alcohol intake and encompasses a clinical spectrum from simple steatosis (NAFL) to non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis[11,8]. In recognition of its strong association with obesity, insulin resistance, and type 2 diabetes, an international panel in 2020 proposed renaming NAFLD to metabolic dysfunction–associated fatty liver disease

(MAFLD)[12]. This shift in terminology reflects a better understanding of the metabolic origins of the disease rather than a sole focus on the exclusion of alcohol as a cause.

The pharmacological properties of *T. arjuna*, notably its antioxidant, anti-inflammatory, and lipid-modulating effects, directly address key mechanisms in NAFLD pathogenesis[13],[7]. Its flavonoids and triterpenoids exhibit potent free-radical scavenging activity and anti-inflammatory effects, potentially mitigating oxidative stress and hepatic inflammation[13],[14]. Additionally, its lipid-lowering actions, which include reducing triglyceride levels and inhibiting cholesterol synthesis, may help prevent hepatic fat accumulation[7].

Experimental studies lend strong support to these mechanisms. In HepG2 cell models, arjunolic acid reduced intracellular triglyceride content by approximately 66% and decreased Oil Red O staining by about 36% at 50 μM compared to controls[8]. In high-fat diet–induced NAFLD mouse models, arjunolic acid administration lowered serum transaminases, improved histological features of steatosis, upregulated hepatic PPARα and FXRα, and downregulated PPARγ—changes consistent with enhanced β-oxidation and reduced lipogenesis. Similarly, aqueous *T. arjuna* bark extracts have been shown to bolster hepatic antioxidant defences in toxin-induced injury models[14]. These extracts demonstrated free-radical scavenging capacity comparable to ascorbic acid, increased activities of superoxide dismutase, catalase, and glutathione, and reduced lipid peroxidation following carbon tetrachloride exposure. Restoration of liver enzyme levels (GPT/ALT, ALP) and protection of hepatic and renal tissues were observed, attributable to the extract's antioxidant and membrane-stabilising effects[14].

Given this evidence, the present study aims to evaluate the curative effects of *T. arjuna* in NAFLD by integrating traditional medicinal knowledge with modern molecular profiling. Specifically, we have employed transcriptomic analysis of samples treated with key phytochemicals present in *T. arjuna* to investigate the gene expression changes they induce. This approach enables us to identify the molecular pathways and regulatory networks potentially responsible for its hepatoprotective effects. By elucidating these mechanisms, we not only provide deeper insight into how *T. arjuna* exerts its therapeutic action but also generate the type of reproducible, mechanistic evidence that is critical for the broader clinical acceptance of plant-derived medicines. Establishing scientifically robust proof of efficacy and mechanism is an essential step toward integrating such herbal therapies into mainstream treatment strategies for metabolic liver diseases.

Chapter 2. Review of Literature

2.1 Non-Alcoholic Fatty Liver Disease (NAFLD) and Limitations of Current Therapies

Development of NAFLD is largely driven by metabolic disturbances such as type 2 diabetes, insulin resistance, and metabolic syndrome. NAFLD affects roughly one-quarter of people worldwide and has a prevalence of 9 % to 32 % within India's population. Its occurrence is particularly elevated in individuals with type 2 diabetes and obesity. It is more common in men than in women and increases with age, peaking between 52 and 60 years for both sexes[15]. Recognizing NAFLD is vital, as it is associated directly or indirectly with conditions such as type 2 diabetes mellitus, obesity, cardiovascular disease, dyslipidemia, and hypertension [16]. Clinically, NAFLD often presents with abdominal discomfort, elevated hepatic enzymes, and unintended weight loss; without lifestyle intervention, it can progress to severe outcomes like liver fibrosis and cirrhosis. While lifestyle modification remains the cornerstone of both prevention and management, pharmacologic options are limited and frequently accompanied by adverse effects, including edema, weight gain, heart failure risk, and potential bladder cancer, underscoring the importance of dietary and exercise-based approaches[8]. Hepatic lipid accumulation (steatosis), insulin resistance, oxidative stress, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, chronic inflammation, altered lipid metabolism, and dysregulated adipokine signaling are the main characteristics of NAFLD (Figure 2.1).

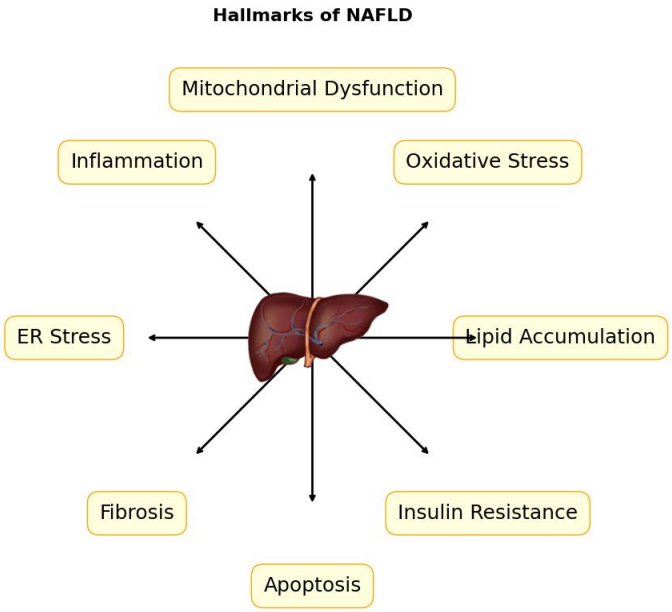

Figure 2.1. Hallmarks of Non-Alcoholic Fatty Liver Disease (NAFLD) include mitochondrial dysfunction, oxidative stress, lipid accumulation, insulin resistance, apoptosis, fibrosis, ER stress, and inflammation.

These interconnected mechanisms cause hepatocellular damage and fibrogenesis while upsetting hepatic homeostasis. For example, oxidative stress brought on by lipid peroxidation exacerbates liver injury by causing mitochondrial damage and the release of inflammatory cytokines. One important factor that worsens hepatic lipogenesis and hinders lipid export is insulin resistance. Furthermore, new research indicates that changes in immune responses and gut flora also play a role in the pathophysiology of NAFLD. It is essential to comprehend these characteristics in order to create focused treatment plans for NAFLD[17].

2.2 Current Approaches for NAFLD Treatment

Efficient treatment of NAFLD is complicated due to its complex origin and difficulty in diagnosing a broad spectrum of disease stages. In addition to this, some situations, such as obesity, diabetes, and lifestyle, significantly affect the management of NAFLD. So, very few treatments are available to get rid of this disease[18].

Medication Treatment

The treatment of NAFLD is inefficient due to a lack of specific medications. Therefore, therapies are specific for diabetes, obesity and lipid disorders to control patient glycemia, liver function, and lipid profile. Pharmacological treatment is required for those people who do not reach anticipated weight loss objectives, and pharmacological therapy is recommended[19]. Here is the list of medications that can be used in treating NAFLD.

Vitamin E: The involvement of oxidative stress in the evolution of NAFLD/NASH has led to substantial research on the therapeutic effects of antioxidants, especially vitamin E (alpha-tocopherol). Vitamin E can decrease inflammation and enhance liver function in non-diabetic patients with biopsy-proven NASH. However, studies on its impact on liver fibrosis have shown conflicting results. In non-diabetic patients with biopsy-proven NASH, vitamin E is advised by American recommendations; however, more research is needed to determine vitamin E's efficacy in diabetic patients[20].

Pioglitazone: It belongs to the class of Thiazolidinedione (TZD) derivatives. Pioglitazone enhances insulin sensitivity and regulates lipid metabolism. It decreases the rate of plasma-free fatty acids, triglycerides and LDL cholesterol and increases the rate of HDL cholesterol. Still, it does not affect fibrosis with this drug; it has potential side effects, including weight gain, fluid retention, and heart failure[21].

Resmetirom: Resmetirom belongs to the class of selective thyroid hormone receptor-beta. It targets the liver THR receptors by reducing the hepatic fat and improving liver function. It also has side effects, including diarrhoea and nausea[22].

Metformin: Metformin, classified under biguanides, is predominantly used as an oral antihyperglycemic agent in treating type 2 diabetes mellitus. It enhances insulin sensitivity, reduces hepatic glucose production, and improves the utilization and uptake of peripheral glucose. This would be more helpful for people with insulin resistance. Apart from its vital role in managing diabetes, metformin has also been explored for its potential advantages in treating NAFLD. It may aid in reducing hepatic lipogenesis and improving liver function. However, clinical studies on the effects and efficacy of metformin in NAFLD patients have yielded mixed results, highlighting the need for further research to fully comprehend its therapeutic potential in this area[23].

Pentoxifylline: Pentoxifylline comes under a class of phosphodiesterase inhibitors, and its possible effect is related to hepatic diseases. The suggested action for Pentoxifylline is its regulation of TNF-alpha and its impact on liver inflammation. But there are some side effects of pentoxifylline, like headache and abdominal cramps[24].

Thiazolidinediones (TZD): Thiazolidinediones (TZDs) are classified as antidiabetic drug. It is also known as an insulin sensitizer. The two main TZDs discussed in the paper are pioglitazone and rosiglitazone which improve insulin sensitivity by activating peroxisome proliferator-activated receptor-γ (PPAR-γ) signalling. Treatment with TZDs caused significant weight gain compared to control groups, yet it did not significantly change body mass index (BMI) or fat percentage. Additionally, TZD therapy did not substantially alter serum cholesterol or triglyceride levels[25].

Statins: Statins, classified as HMG-CoA reductase inhibitors, are used to treat NAFLD. It is used to treat NAFLD. These medications reduce lipids by inhibiting the HMG-CoA reductase enzyme, which is crucial in cholesterol production, and have anti-lipid and anti-inflammatory effects. It also shows some side effects such as Muscle pain, tenderness or weakness, Increased risk of diabetes, Liver enzyme elevations, Digestive problems(constipation, diarrhoea, nausea), Headaches, and Liver enzyme elevations [26].

Omega-3 Fatty Acids: Omega-3 fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are primary polyunsaturated fats found in fish oil and certain algae and are known for their cardiovascular and anti-inflammatory benefits. They play a

significant role in managing NAFLD by enhancing insulin sensitivity, reducing inflammation, and decreasing fat accumulation in the liver through the negative regulation of hepatic lipogenesis. Omega-3s also activate PPARs to promote fatty acid oxidation and improve lipid profiles by lowering triglycerides and increasing high-density lipoprotein (HDL) levels. While generally safe,omega-3 supplements can cause side effects such as gastrointestinal issues, increased bleeding risk, allergic reactions, and their potential impact on people with diabetes[27].

2.3 Overview of *Terminalia arjuna*: Uses and Phytochemistry

2.3.1 Characteristics *of Terminalia arjuna*

The tree *Terminalia arjuna*, a prominent medicinal species in the Ayurvedic, Unani, and Siddha traditions, belongs to the Combretaceae family. It is a large, spreading tree with a buttressed trunk, oblong leaves, and clusters of white flowers, often attaining heights between 60 and 80 feet. Its fruit is characterized by a hard, fibrous exterior. Naturally*, T. arjuna* thrives along riverbanks, streams, and seasonally dry watercourses throughout the Indo-sub-Himalayan belt—covering areas such as Uttar Pradesh, Chota Nagpur, southern Bihar, Madhya Pradesh, Delhi, and the Deccan—as well as in parts of Burma, Mauritius, and Sri Lanka. While it adapts to a wide range of soils, it prefers red lateritic or moist, fertile loams and can tolerate short periods of partial flooding. Propagation occurs through seeds, which typically germinate within 50–70 days, achieving a success rate of 50–60% [28]. The species is renowned for its extensive therapeutic properties and is vital in numerous biological processes.

2.3.2 Ethnomedical Applications

Traditionally called Arjuna, Indradru, Partha, and Veeravriksha, this tree's bark has long been used in Ayurveda to treat cardiovascular disorders (e.g., angina, heart failure, atherosclerosis), metabolic imbalances (hypolipidemia, hypertension, diabetes), inflammation, and liver ailments. Early texts by Vagabhatta and Chakradatta document its bark powder and ghee-based decoctions for heart protection [28]. Regional folk practices employ bark and leaf extracts to relieve headaches, dental parasites, earache, and urinary complaints[13]. Medicinal plants have been a main source of therapeutic agents from ancient times to cure diseases. *Terminalia arjuna* is one of the most accepted and beneficial medicinal plants in the indigenous system of medicine for the treatment of various critical diseases, such as anti-carcinogenic, anti-mutagenic and gastroprotective effects.

2.3.3 Phytoconstituents of arjuna

The complete chemical composition of *Terminalia arjuna* bark remains unknown, with variations likely due to differences among plant varieties. One study reports that the bark contains approximately 34% ash, which includes calcium and sodium carbonates along with trace amounts of alkaline chlorides. It also contains about 15% tannins, including glucotannic acid, a glucosidal compound, and a coloring agent. Other studies have found around 12% tannins, predominantly pyrocatechol tannins, along with a phytosterol, an easily hydrolyzed organic ester, organic acids, coloring agents, and sugars. Some analyses have not detected glucosides, alkaloids, or essential oils. Additionally, the bark is rich in calcium salts and contains trace amounts of magnesium and aluminum salts. The root, in contrast, has an ash content of approximately 30% and a tannin content of around 12%. It also contains sugars, tannins, coloring agents, a glucoside-like compound, calcium and sodium carbonates, and trace levels of alkali metal chlorides. Notably, the bark is rich in co-enzymes that may aid in heart protection.

Terpenoids, Ursane Triterpenoids, and Glycosides

Preliminary research on the benzene extract of the bark identified an oleanane-type triterpenoid named arjunin, while the alcoholic extract contained a lactone called arjunetin. Subsequent studies revealed additional compounds such as arjunglucoside I, arjunglucoside II, arjunic acid, and arjungenin in the stem bark. Further investigations uncovered two more compounds: arjunoside III and arjunoside IV, as well as a triterpene carboxylic acid, terminic acid, in the root's ethyl acetate extract. When the heartwood of *T. arjuna* was extracted using n-hexane, β-sitosterol and terminic acid were also identified. Notably, terminic acid was the first lupane derivative discovered in any Terminalia species and represents the first known example of a lup-20(29)-en derivative in nature with a hydroxyl group at the uncommon C-13 position. The acetone fraction of an ethanolic stem bark extract yielded terminoside A, another oleanane triterpene. The stem's hexane extraction confirmed the presence of terminic acid and β-sitosterol. The structure of terminoside A, another oleanane-type triterpane, was determined after extracting it from the acetone fraction of the ethanolic stem bark. They identified it as olean-1α,3β,22β-triol-12-en-28-oic acid-3β-d-glucopyranoside, which was shown to reduce nitric oxide generation in lipopolysaccharide-stimulated macrophages and inhibit inducible nitric oxide synthase [29].

Moreover, several glycosyl esters were identified, including 2α,3β,23-trihydroxyurs-12,18-dien-28-oic acid and its 28-O-β-D-glucopyranosyl ester, quadranoside VIII, kajiichigoside, and 2α,3β,23-trihydroxyurs-12,19-dien-28-oic acid. Additionally, five ursane-type triterpene glucosyl esters were identified, one of which is a novel compound, 2α,3β-dihydroxyurs-12,18-dien-28-oic acid. Other derivatives such as 28-O-β-D-glucopyranoside and 3-O-β-D-glucopyranosyl-2α,3β,19α-trihydroxyolean-12-en-28-oic acid were identified using advanced spectrochemical techniques. Two additional glycosides, Termiarjunoside I and II, were found in ethanolic extracts of the bark, along with Arjunglucoside IV and V and Arjunasides A–E. Another compound, olean-3β,22β-diol-12-en-28-β-D-glucopyranoside-oic acid, was identified via chromatography [29].

Flavonoids

The bark of *T. arjuna* is known for its high flavonoid content, which stands out when compared to many other medicinal plants. Key flavonoids identified in the bark include arjunolone, flavones, bicalein, quercetin, kaempferol, and pelargonidin. Arjunolone is chemically described as 6,4-dihydroxy-7-methoxy flavone, and bicalein is 5,6,7-trihydroxy flavone. A newer addition to this list is luteolin, isolated from the 1-butanol extract, which has shown significant antimutagenic and antibacterial properties. Luteolin effectively suppresses the growth of *Neisseria gonorrhoeae*, a Gram-negative bacterium, with concentrations ranging from 12.5 to 25 μg/disk. Its anticancer activity is enhanced when used alongside compounds like ethyl gallate and gallic acid [29].

In a study comparing the flavonoid content of common dietary plants, *T. arjuna* bark was found to have an exceptionally high concentration of flavonoids—5698 ± 531 mg/100 g—far surpassing cinnamon (1914 ± 106), tea leaves (1255 ± 119), capsicum (1302 ± 55), and turmeric (487 ± 23). Further research analyzing the antioxidant and phenolic content across *Terminalia* species (*T. arjuna, T. bellerica, T. chebula, and T. muelleri*) found substantial phenolic levels in *T. arjuna* leaves, bark, and fruits, ranging from 72.0 to 167.2 mg/kg [29].

Flavonoids are well known for their broad health benefits. They neutralize free radicals, prevent LDL cholesterol oxidation, boost endothelial nitric oxide production, reduce endothelial cell activation, and limit platelet aggregation. They also have the potential to inhibit cyclooxygenases, reducing the likelihood of thrombosis. Regular intake of flavonoid-rich foods is inversely related to coronary artery disease, and the high flavonoid content in *T. arjuna* suggests it plays a significant role in its cardiovascular protective effects [29].

Tannins

In addition to flavonoids, *T. arjuna* bark contains a broad range of tannins. Among the well-known hydrolyzable tannins are pyrocatechols, punicallin, punicalagin, terchebulin, terflavin C, castalagin, casuariin, and casuarinin. In total, about 15 different tannins and related compounds have been isolated from the bark. These tannins are known to increase nitric oxide production and relax blood vessels that have been pre-contracted with norepinephrine, which may explain the blood pressure-lowering effects of *T. arjuna* bark observed in traditional medicine. Tannins are also believed to contribute to the bark's astringent properties and are associated with wound healing and antimicrobial activity [29].

Table 1 lists some of the most abundant phytochemicals identified in different parts of *T. arjuna*.

Table 1. Phytochemicals found in different parts of *Terminalia arjuna.*

S. No.	Phytochemical Name	Plant parts
1	(+)-Catechol, (+)	Bark
2	(+)-Gallocatechol	Bark
3	(+)-Leucocyanidin	Bark
4	(+)-Leucodelphinidin	Heart Wood, Wood
5	1,2,3,4,6-Pentagalloylglucose	Leaf
6	2',4',5,7-Tetrahydroxyflavone	Fruit
7	2,3(S)-HHDP-6-O-Galloyl-D-Glucose	Bark
8	2,3(S)-HHDP-D-Glucoside	Bark
9	2,3,4,6-Tetragalloyl-Glucose	Leaf
10	8-Hydroxy-Hexadecanoic-Acid	Root Bark
11	Afrormosin	Fruit
12	Arachidic-Stearate	Fruit
13	Arjunagenin	Bark
14	Arjunetin	Bark, Leaf, Root Bark
15	Arjungenin	Bark
16	Arjunglucoside I	Bark, Root Bark
17	Arjunglucoside-II	Bark, Leaf
18	Arjunglucoside-III	Bark
19	Arjunglucosides	Plant
20	Arjunic-Acid	Bark, Flower, Leaf, Root Bark, Seed, Stem
21	Arjunin	Leaf
22	Arjunoliatin	Flower, Leaf, Seed, Stem
23	Arjunolic acid	Bark, Flower, Heart Wood, Leaf, Root Bark, Seed, Stem, Wood

24	Arjunolic-Acid-Saponin	Heart Wood, Root Bark
25	Arjunolone	Stem Bark
26	Arjunoside-I	Root Bark
27	Arjunoside-II	Root Bark
28	Arjunoside-III	Root Bark
29	Arjunoside-IV	Root Bark
30	Ash	Leaf
31	Baicalein	Stem Bark
32	Beta-Amyrin	Leaf
33	Beta-Sitosterol	Bark, Fruit, Heart Wood, Leaf, Root, Root Bark
34	Castalagin	Bark
35	Casuariin	Bark
36	Casuarinin	Bark
37	Daucosterol	Fruit
38	Ellagic acid	Bark, Heart Wood, Leaf, Root Bark
39	Epicatechol	Bark
40	Epigallocatechol	Bark
41	Fiber	Leaf
42	Friedelin	Bark
43	Gallic acid	Leaf, Root Bark, Shoot
44	Gallic-acid-Ethyl-Ester	Shoot
45	Leucocyanidin	Root Bark
46	Luteolin	Shoot
47	Mannitol	Fruit
48	Maslinic acid	Leaf
49	Myristyl-Oleate	Fruit
50	N-Hentriacontane	Fruit
51	Oleanolic acid	Flower, Leaf, Root Bark, Seed, Stem
52	Oxalic acid	Bark
53	Potassium chloride	Fruit
54	Protein	Leaf
55	Psidinin-C	Bark
56	Punicalagin	Bark
57	Punicalin	Bark, Leaf
58	Quercetin-7-O-Rhamnoside	Fruit
59	Sugars	Fruit
60	Tannin	Bark, Fruit
61	Terchebulin	Bark
62	Terflavin-C	Bark
63	Terminic acid	Root, Root Bark, Bark, Plant
64	Terminolitin	Fruit

2.4 Hepatoprotective Effects of Arjuna

Arjuna exhibits significant hepatoprotective effects, particularly when administered as an ethanolic extract. In experimental models of paracetamol-induced toxicity, this extract markedly lowers serum levels of ALT, AST, ALP and total bilirubin, indicating improved liver function. Its potent antioxidant activity—mediated by upregulation of key defence enzymes such as SOD, CAT and GPx—helps scavenge free radicals and shields hepatocytes from oxidative injury. The presence of flavonoids, tannins, phenols, glycosides and saponins in Arjuna is believed to contribute to these protective effects by reducing lipid peroxidation and stabilizing cell membranes[30].

Further investigation into arjunolic acid, a principal triterpenoid in Arjuna, has demonstrated promising anti-NAFLD activity. In HepG2 cells, concentrations up to 100 μM were non-cytotoxic, with a GI25 of 379.9 μM, and at 50 μM, it reduced Oil Red O staining by 35.98 % and intracellular triglycerides by 66.36 % ($P < 0.005$). Concurrently, ALT and AST leakage decreased by 61.11 % and 48.29 %, respectively ($P < 0.005$). In high-fat-diet-fed rats, oral arjunolic acid (25–50 mg/kg) significantly lowered serum transaminases, ALP and GGT ($P < 0.005$), upregulated PPARα and FXRα expression, downregulated PPARγ, and ameliorated hepatic steatosis and inflammatory infiltration on histology [8].

Aqueous extracts of Arjuna also confer broad hepatoprotection against toxins such as CCl_4, cadmium, isoniazid and sodium fluoride. These extracts boost antioxidant defences, elevating SOD, CAT, GST and GSH, while attenuating lipid peroxidation. In models of cisplatin-induced liver injury, they reduce serum ALT, TBARS and MDA levels, thereby preserving membrane integrity and preventing oxidative protein damage [31].

Although *T. arjuna* exhibits well-documented lipid-lowering and antioxidant properties, mechanistic research assessing its effect on NAFLD remains limited. This creates a knowledge gap regarding the precise biochemical and molecular mechanisms through which *T. arjuna* may confer hepatoprotective benefits. To address the same, the following objectives were designed for this thesis.

Objectives

1. To systematically examine and evaluate the body of research to determine the main phytochemical components of *Terminalia arjuna* that may have therapeutic value.

2. To use transcriptome data to study the molecular mechanisms and biological activities of phytochemicals obtained from *T. arjuna*, especially about liver diseases such non-alcoholic fatty liver disease (NAFLD).

Chapter 3. Methods and Data Sources

3.1 RNA seq data analysis

Through a literature review, Transcriptomic (RNA-seq) data were identified to gain a deeper understanding of the current body of knowledge on the topic. After gathering phytochemical data from public sources, pertinent RNA-seq datasets were retrieved for additional examination.

3.1.1 Data Availability

Six phytochemicals were analyzed; each linked to its own RNA-seq dataset. Listed below are the phytochemicals alongside their corresponding accession numbers.

Table 2. Accession numbers of selected phytochemicals used for transcriptomic data analysis.

Phytochemical	Accession Number
Maslinic acid	PRJNA761113
Phytosterol	PRJNA759779
Gallic acid	PRJNA1169526
Ellagic Acid	PRJNA719116

3.1.2 Quality Control and Preprocessing

Raw sequencing reads in FASTQ format were subjected to quality control using FastQC (v0.11.9) to assess read quality, GC content, and duplication levels. Adapters and low-quality bases ($Q < 20$) were removed using Trimmomatic (v0.39). Clean reads were retained for further analysis.

3.1.3 Read Alignment

The cleaned reads were aligned to the respective reference genome using STAR aligner (v2.7) with default parameters. Genome indices were built using the STAR index generation mode. The alignment quality was assessed based on mapping rate, read distribution, and duplication metrics.

3.1.4 Quantification of Gene Expression

Aligned reads were quantified at the gene level using featureCounts (v2.0.1) from the Subread package with respective gene annotation in GTF format. Raw count matrices were generated for all samples.

3.1.5 Differential Gene Expression Analysis

Raw count data were imported into the DESeq2 (v1.36) package in R for differential expression analysis. Genes with low read counts across all samples were filtered out prior to normalization. The DESeq2 pipeline was used to estimate size factors, dispersion, and perform hypothesis testing. Differentially expressed genes (DEGs) were identified based on an *p*-value.

3.1.6 Functional and Pathway Enrichment Analysis

To elucidate the biological relevance of differentially expressed genes, functional annotation and pathway enrichment analysis were conducted using Gene Set Enrichment Analysis (GSEA) within the R programming environment.

Gene Ontology (GO) Enrichment

GO enrichment analysis was carried out using the clusterProfiler package, covering all three GO domains: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC). A ranked gene list was constructed by associating each gene symbol with its $\log_2$FC value. Gene symbols were converted to Entrez IDs using the bitr() function, and invalid or unmapped entries were removed. The resulting enriched GO terms were saved as CSV files for visualization and interpretation.

KEGG Pathway Enrichment

In parallel, KEGG pathway enrichment was conducted using the gseKEGG() function, applying the same parameters as above. This analysis identified key signalling pathways, metabolic routes.

Both GO and KEGG enrichment outputs provided insights into the molecular functions, cellular locations, and biological pathways significantly affected by the intervention.

Chapter 4. Maslinic Acid Ameliorates NAFLD Pathogenesis

4.1 Introduction

Phytochemical analyses of *T. arjuna* has revealed richness in oleanane-type triterpenoids, which are predominantly found in its bark and leaves[32]. Among these, maslinic acid stands out as a pentacyclic triterpenoid, specifically known as 2α,3β-dihydroxiolean-12-en-28-oic acid, and is recognized for being widely distributed across various plant species[33]. Although maslinic acid was first discovered in the peels of olives, it is also present in *T. arjuna* and several other botanical sources[34]. The increasing scientific interest in maslinic acid stems from its broad range of biological activities and promising therapeutic potential.

The following section provides an overview of maslinic acid's biochemical and pharmacological characteristics and discuss its relevance as a therapeutic agent, with particular emphasis on its effects in non-alcoholic fatty liver disease (NAFLD).

4.2 Biological and pharmacological properties of Maslinic acid

Maslinic acid is a pentacyclic triterpene belonging to the oleanane group, distinguished by two hydroxyl groups at the C-2 and C-3 positions, making it a dihydroxy derivative of oleanolic acid—a notable constituent in *T. arjuna*[32]. Its chemical structure, $C_{30}H_{48}O_4$, sets it apart from oleanolic acid due to the extra hydroxyl group on C-2[35]. In its pure form, maslinic acid appears as a white amorphous solid, insoluble in water but dissolvable in organic solvents. Importantly, it demonstrates a high degree of safety in animal studies, as even large doses have not led to toxic effects, reinforcing its status as a naturally safe compound.

Maslinic acid displays an impressive array of pharmacological effects. Its activities span anti-inflammatory, antioxidant, anti-tumor, hypoglycaemic, and neuroprotective domains[35]. At the molecular level, it influences several signalling pathways, notably by suppressing inflammation through the downregulation of NF-κB activation, thereby curtailing the release of inflammatory mole[35]. Repeatedly, maslinic acid treatment has been shown to reduce pro-inflammatory cytokines, such as TNF-α and various interleukins, in experimental models of inflammation[35]. Additionally, it bolsters antioxidant mechanisms through Nrf2 activation, which increases the expression of the body's own antioxidant enzymes, defending against oxidative[35]. Its anti-cancer properties have been attributed to its ability to hinder cancer cell growth and trigger programmed cell death in multiple cancer cell types[35]. Regarding metabolism, maslinic acid acts as a hypoglycaemic and insulin-sensitizing agent by promoting

glucose uptake and glycogen storage, as well as by suppressing glycogen phosphorylase activity. This effect is likely due to its facilitation of insulin signaling through enhanced phosphorylation of IRβ, Akt, and GSK3β [36]. The wide-ranging bioactivities of maslinic acid suggest that it is effective against complex disorders characterized by inflammation, oxidative imbalance, and metabolic disruption.

Owing to its varied biological activities, maslinic acid has been evaluated for therapeutic roles in several disease models. For example, it has demonstrated a protective effect on the heart in diabetic mice by reducing oxidative stress and glycation-related injury[35]. It also exhibits anti-angiogenic and anti-proliferative actions in experimental models of liver cancer[35]. These findings underline Maslinic acid's therapeutic promise for multiple organ diseases[35]. Among these uses, Maslinic acid's potential in addressing NAFLD, a common metabolic liver disorder currently lacking any approved medications, stands out [37].

4.3 Maslinic Acid for Non-Alcoholic Fatty Liver Disease (NAFLD)

NAFLD involves the excessive accumulation of fat in liver cells (steatosis), with the possibility of progression to more severe conditions such as inflammation (NASH) and fibrosis[33]. Maslinic acid has shown noteworthy liver-protective properties in animal models of NAFLD. In studies with obese, diet-induced mice, administration of maslinic acid led to significant reductions in overall body weight, liver mass, and liver fat content, as well as clear histological improvements with less steatosis[33]. These animals also had lower levels of serum triglycerides, glucose, and leptin, alongside an increase in adiponectin, compared to untreated mice fed a high-fat diet[33]. Such metabolic benefits suggest that maslinic acid improves key features of NAFLD, possibly by boosting insulin sensitivity and modifying adipokine levels. Indeed, maslinic acid-treated mice showed alleviated insulin resistance, increased liver glycogen stores, and decreased hepatic fat, all indicating restored liver metabolic health[36].

On a mechanistic level, maslinic acid influences several pathways related to fat metabolism and liver damage in NAFLD. A central effect is the activation of the Sirt1/AMPK signaling cascade in the liver[33]. Maslinic acid upregulates Sirtuin 1, an enzyme that senses cellular energy status, which in turn activates AMPK through phosphorylation[33]. This Sirt1/AMPK pathway is crucial for maintaining lipid balance, and its stimulation by maslinic acid inhibits acetyl-CoA carboxylase via phosphorylation, effectively reducing the body's own fat synthesis[33]. Correspondingly, NAFLD mice treated with maslinic acid exhibit decreased levels of fat-producing transcription factors (such as SREBP-1c and PPARγ) and enzymes like fatty acid

synthase in the liver[33]. At the same time, maslinic acid accelerates the breakdown of fats by enhancing the hepatic expression of lipolytic enzymes - adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), as well as carnitine palmitoyltransferase-1 and -2 (CPT-1, CPT-2), which are essential for mitochondrial fatty acid oxidation[33]. This multi-pronged approach helps shift the liver from fat storage to fat burning and clearance.

Additionally, maslinic acid supports autophagy in the liver, particularly the lipophagy process (where lipid droplets are broken down via autophagy). Experimental NAFLD models treated with maslinic acid show upregulation of autophagy markers such as Beclin-1 and ATG1, demonstrating enhanced autophagic activity. Increased lipophagy contributes to the removal of excess fat within liver cells, reducing steatosis. Furthermore, maslinic acid delivers anti-inflammatory and anti-apoptotic effects in fatty liver models. Mice on a high-fat diet and treated with maslinic acid display lower levels of inflammatory cytokines (like TNF-α and IL-1β) and decreased levels of pro-apoptotic proteins (e.g., Bax, cleaved caspase-3), while the anti-apoptotic protein Bcl-2 is increased. Collectively, these changes are associated with less liver inflammation and reduced cell death [38].

In summary, by inhibiting fat production, enhancing fat breakdown and oxidation, stimulating lipophagy, and suppressing inflammation and apoptosis, maslinic acid significantly improves NAFLD pathology[33],[38]. Studies conclude that maslinic acid ameliorates hepatic steatosis through the coordinated regulation of lipid metabolism enzymes and pathways governing lipolysis and fatty acid oxidation in the liver[33]. By directly targeting the core metabolic disruptions and inflammatory injuries that define NAFLD, maslinic acid emerges as a promising candidate for treating this increasingly common disease [38].

The RNA seq data analysis utilised 6 pancreatic cell sample of human where 3 samples were untreated and 3 samples were maslinic acid-treated downloaded from NCBI BIOPROJECT (PRJNA761113). The GTF file and FASTA sequence of the human were taken from the genome section of NCBI.

4.4 Result

4.4.1 Identification of Differentially Expressed Genes (DEGs)

To evaluate the transcriptional differences between experimental and control groups, a volcano plot (Figure 4.1A) was constructed using thresholds of $\log_2FC \geq 1$ for identifying upregulated genes, $\log_2FC \leq -1$ for downregulated genes, and a significance level of $p < 0.05$ ($-\log_{10}(p) \geq$

1.3). The analysis revealed 10 genes with significant upregulation and 34 genes with significant downregulation, indicating a broad transcriptional response characterized by a dominance of gene activation. The complete list of DEGs is provided in Supplementary file 1, and an overview of global changes in the expression of genes is shown as a heatmap in Figure 4.1B.

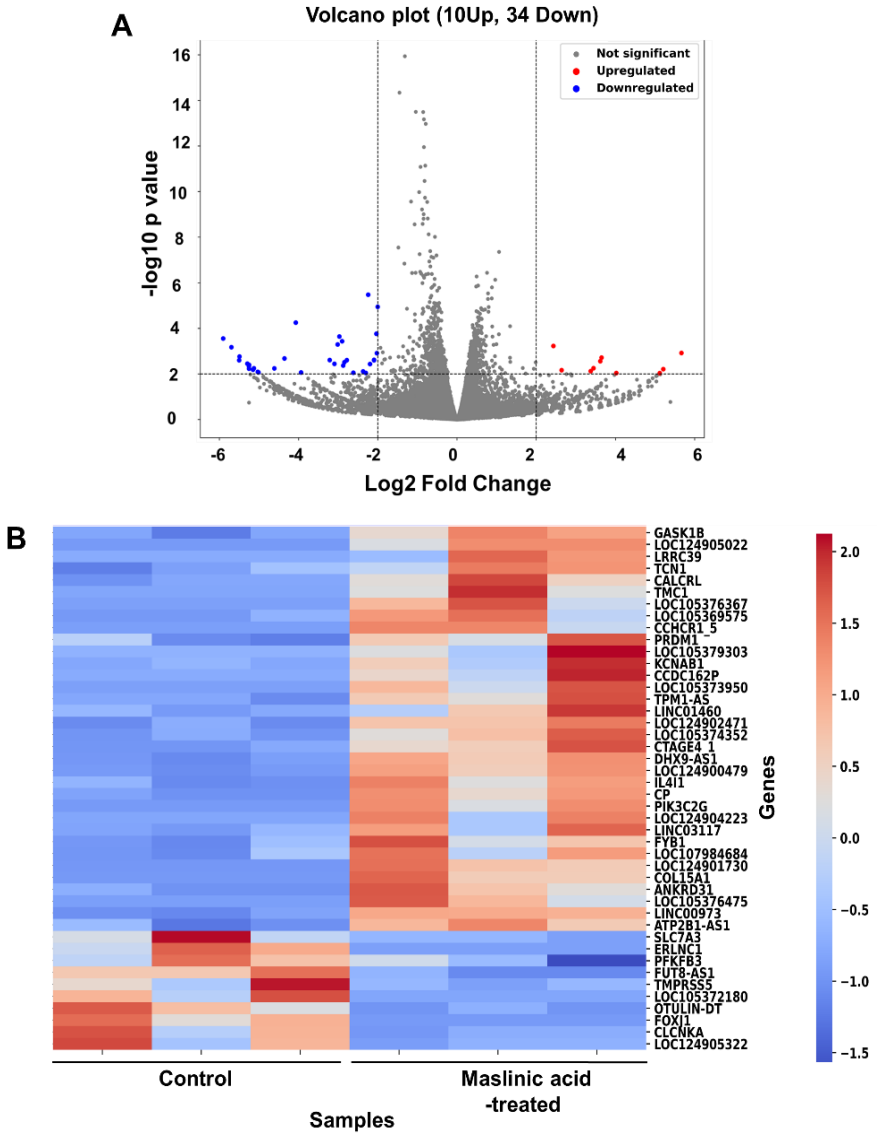

Figure 4.1. Overall changes in the expression of genes upon treatment with Maslinic acid. (A) Volcano plot depicting differential gene expression between control and Maslinic acid-treated samples. Each point represents a single gene. Significantly upregulated genes (adjusted $p < 0.05$, $|\log_2 FC| > 1$) are shown in green, while significantly downregulated genes are shown in red. The vertical dashed lines indicate ±1 $\log_2$ fold change, and the horizontal dashed line corresponds to $-\log_{10}$(adjusted p) = 2. (B) Heatmap of z score–normalized expression for all the genes across three controls and three treated groups. Red and blue denote higher and lower expression, respectively, with hierarchical clustering grouping genes by similar patterns.

4.4.2 Understanding Changes in Gene Expression and Associated Biological Responses

The KEGG pathway enrichment plot (Figure 4.2A) revealed multiple metabolic and signaling pathways associated with non-alcoholic fatty liver disease (NAFLD).

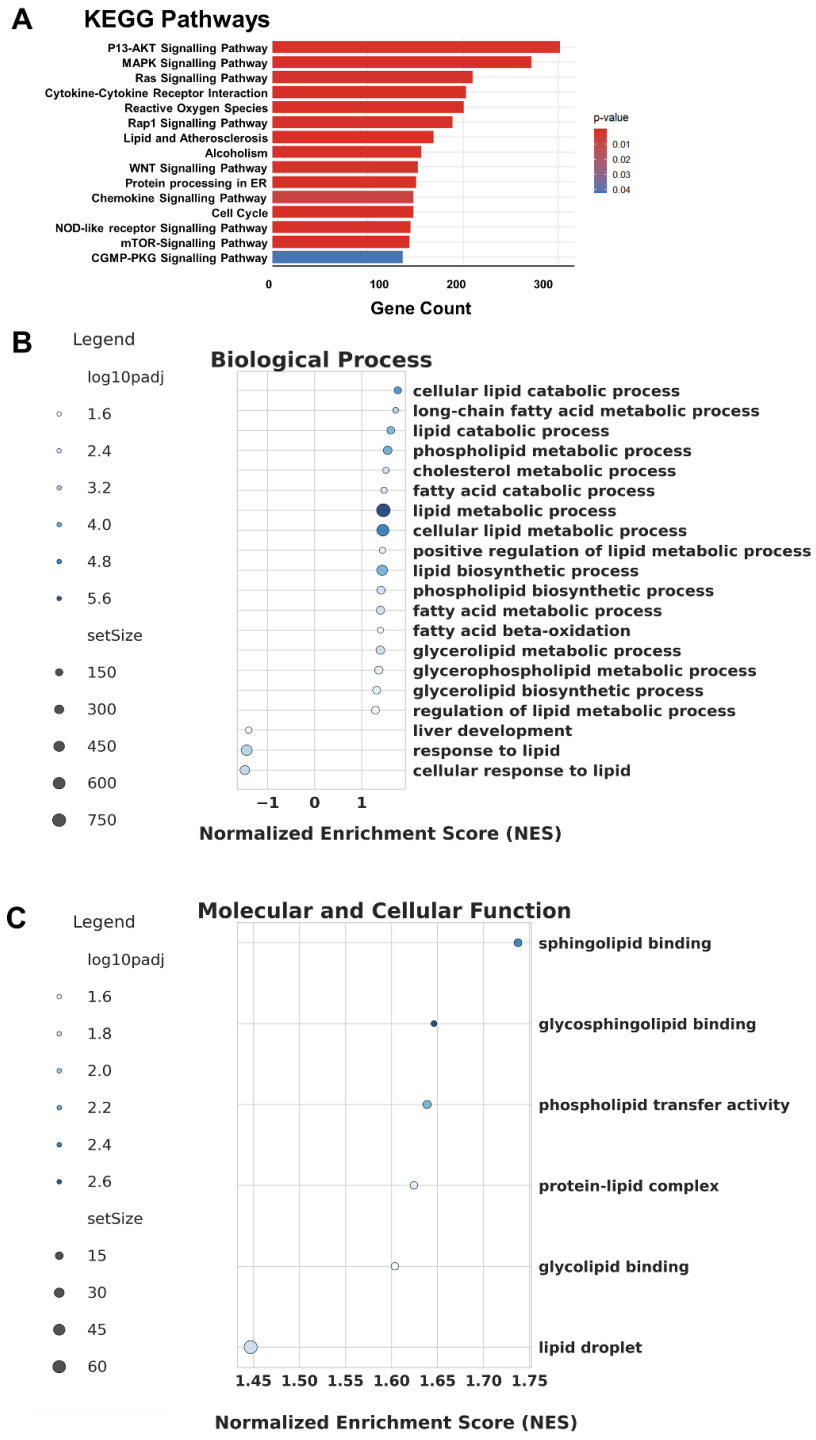

Figure 4.2. Enrichment analysis of differentially expressed genes in Maslinic-treated samples, showing (A) KEGG pathways, (B) GO – Biological Processes, and (C) GO – Molecular and Cellular Functions.

As expected, oxidative phosphorylation was significantly impaired in the diseased state. In NAFLD, excessive free fatty acids inundate liver mitochondria, promoting heightened reactive oxygen species (ROS) formation, oxidative stress, and cellular injury, alongside notable declines in electron transport chain (ETC) complexes I and IV [39].

Upon treatment with maslinic acid, a protective transcriptional reprogramming was observed. Catalase expression increased by 1.43 $\log_2$FC, facilitating hydrogen peroxide breakdown and preventing lipid peroxidation [40]. Nrf1 was upregulated by 3.10 $\log_2$FC, inducing downstream antioxidant enzymes (HO-1, NQO1, GSTs) and enhancing mitochondrial biogenesis through PGC1α [39]. Similarly, GPX1 was elevated by 2.15 $\log_2$FC, bolstering peroxide detoxification [41], while TFAM increased by 1.37 $\log_2$FC, supporting mitochondrial DNA integrity and reducing ROS [42]. Collectively, these changes suggest that maslinic acid ameliorates oxidative stress and restores mitochondrial function in NAFLD.

Regulation of Lipid Metabolism and Insulin Resistance

Other enriched pathways were linked to insulin resistance, lipid handling, and inflammation, hallmarks of NAFLD progression [43]. Maslinic acid treatment resulted in a 1.22 $\log_2$FC upregulation of AdipoR1, strengthening adiponectin's LKB1–AMPK signaling cascade to inhibit acetyl-CoA carboxylase (suppressing lipogenesis) and activate CPT1 (enhancing fatty acid oxidation) [44]. PPARα expression rose by 2.24 $\log_2$FC, leading to upregulation of CPT1, ACOX1, and MCAD—key drivers of mitochondrial/peroxisomal β-oxidation and VLDL secretion [45]. Similarly, PPARδ was up by 1.08 $\log_2$FC, further promoting fatty acid utilization and lipophagy [46]. Anti-inflammatory regulation was also evident, with IL1RN (2.06 $\log_2$FC) blocking IL-1 signaling [47], and NQO1 (1.33 $\log_2$FC) enhancing insulin sensitivity and reducing immune infiltration in the liver [48].

Metabolic Rewiring in NAFLD and Maslinic Acid's Corrective Action

Beyond lipid metabolism, maslinic acid influenced other KEGG pathways. Amino acid biosynthesis, often disrupted in NAFLD via branched-chain amino acid dysregulation [49], showed corrective shifts. In glycolysis/gluconeogenesis, NAFLD typically skews metabolism toward gluconeogenesis with upregulated PEPCK and G6Pase [50]; here, maslinic acid activated AMPK/SIRT1 (2.24 $\log_2$FC), reducing glucose, leptin, and free fatty acids while elevating adiponectin and downregulating lipogenic factors [33].

The TCA cycle, frequently disrupted in NAFLD by citrate export for lipogenesis [51], also showed normalization. Similarly, the PI3K-Akt pathway, suppressed in NAFLD [52], was partially restored by maslinic acid. Dysregulated lipid and atherosclerosis pathways, marked by reduced ABCA1 (–1.06 log_2FC) [54], ABCG5/8 (–1.39 log_2FC) [55], and ANGPTL3 (–3.39 log_2FC) [56], suggested improved cholesterol efflux and reduced lipid burden upon treatment. Disturbances in steroid biosynthesis [57] and glycerophospholipid metabolism [58], both implicated in fibrosis and ER stress, were also moderated.

GO Biological Processes and Lipid Homeostasis

Gene ontology analysis (Figure 4.2B) highlighted lipid metabolic processes as major contributors to NAFLD pathology. Insulin resistance-induced adipose lipolysis increases hepatic fatty acid influx, while aberrant insulin signaling keeps SREBP1c/ChREBP active, fueling de novo lipogenesis [59]. Consistent with this, CD36 was upregulated by 0.90 log_2FC, correlating with hepatic lipid accumulation [60]. Positive regulation of lipid metabolism, linked to PPARγ and SREBP1c activity [61], showed a notable rise in SCD1 expression (3.29 log_2FC), which—though paradoxically promoting unsaturated fat synthesis—may confer resistance to lipotoxic apoptosis [62].

Maslinic acid also improved β-oxidation capacity. CPT1A, an isoform resistant to malonyl-CoA inhibition, increased by 0.98 log_2FC, offering protection against diet-induced steatosis [64]. Autophagy-related clearance of lipid droplets was evident through Atg2 upregulation (2.21 log_2FC) [68], suggesting that maslinic acid enhances lipophagy to alleviate hepatocellular fat accumulation.

Cellular Stress, Inflammation, and Survival Pathways

NAFLD progression is driven by ER stress, NF-κB/JNK-mediated inflammation, and apoptosis [70]. Here, BAX was elevated by 1.14 log_2FC, consistent with pro-apoptotic signaling [71], though reductions in fibrosis-associated MMP9 (–0.99 log_2FC) [73] may reflect protective remodeling. Genes regulating hepatocyte differentiation, such as HNF4α, remained suppressed in NAFLD models [72], but maslinic acid may counterbalance this by supporting regenerative potential.

Molecular and Cellular Functions

In molecular function analysis (Figure 4.2C), maslinic acid was shown to influence processes linked to lipid droplet stability, phospholipid transfer, and VLDL secretion. Overexpression of PLIN2 and defective ATGL/PNPLA2-mediated mobilization in NAFLD promote triglyceride

retention [74]. Maslinic acid modulated these pathways to restore lipid trafficking. Moreover, disruption of glycolipid and sphingolipid metabolism, linked to insulin resistance and inflammation [75],[76], was alleviated. VLDL assembly and secretion, often impaired in NAFLD due to APOB100 defects [77], were supported by maslinic acid, preventing hepatocellular fat trapping.

The further details for gene ontology analysis with biological processes, molecular and cellular function, and KEGG pathway enrichment analysis are given in Supplementary file 2 and Supplementary file 3, respectively.

Network Analysis of Maslinic Acid Targets

Finally, Cytoscape-based network visualization (Figure 4.3) provided an integrated perspective of maslinic acid's effects. Functional enrichment maps highlighted clusters in oxidative stress, lipid metabolism, inflammation, and immune regulation. Central hub genes such as PIK3C2G, IL1B, SYT13, and CHRNA1 emerged as critical regulators of maslinic acid's pleiotropic actions. Peripheral clusters suggested roles in DNA stability and protein processing, underscoring broader cytoprotective functions.

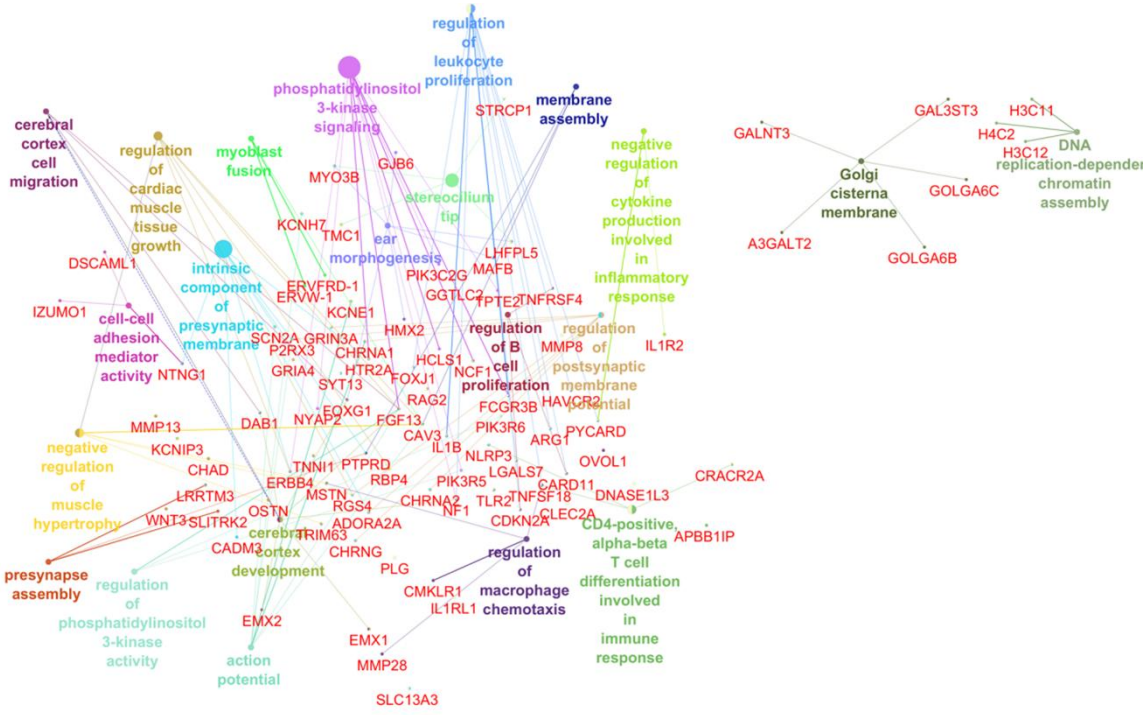

Figure 4.3. Cytoscape network of maslinic acid targets highlighting key genes, pathways, and biological processes based on GO enrichment analysis.

Altogether, precise adjustments in transporters, nuclear receptors, antioxidants, cytokine modulators, autophagy regulators, and receptors form a multipronged strategy to reverse NAFLD—restoring metabolism, mitigating oxidative damage, resolving inflammation, and preserving hepatocyte health. For instance, VDR was found to be downregulated by 0.99 log_2FC, aligning with studies showing that vitamin D receptor knockout mice exhibit resistance to steatosis, suggesting that its suppression may have a paradoxical protective role in NAFLD [78]. Similarly, ChREBP demonstrated context-dependent regulation—while its activation promotes de novo lipogenesis under nutrient-rich conditions, it has also been reported to improve insulin sensitivity by facilitating glycolytic flux and hepatic lipid partitioning [79]. These nuanced regulatory effects highlight the complexity of NAFLD pathophysiology and underscore how maslinic acid fine-tunes multiple interconnected pathways to achieve metabolic restoration.

Taken together, these findings demonstrate that maslinic acid exerts broad and coordinated regulatory effects on oxidative stress defense, lipid and glucose metabolism, inflammatory signaling, and hepatocellular survival. By targeting both upstream sensors and downstream effectors across multiple pathways, maslinic acid mitigates mitochondrial dysfunction, alleviates steatosis, and restores metabolic flexibility. The network-level insights further reveal hub genes and processes that may serve as biomarkers of therapeutic efficacy. Overall, maslinic acid emerges as a promising natural compound with multifaceted potential for the management of NAFLD and its associated metabolic complications.

Chapter 5. Therapeutic Potential of Phytosterol in NAFLD

5.1 Introduction

Phytosterols, comprising plant-derived sterols and stanols, are naturally occurring compounds integrated into plant cell membranes. Structurally, they resemble cholesterol but possess distinguishing ethyl or methyl substitutions in their side chains. This similarity enables phytosterols to compete with cholesterol for incorporation into intestinal micelles, thereby markedly reducing cholesterol absorption. While primary dietary sources include vegetable oils, seeds, legumes, and grains, phytosterols are also abundantly present in medicinal botanicals such as *Terminalia arjuna*[80].

5.2 Pharmacological Properties of Phytosterols

Beyond their cholesterol-lowering ability, phytosterols exhibit diverse bioactive properties, including antioxidant, anti-inflammatory, immunomodulatory, and hepatoprotective effects[80]. The most extensively studied phytosterols—β-sitosterol, campesterol, and stigmasterol—reduce intestinal cholesterol uptake by competitively displacing it within micelles[81]. In addition, these compounds inhibit activation of the nuclear factor kappa B (NF-κB) signalling pathway, thereby suppressing the release of inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)[82]. Phytosterols also strengthen antioxidant defenses by upregulating enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, thus safeguarding hepatocytes from oxidative injury[83].

Terminalia arjuna, a cornerstone of Ayurvedic medicine, is particularly rich in phytosterols—most notably β-sitosterol—which significantly contributes to its hepatoprotective activity[84]. In the context of non-alcoholic fatty liver disease (NAFLD), these phytosterols exert multifaceted benefits by modulating lipid metabolism, reducing hepatic cholesterol accumulation, and attenuating inflammatory responses. In murine NAFLD models, supplementation with *T. arjuna* bark extract was shown to downregulate key lipogenic genes such as FASN, SREBP-1c, and ACC1, while enhancing the expression of fatty acid oxidation regulators including PPARα and CPT1A[84]. This intervention improved liver function parameters by lowering serum ALT and AST levels, alleviated oxidative stress, and ameliorated histopathological features of steatosis.

Moreover, phytosterols from *T. arjuna* inhibited the NF-κB pathway, reducing hepatic expression of inflammatory mediators such as TNF-α, IL-6, and iNOS, thereby limiting

inflammation-associated hepatic damage. Collectively, these findings suggest that phytosterol-rich preparations from *T. arjuna* may hold therapeutic value for NAFLD, particularly during its early metabolic and inflammatory phases.

Given the well-established antioxidant and lipid-regulatory effects of *T. arjuna*-derived phytosterols—and their influence on central molecular regulators of lipid homeostasis and inflammation—it is essential to investigate the mechanistic basis of these hepatoprotective actions in NAFLD. While earlier pharmacological studies have reported favorable biochemical and histological outcomes, the comprehensive transcriptional impact of phytosterol intervention remains poorly understood. RNA sequencing (RNA-Seq), as a high-resolution genomic approach, enables systematic profiling of hepatic gene expression changes in response to treatment. Accordingly, we employed RNA-Seq to delineate differentially expressed genes and enriched pathways modulated by *T. arjuna* phytosterols, thereby providing deeper insights into the molecular framework underlying their therapeutic potential in NAFLD.

5.3 Result

5.3.1 Identification of Differentially Expressed Genes

To assess the transcriptional differences between the control and phytosterol-treated samples, a volcano plot (Figure 5.1A) was created using thresholds of $\log_2FC \geq 1$ for identifying upregulated genes, $\log_2FC \leq -1$ for downregulated genes, and a significance level of $p < 0.05$ ($-\log_{10}(p) \geq 1.3$). This analysis identified 65 genes that were significantly upregulated and 40 genes that were significantly downregulated, highlighting a widespread transcriptional response dominated by gene activation. Most differentially expressed genes exhibited moderate expression changes, with $\log_2FC$ values falling between ±1 and ±2, indicating the involvement of finely tuned regulatory mechanisms impacting crucial cellular functions. The complete list of DEGs is provided in Supplementary file 4, and an overview of global changes in the expression of genes is shown as a heatmap in Figure 5.1B.

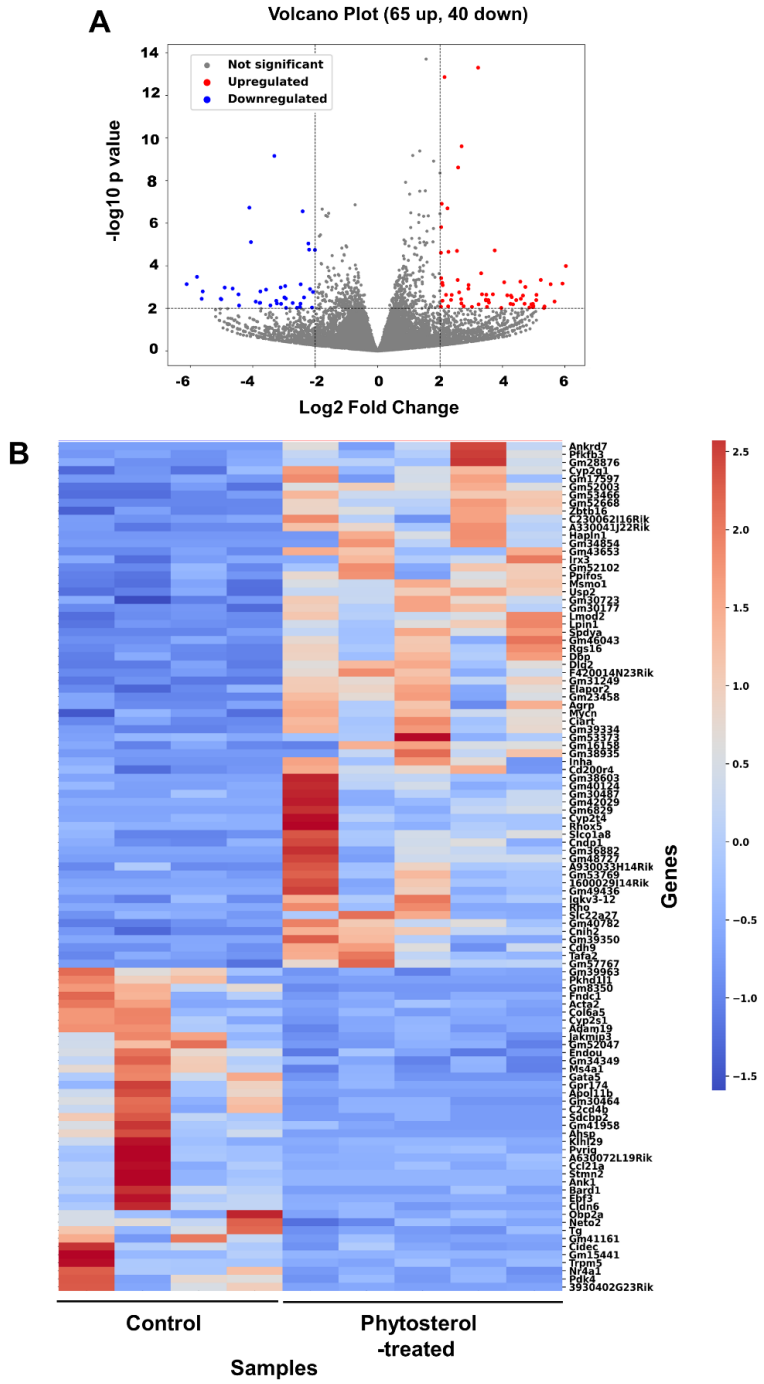

Figure 5.1. Overall changes in the expression of genes upon treatment with Phytosterol. (A) Volcano plot of gene expression changes between phytosterol-treated and control samples. Each dot represents a gene; green dots indicate significant up-regulation and red dots significant down-regulation (adjusted $p < 0.05$, $|\log_2 FC| > 1$), while gray dots are non-significant. Vertical lines at ±1 $\log_2$ fold-change and a horizontal line at $-\log_{10}(\text{adjusted } p) = 2$ denote significance thresholds. (B) Heatmap of z-score–normalized expression for dysregulated genes control and phytosterol-treated replicates. Red denotes higher expression and blue lower; hierarchical clustering groups genes with similar expression profiles.

5.3.2 Gene Set Enrichment Analysis Results

The KEGG pathway analysis highlighted multiple biological processes associated with the onset and progression of non-alcoholic fatty liver disease (NAFLD) (Figure 5.2A).

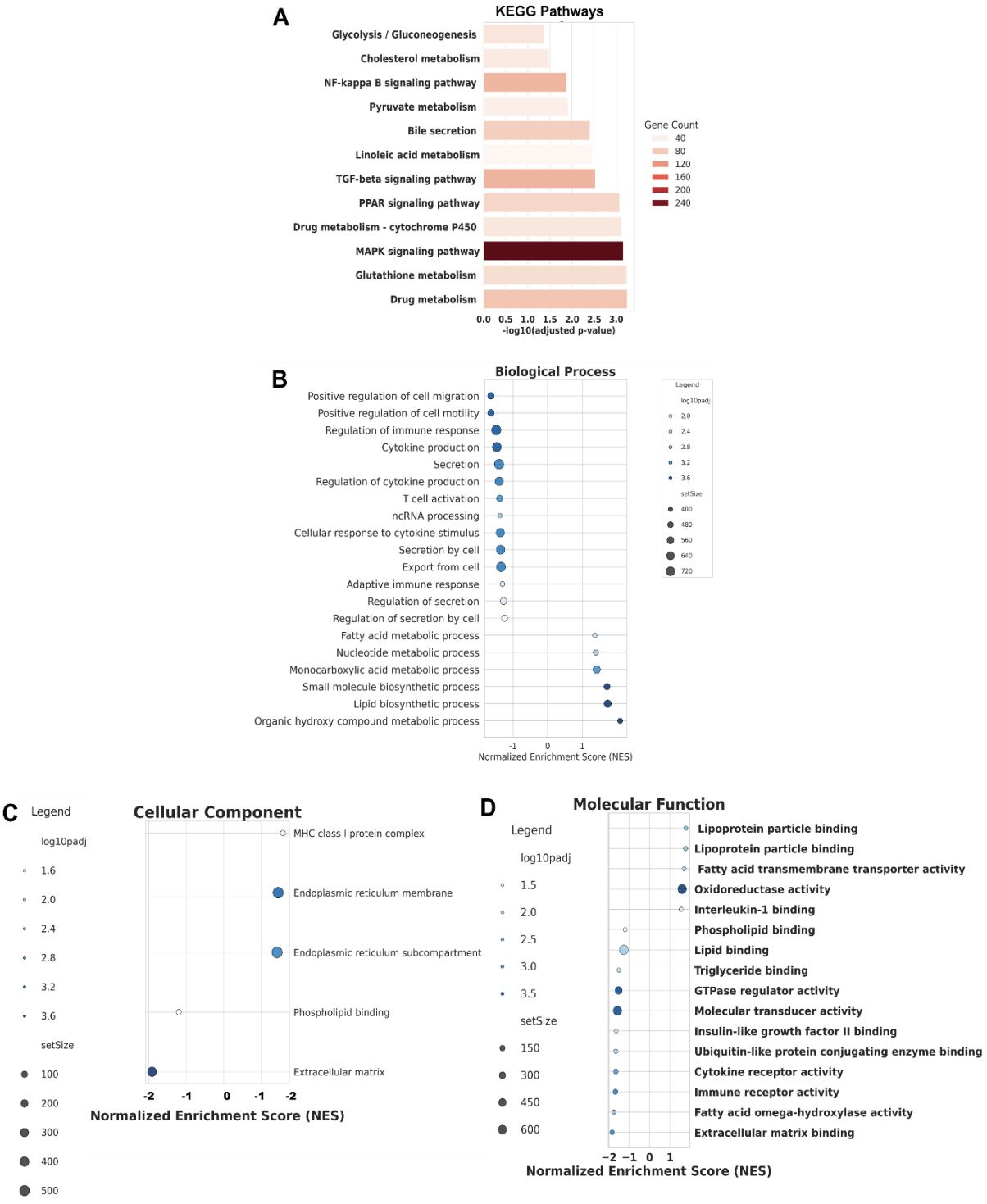

Figure 5.2. Enrichment analysis of differentially expressed genes in Phytosterol-treated samples. (A) Top ten KEGG pathways enriched among differentially expressed genes. Bar

lengths correspond to $-\log_{10}$(adjusted p) and shading reflects gene count. Prominent pathways include MAPK signaling, cytochrome P450–mediated drug metabolism, PPAR signaling, and glutathione metabolism. (B) GO Biological Process bubble plot arranged by normalized enrichment scores (NES). Enriched processes cover organic hydroxy compound metabolism, lipid biosynthesis, small-molecule biosynthetic pathways, immune response regulation, and cytokine production. (C) GO Molecular Function enrichment bubble plot. The x-axis shows NES, bubble size indicates the number of genes in each set, and color depth represents $-\log_{10}$(adjusted p). Key enriched functions include lipoprotein particle binding, oxidoreductase activity, and phospholipid binding. (D) GO Cellular Component enrichment bubble plot, sorted by NES. Major components include the MHC class protein complex, endoplasmic reticulum membrane and subcompartment, and the extracellular matrix.

Among these KEGG pathways, the MAPK signalling pathway was markedly enriched, consistent with its established role in promoting inflammation, apoptosis, and insulin resistance—pathophysiological features observed in NAFLD and accentuated in non-alcoholic steatohepatitis (NASH)[85]. Likewise, the TGF-β signalling pathway was prominent, reflecting its contribution to hepatic fibrosis through the activation of stellate cells and excessive extracellular matrix production[86].

Differential gene expression analysis further supported these findings. REV-ERB (NR1D1) was upregulated (~1.3 log2 fold), consistent with its protective role in inhibiting fibrosis-related genes and delaying NASH progression[87]. Additionally, FGF7 displayed robust upregulation (>3 log2 fold), while FGF18 also increased (>1.0 log2 fold). These changes align with previous evidence demonstrating that FGF7 therapy and elevated FGF18 expression promote hepatocyte survival, attenuate fibrosis and inflammation, and suppress stellate cell activation[88],[89].

Conversely, the NF-κB pathway remained partially active, enhancing pro-inflammatory mediators such as TNF-α and IL-6[90]. Interestingly, CRLF1 expression was markedly elevated (>4.5 log2 fold), although this appears to lack pathogenic relevance, as prior studies indicate CRLF1 does not directly contribute to inflammation or liver injury[91]. In contrast, IL-10 was significantly upregulated (~3 log2 fold), reinforcing its crucial anti-inflammatory role, given that reduced IL-10 levels exacerbate insulin resistance and hepatic inflammation. Similarly, downregulation of KLRB1 (~2 log2 fold) corroborates its protective effect by reducing liver inflammation[92].

Oxidative Stress and Metabolic Regulation

NAFLD is characterized by profound metabolic derangements, including oxidative stress. Disruptions in cytochrome P450-mediated drug metabolism impair hepatic detoxification and promote oxidative burden[93], while abnormalities in bile secretion further aggravate lipid imbalance and inflammation[94].

Our dataset revealed dysregulation of the PPAR signalling pathway, a key regulator of glucose and lipid homeostasis. Its modulation is therapeutically relevant, as PPAR activation enhances insulin sensitivity and reduces hepatic steatosis[95]. Antioxidant defense was also impaired, with GPX5 expression markedly reduced (~2.8 log2 fold), reflecting compromised glutathione metabolism, a hallmark of NAFLD[96]. Although counterintuitive, this downregulation may represent a compensatory adaptation, potentially mitigating DNA damage and lipid peroxidation[97].

Metabolic transcriptional regulators were also altered. HDAC9 was downregulated (~1.0 log2 fold), consistent with previous reports showing that HDAC9 suppression reduces gluconeogenesis and improves glucose handling[98]. Additional perturbations in pyruvate, linoleic acid, and cholesterol metabolism further contributed to intracellular stress, lipid accumulation, and inflammation[99–101].

Genes Associated with Lipid Accumulation

Several genes implicated in lipid regulation were differentially expressed. CD9 expression increased (~0.9 log2 fold), a change linked to lower hepatic triglyceride accumulation, improved liver function, and reduced liver/body weight[102]. Folate metabolism showed a striking upregulation (>3.2 log2 fold), consistent with its role in decreasing hepatic lipid deposition and potential utility as a diagnostic or therapeutic target[103]. In contrast, WISP1 (Ccn4) (~1.0 log2 fold downregulation) was associated with reduced lipid accumulation[104], while HHEX (~1.0 log2 fold downregulation) likely supported lipid droplet integrity and balanced lipophagy[105]. Downregulation of Zfp423 (~1.0 log2 fold) further promoted healthy adipocyte development, protecting against excessive hepatic lipid burden.

Together, these results underscore that phytosterol treatment modulates multiple lipid-regulating and fibrogenic pathways, reinforcing its therapeutic relevance in NAFLD.

Biological Processes Enrichment

Biological processes enrichment analysis revealed disruptions in lipid biosynthesis, fatty acid metabolism, small molecule biosynthesis, and organic hydroxy compound turnover (Figure 5.2B). These findings highlight fundamental impairments in hepatic metabolic capacity[106]. Consistent with NAFLD pathology, oxidative stress, mitochondrial dysfunction, and hepatic lipid accumulation emerged as central features. Perturbations in monocarboxylic acid and nucleotide metabolism further suggested impaired energy generation and nucleic acid turnover, potentially hindering hepatocyte regeneration[107],[108].

Immune-related pathways, including T-cell activation, adaptive immunity, and cytokine signaling, were also enriched, reflecting the key role of immune dysregulation in progression to NASH[109]. Dysregulated cytokine regulation and secretion amplified metabolic stress[110],[111], while pathways linked to cell motility and migration pointed to fibrotic remodeling and immune cell infiltration[112],[113]. Notably, enrichment of ncRNA processing pathways suggested an additional regulatory layer in inflammation and lipid metabolism[114].

Molecular Function Alterations

Molecular function analysis (Figure 5.2C) revealed impairments in lipid handling, including fatty acid transport, triglyceride interactions, lipoprotein binding, and general lipid binding[115–117]. These defects underlie hepatocellular fat accumulation. Increased oxidoreductase activity indicated enhanced oxidative stress, a known driver of hepatocyte damage[118]. Immune-related molecular functions, such as cytokine receptor signaling and interleukin-1 binding, further highlighted inflammatory activity during advanced NAFLD/NASH[119–120]. Disruptions in GTPase signaling suggested impaired insulin responsiveness and inflammation[121]. Additionally, enrichment of extracellular matrix-binding functions was consistent with fibrosis[122], while altered ubiquitin interactions reflected impaired protein turnover[62].

Cellular Compartment Analysis

Cellular compartment enrichment (Figure 5.2D) identified the endoplasmic reticulum (ER) as a major site of disruption. As a hub of lipid synthesis, detoxification, and protein folding, ER dysfunction in NAFLD promotes unfolded protein response activation, inflammation, and hepatocyte injury[123]. Elevated caspase-12 (~1.0 log2 fold) suggested a protective adaptation against ER stress-mediated apoptosis[124]. Genes involved in phospholipid binding were also affected, indicating changes in membrane composition that could impair insulin signaling and

promote inflammation[78]. Furthermore, enrichment of MHC class I proteins pointed to increased immune surveillance, consistent with immune-mediated hepatocyte damage in NAFLD[125]. Extracellular matrix-associated changes further indicated fibrotic remodeling[126].

The complete gene ontology enrichment data has been provided in Supplementary file 5, and KEGG pathway enrichment data in Supplementary file 6.

Gene Interaction Network

The Cytoscape gene interaction network (Figure 5.3) revealed interconnected clusters spanning metabolism, immune regulation, and signaling. Key clusters included ERK1/ERK2 cascade, Ras protein signal transduction, retinoid binding regulation, potassium and calcium ion channel activity, and lymphocyte homeostasis. Intriguingly, clusters related to mesonephros development and dendritic extension suggested systemic effects extending beyond hepatic regulation. Central network nodes included Erbb4, Cdh11, Fgfr, Cyp2c family members, and immunoglobulin heavy chain variants (e.g., *Ighv1-7*, *Ighv10-1*), underscoring the pleiotropic nature of phytosterol action. Peripheral modules such as reciprocal meiotic recombination and cell wall disintegration reflected more specialized or condition-specific responses. Collectively, this network highlights the multi-target, pleiotropic therapeutic potential of phytosterols in NAFLD management.

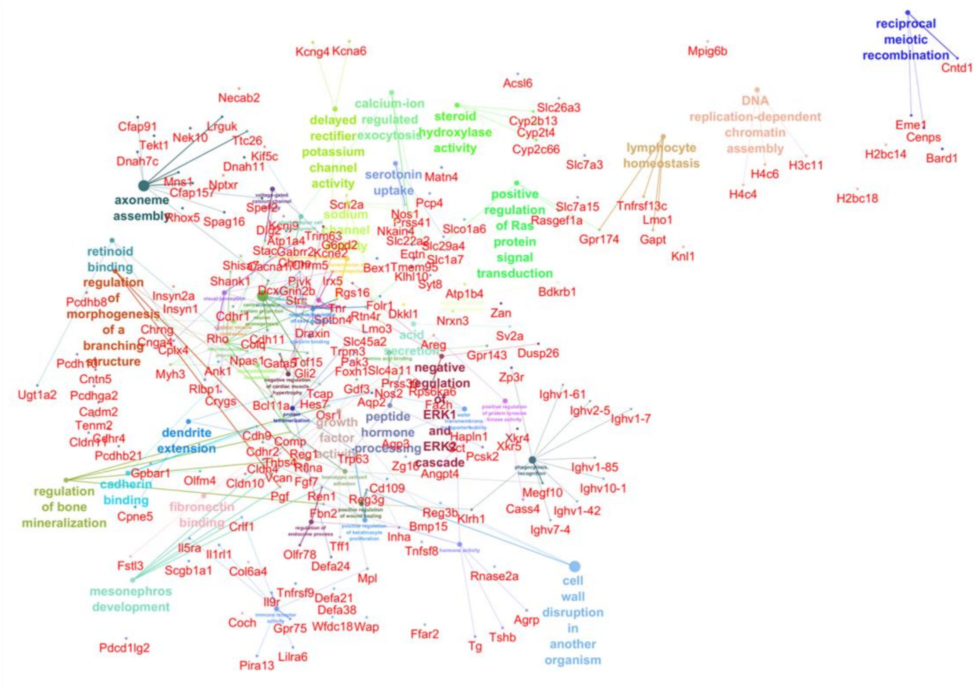

Figure 5.3. Cytoscape-derived interaction network depicting phytosterol-induced reprogramming of biological processes.

In conclusion, phytosterols derived from *Terminalia arjuna* represent a promising therapeutic avenue for the management of NAFLD. By simultaneously regulating lipid metabolism, enhancing antioxidant defenses, and suppressing inflammatory signalling, these bioactive compounds offer a multipronged approach to hepatoprotection. The observed improvements in biochemical, molecular, and histopathological markers highlight their potential to intervene in both the metabolic and inflammatory phases of NAFLD progression. Importantly, RNA-Seq–based transcriptional profiling provides an opportunity to uncover the molecular networks driving these protective effects, thereby laying the foundation for developing phytosterol-based therapeutic strategies in the clinical management of NAFLD.

Chapter 6. Therapeutic Role of Ellagic Acid in NAFLD

6.1 Introduction

Terminalia arjuna bark is rich in polyphenols, notably free ellagic acid and its glycosidic derivatives, such as 3-O-methyl ellagic acid 4-O-β-D-xylopyranoside and ellagic acid 3-O-rhamnoside. In addition, the bark extracts contain a variety of phenolic acids, including gallic acid, ellagic acid, and their derivatives, with ellagitannins identified as the predominant constituents through HPLC analysis[127,128]. The presence of ellagitannins in *T. arjuna* bark has further been confirmed by qualitative profiling using HPLC-ESI-QTOF-MS/MS across multiple plant tissues[128].

6.2. Therapeutic Effects of Ellagic Acid

Ellagic acid, owing to its polyphenolic framework, exhibits a broad spectrum of pharmacological activities. It acts as a potent scavenger of reactive oxygen species (ROS) and activates the Keap1–Nrf2–ARE signaling axis, thereby enhancing antioxidant defenses and preventing lipid peroxidation[129,130]. Ellagic acid also exerts strong anti-inflammatory effects by inhibiting NF-κB and MAPK signaling pathways, which suppresses the production of pro-inflammatory cytokines such as TNF-α and IL-6 in metabolic tissues[129]. In experimental models of liver fibrosis, Ellagic acid has been shown to inhibit the transdifferentiation of quiescent hepatic stellate cells into myofibroblast-like cells, thereby limiting extracellular matrix deposition and directly demonstrating anti-fibrotic potential[131]. Furthermore, ellagic acid derived from *Terminalia arjuna* fruit has been reported to protect human lymphocytes from chromium- and cobalt-induced cytotoxicity by restoring cell viability and proliferation, reducing apoptosis, and normalizing cytokine secretion profiles[132].

6.3 Mechanisms by Which Ellagic Acid Ameliorates NAFLD

Ellagic acid exerts protective effects against non-alcoholic fatty liver disease (NAFLD) through multiple synergistic mechanisms. Ellagic acid activates AMP-activated protein kinase (AMPK), which promotes nuclear translocation of Nrf2 and enhances the expression of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione (GSH). This cascade reduces lipid peroxidation and mitigates oxidative stress in hepatocytes. Concurrently, Ellagic acid suppresses NF-κB signaling, leading to reduced secretion of pro-inflammatory cytokines including TNF-α and IL-6, as well as decreased macrophage infiltration in liver tissue.

In addition to its direct hepatic effects, Ellagic acid and its gut-derived metabolite urolithin A further activate AMPK, thereby inhibiting acetyl-CoA carboxylase, suppressing de novo lipogenesis, and enhancing fatty acid β-oxidation[133]. The biotransformation of Ellagic acid into urolithins by commensal gut microbiota contributes additional benefits by improving insulin sensitivity, modulating bile acid metabolism, and strengthening intestinal barrier integrity, which collectively reduce endotoxin-mediated hepatic injury via the gut–liver axis[134].

Clinical evidence also supports these mechanisms. In a randomized, double-blind trial, supplementation with 180 mg/day of Ellagic acid for eight weeks in NAFLD patients resulted in significant reductions in serum ALT, AST, triglycerides, LDL cholesterol, oxidative stress markers, and insulin resistance compared with placebo[135].

6.4 Result

6.4.1 Identification of Differentially Expressed Genes

As shown in Figure 6.1A, transcriptome profiling detected 27,270 transcripts, of which 2,370 were significantly up-regulated and 1,220 were significantly down-regulated. The complete dataset is available in Supplementary file 7.

The volcano plot provides a global view by plotting effect size against statistical confidence. Along the x-axis, $\log_2$ fold change indicates direction and magnitude of regulation: positive values (right) represent up-regulation, while negative values (left) indicate down-regulation. The y-axis represents $-\log_{10}$(p-value), where higher values correspond to stronger statistical support. Thresholds are marked by vertical dashed blue lines at ±1 (≥2-fold change) and a horizontal blue line at $-\log_{10}(0.05) \approx 1.3$ ($p < 0.05$). Genes meeting both criteria appear as green points (up-regulated, upper right) or red points (down-regulated, upper left), while grey points clustered near the centre represent non-significant changes.

Altogether, the analysis highlights a robust subset of differentially expressed genes, forming a strong basis for downstream enrichment and pathway analyses. Global changes in gene expression are further illustrated as a heatmap in Figure 6.1B.

6.4.2 Gene Set Enrichment Analysis Results

Despite consuming little alcohol, triglycerides accumulated in hepatocytes, causing non-alcoholic fatty liver disease (NAFLD), which set off a series of events that included insulin

resistance, chronic inflammation, and ultimately fibrosis. Enriched KEGG pathways revealed that NAFLD arose from continuous disruptions in inflammatory signalling, cellular stress, and metabolic regulation (Figure 6.2A). In addition, abnormalities in endo-lysosomal trafficking inhibited autophagy and lipid processing, exacerbating fat formation[136].

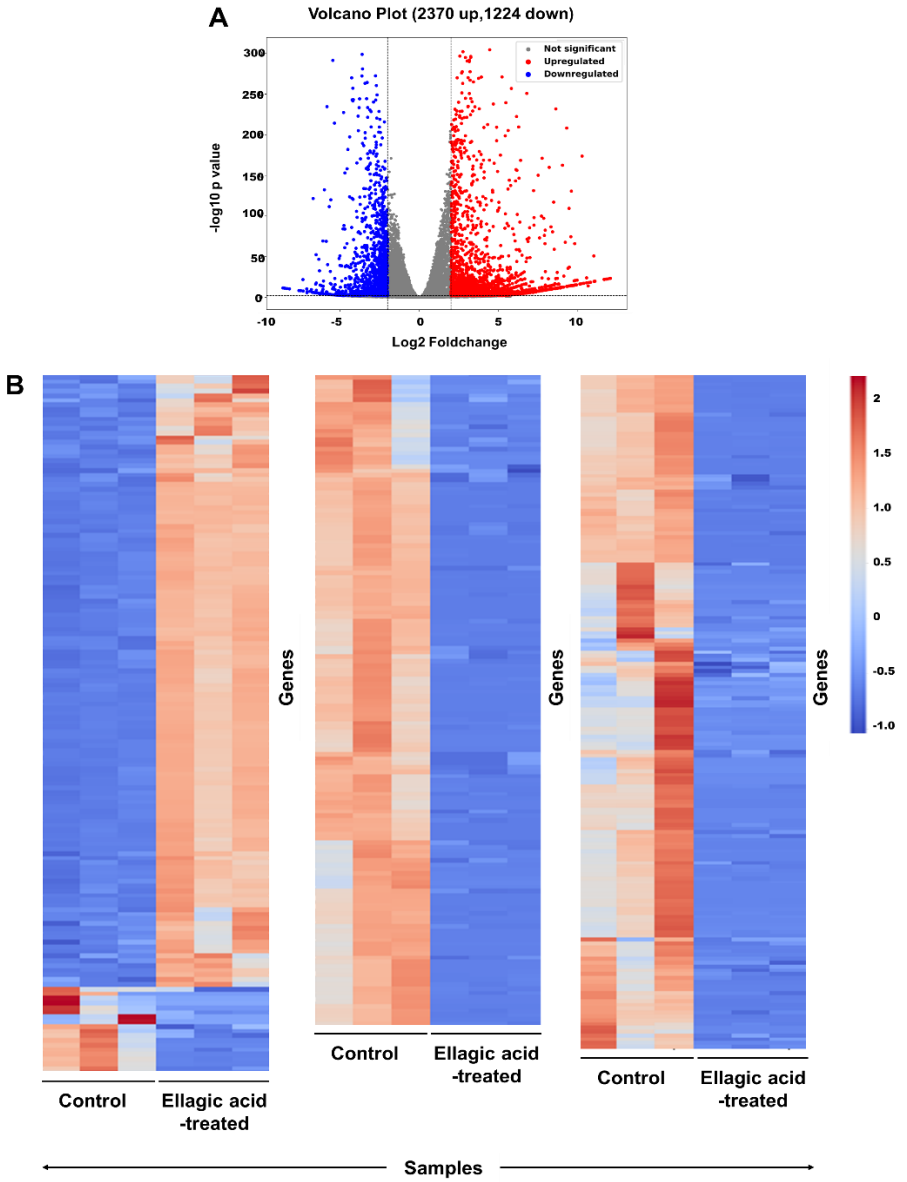

Figure 6.1. Overall changes in the expression of genes upon treatment with Ellagic acid. (A) Volcano plot of gene expression changes between Ellagic acid-treated and control samples. Each dot represents a gene; green dots indicate significant up-regulation and red dots significant down-regulation (adjusted $p < 0.05$, $|\log_2 FC| > 1$), while gray dots are non-significant. Vertical lines at ±1 $\log_2$ fold-change and a horizontal line at $-\log_{10}$(adjusted p) = 2 denote significance thresholds. (B) Heatmap of z-score–normalized expression for dysregulated genes control and Ellagic acid-treated samples. Red denotes higher expression

and blue lower; hierarchical clustering was used to group the genes with similar expression profiles.

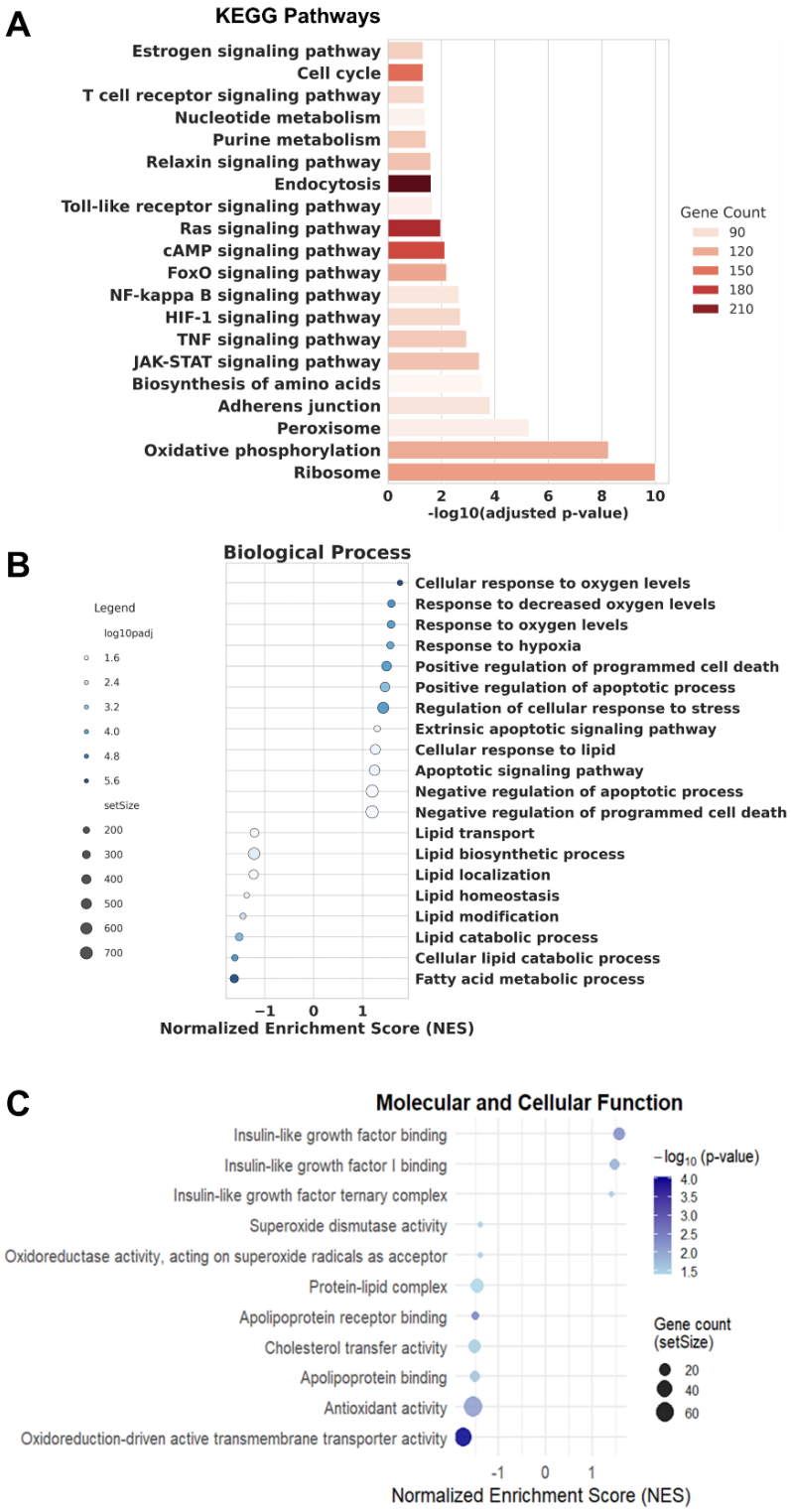

Figure 6.2. Enrichment analysis of differentially expressed genes in Phytosterol-treated samples. (A) Bar chart of the most significantly enriched KEGG pathways among all

differentially expressed genes. Bar length represents –log₁₀(adjusted *p*), and color intensity corresponds to the number of genes in each pathway. Prominent pathways include ribosome, oxidative phosphorylation, peroxisome, and JAK–STAT signaling. (B) Bubble plot of GO Biological Process enrichment. the x-axis is the normalized enrichment score (NES), bubble size reflects the number of genes in each term, and color depth indicates –log₁₀(adjusted *p*). Highlighted processes cover oxygen response, apoptosis regulation, lipid transport, and fatty acid metabolism. (C) Bubble plot of GO Molecular and Cellular Function enrichments, ordered by NES. Bubble size denotes set size and color intensity denotes –log₁₀(adjusted *p*). Key functions include IGF binding, oxidoreductase activity, protein–lipid complexes, and cholesterol transfer.

When SCD1 expression was reduced by slightly more than 2 on the log2 scale in our SCD1 knockout dataset, hepatocytes were driven towards enhanced fatty acid breakdown. This shift increased carnitine palmitoyltransferase activity while suppressing lipogenic genes, thereby reducing VLDL secretion and triglyceride synthesis[62]. Dysregulated cAMP signalling further disrupted lipolysis and insulin sensitivity, though phosphodiesterase inhibitors could aid in re-establishing equilibrium[137]. Altered FoxO transcription factors reduced fatty acid oxidation and antioxidant defences, leading to oxidative damage and lipid overload[138]. In steatotic hepatocytes, mitochondrial oxidative phosphorylation defects caused excessive reactive oxygen species generation and an imbalance in energy homeostasis[139].

In our liver samples, forced overexpression of KLF11 and KLF16—KLF11 increasing slightly more than 2 units and KLF16 approximately 1.25 on the log2 scale—activated PPARα signalling. This was confirmed by real-time PCR and Western blots, which showed upregulation of CPT1a, MCAD, CYP4A10, and CYP4A14, elevated serum ketones, and accelerated mitochondrial and peroxisomal β-oxidation, without altering SREBP-1c, FAS, or ACC levels[140,46]. Similarly, PPARδ increased by about 2.04 log2 units in hepatocytes and adipose tissue from our PPARD intervention data. This upregulation of Cpt1a and other oxidative genes by nearly one log2 unit reduced lipid droplet accumulation in db/db and high-fat diet mouse models[141].

Sex-specific differences in NAFLD susceptibility were reflected by oestrogen receptor activation, which prevented fat deposition and inflammation[142], whereas relaxin signalling added additional anti-steatotic and anti-fibrotic effects[143]. Conversely, altered purine metabolism aggravated oxidative and inflammatory damage by increasing uric acid levels[144],

while aberrant JAK-STAT and NF-κB/TNF/TLR cascades sustained prolonged hepatic inflammation and insulin resistance[145].

Steatosis was further alleviated by lowering lipogenic drivers. For instance, PROTAC3-mediated clearance of the I148M PNPLA3 variant reduced hepatic triglyceride overload in sucrose-fed mice, while antisense oligonucleotides against PNPLA3 increased PNPLA3 knockdown by ~2.73 log2 units in our dataset, leading to a ~20% reduction in liver fat and improved insulin sensitivity[146]. In our research, ACC1 suppression lowered its expression by 1.28 log2 units, inhibiting *de novo* lipogenesis and total adiposity[147]. Fasn knockout in ob/ob mice resulted in an increase of 1.51 log2 units in Fasn expression; unexpectedly, this loss of function reduced VLDL secretion and improved glucose tolerance[148]. DGAT2 silencing elevated DGAT2 expression by 2.50 log2 units in our deseq data, whereas GPAT1 ablation in obese models reduced its expression by ~1.76 log2 units, thereby decreasing triglyceride storage[149]. These interventions suppressed SREBP-1c lipogenesis and promoted β-oxidation, lowering diacylglycerol levels and PKC activation[150]. Together, these overlapping perturbations illustrated the complex progression from steatosis to fibrosis and steatohepatitis. Complete data has been provided in Supplementary file 8.

Biological process enrichment (Figure 6.2B) further highlighted continuous abnormalities in lipid uptake, stress adaptation, hypoxia signalling, and cell death regulation. In our JAK2L/high-fat model, hepatocyte-specific deletion of Cd36 reduced CD36 expression by more than three log2 units, thereby suppressing inflammation, restoring insulin sensitivity, and decreasing oleic acid uptake[70]. Steatosis and insulin resistance were also alleviated by increased LDLR expression (~1 log2 unit), which accelerated lipoprotein clearance[151]. Moreover, PCSK9 suppression by 3.84 log2 units conferred protection against both NAFL and NASH without impairing liver regeneration. ApoC3 knockdown reduced plasma triglycerides by >90% and lowered its levels by 4.56 log2 units, while also decreasing LDL-C and thereby lessening hepatic fat burden[152].

Hypoxia-related enrichments (e.g., “cellular response to oxygen levels” and “response to hypoxia”) suggested that fat-laden livers underwent hypoxic stress, which aggravated lipid imbalance and promoted fibrosis through HIF pathways[153]. Transition from benign steatosis to steatohepatitis was associated with apoptosis-related enrichments (“positive regulation of apoptotic process” and “extrinsic apoptotic signalling pathway”), reflecting enhanced hepatocyte death[154]. Oxidative stress, highlighted by “regulation of cellular stress response,”

underscored lipid overload–induced mitochondrial and ER dysfunction, which increased ROS, hepatocyte injury, and inflammation[155]. Lipid enrichment terms indicated reduced β-oxidation (negative NES for "fatty acid metabolic process") alongside increased lipogenesis and uptake (positive NES for "lipid biosynthetic process" and "lipid transport"). The coexistence of pro- and anti-apoptotic terms suggested that while lipotoxic stress enhanced death signals, survival mechanisms were insufficient to counterbalance the damage. Collectively, these results illustrated how continuous disruptions in fatty acid breakdown, lipid synthesis/transport, hypoxia signalling, oxidative stress, and apoptotic processes drove progression from steatosis to fibrosis, inflammation, and cellular injury.

Molecular and cellular function analysis (Figure 6.2C) identified antifibrotic targets. In I148M knock-in mice, PNPLA3 suppression reduced collagen deposition and steatosis, while HSP47 (Serpinh1) inhibition lowered its expression by ~2.47 log2 units, preventing collagen synthesis and halting cirrhosis progression[156]. Reduced IGFBP levels reflected more severe NAFLD, with insulin resistance and fat accumulation correlating with disrupted IGF-binding protein regulation[78]. Antioxidant defences such as superoxide dismutase were initially activated but were eventually overwhelmed by mitochondrial dysfunction, as observed with EC-SOD dysregulation[157]. Hepatic lipid deposition was further promoted by abnormalities in ApoA-I and ApoE, as well as decreased HDL-mediated cholesterol efflux. Lipoprotein interactions—including protein–lipid complex formation and apolipoprotein receptor binding—also played crucial roles[158]. Disruption of oxidoreduction-driven transmembrane transporters and mitochondrial electron transport complexes impaired ATP synthesis, increased ROS, and drove metabolic collapse in fatty hepatocytes[159]. These findings emphasized that NAFLD progression involved interwoven molecular pathways, including growth factor signalling, lipid transport, antioxidant defence, and redox homeostasis.

Full data for gene ontology enrichment has been provided in Supplementary file 9.

Functional enrichment of differentially expressed genes targeted by ellagic acid (Figure 6.3) provided further insight. The Cytoscape network highlighted ellagic acid's regulation of several critical biological processes. Clusters of digestion and absorption of carbohydrates, chemical carcinogenesis, and retinal metabolism (purple cluster) indicated its role in metabolic control. A prominent green cluster, involving zymogen activation and acute-phase response, pointed to anti-inflammatory potential. Genes linked to salivary secretion and gastrointestinal epithelium maintenance formed another cluster, suggesting a role in digestive and mucosal defence. A

light-blue cluster associated with late envelope proteins and cornified envelope binding indicated possible contributions to epithelial development. Additional network associations—such as those with nodes of Ranvier, epidermal barrier development, and negative regulation of interleukin-2 production—suggested immunomodulatory and neuroprotective roles. Taken together, ellagic acid exhibited pleiotropic effects on immunological pathways, inflammation, metabolism, and epithelial integrity, underscoring its potential as a multifactorial therapeutic agent in NAFLD.

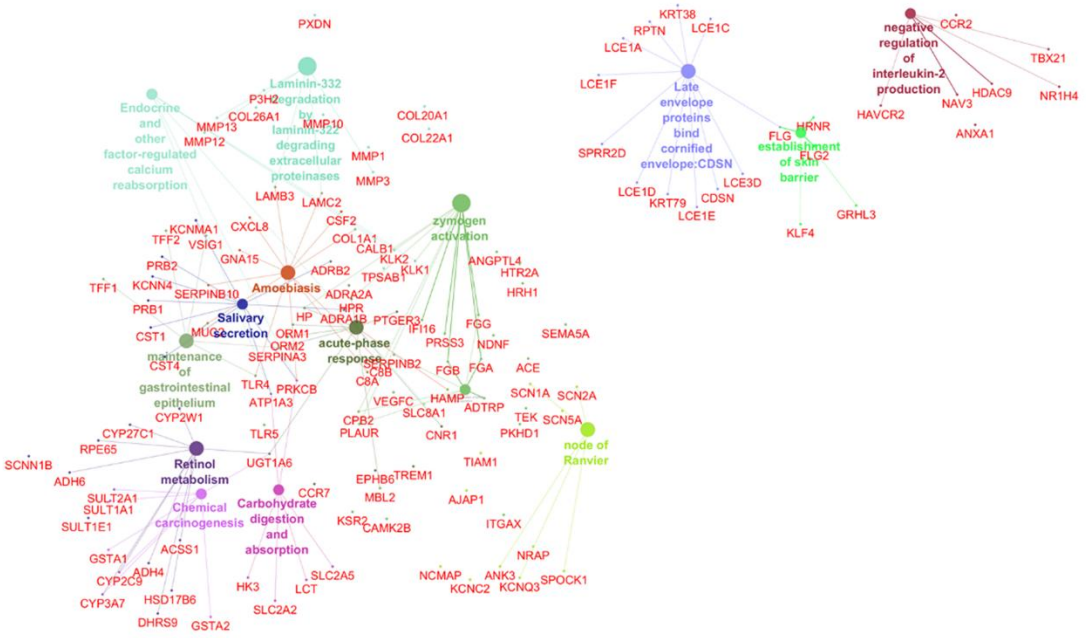

Figure 6.3. Cytoscape-derived interaction network depicting phytosterol-induced reprogramming of biological processes.

The findings demonstrated that ellagic acid treatment exerted multifaceted protective effects against NAFLD by modulating metabolic, inflammatory, oxidative, and fibrotic pathways. Through its ability to activate fatty acid oxidation, suppress lipogenesis, enhance antioxidant defences, and restore cellular homeostasis, ellagic acid counteracted the pathological processes driving progression from steatosis to NASH and fibrosis. Moreover, network-level analyses revealed its pleiotropic roles in metabolic regulation, immunomodulation, and epithelial maintenance, further reinforcing its therapeutic potential. Collectively, these results suggested that ellagic acid represented a promising candidate for managing NAFLD through multi-targeted mechanisms.

Chapter 7. Gallic acid mitigates the effects of NAFLD

7.1 Introduction

One of its key bioactive constituents of *Terminalia arjuna* is gallic acid (3,4,5-trihydroxybenzoic acid), a prominent phenolic compound found abundantly in its bark extract [160]. Gallic acid, a small-molecule polyphenol found in various plants and fruits, is particularly noted for its potent antioxidant and anti-inflammatory activities [161]. Research has shown that the bark of *T. arjuna* is rich in polyphenolic compounds, with gallic acid being a major contributor to its therapeutic actions, including hepatoprotection, lipid regulation, and enhancement of metabolic health [161,162].

7.2 Therapeutic effects of NAFLD

Gallic acid demonstrates a broad spectrum of therapeutic benefits, largely attributed to its strong antioxidant, anti-inflammatory, and metabolic regulatory effects. It neutralizes reactive oxygen species and boosts the activity of key antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase [163]. Additionally, gallic acid mitigates inflammation by lowering the expression of pro-inflammatory cytokines like TNF-α, IL-6, and IL-1β, primarily through suppression of the NF-κB signalling pathway.

Furthermore, gallic acid protects liver cells by inhibiting lipid peroxidation and preserving hepatocyte integrity under conditions of chemical or metabolic stress. It also plays a beneficial role in regulating metabolic processes by enhancing insulin sensitivity, decreasing levels of blood cholesterol and triglycerides, and reestablishing normal glucose metabolism. These actions are typically driven by the activation of important molecular pathways such as AMPK (AMP-activated protein kinase), PI3K/Akt, and PPARγ. Evidence from multiple studies involving high-fat diet animal models indicates that gallic acid can help reduce body weight gain, lower serum lipid concentrations, and control systemic inflammation, key contributors to metabolic syndrome and liver-related disorders [161].

7.3 Gallic Acid in the Treatment of NAFLD

Gallic acid had been extensively investigated for its ability to mitigate the pathological conditions associated with NAFLD. In vitro experiments using fatty acid-loaded HepG2 liver cells demonstrated that gallic acid effectively reduced lipid accumulation and prevented cell death by stimulating the AMPK signalling pathway[164]. Moreover, in co-culture systems involving hepatocytes and macrophages, gallic acid lowered the secretion of inflammatory

cytokines, suggesting its potential to halt the transition from simple steatosis to more severe steatohepatitis[164].

Animal studies further confirmed the hepatoprotective effects of gallic acid in NAFLD. In mice subjected to a high-fat diet, daily administration of gallic acid (50–100 mg/kg) significantly improved metabolic parameters, including body weight, liver triglyceride content, insulin responsiveness, and histological liver condition[165]. Mechanistic investigations revealed that gallic acid activated the AMPK–ACC–PPARα signalling pathway, thereby enhancing fatty acid oxidation while suppressing de novo lipogenesis. Another study highlighted its role in upregulating interferon regulatory factor 6 (IRF6), which downregulated PPARγ expression, thereby restricting lipid accumulation and adipocyte formation in hepatocytes. In addition, in a rat model combining HFD with environmental dust exposure, gallic acid effectively reduced oxidative damage and suppressed the activation of inflammatory mediators such as NF-κB, TNF-α, and IL-6, thus protecting against hepatic inflammation and fibrosis[161].

Overall, the evidence indicated that gallic acid—whether administered as a purified compound or as a component of *Terminalia arjuna* extract—exerted comprehensive therapeutic effects in NAFLD. By modulating lipid regulatory pathways, alleviating oxidative and inflammatory stress, and improving overall metabolic function, gallic acid emerged as a promising natural agent for the prevention and treatment of NAFLD.

7.4 Results

7.4.1 Identification of Differentially Expressed Genes

As shown in Figure 7.1A, the dataset comprising 28,364 genes revealed that 557 were significantly upregulated and 18 were significantly downregulated, indicating substantial transcriptional alterations under the experimental condition. The complete dataset is provided in Supplementary file 10. A global view of changes in the expression of genes upon Gallic acid treatment has been shown as a heatmap in Figure 7.1B.

7.4.2 Gene Set Enrichment Analysis Results

KEGG pathway enrichment revealed a complex network underlying NAFLD (Figure 7.2A). Several critical pathways were implicated, including Wnt/β-catenin signalling, which played a pivotal role in lipid homeostasis and liver repair but became dysregulated during steatosis and fibrosis[166]. Overactivation of mTOR promoted de novo lipogenesis, thereby aggravating

hepatic steatosis[167]. Impaired oxidative phosphorylation enhanced mitochondrial ROS production, which in turn damaged hepatocytes[168].

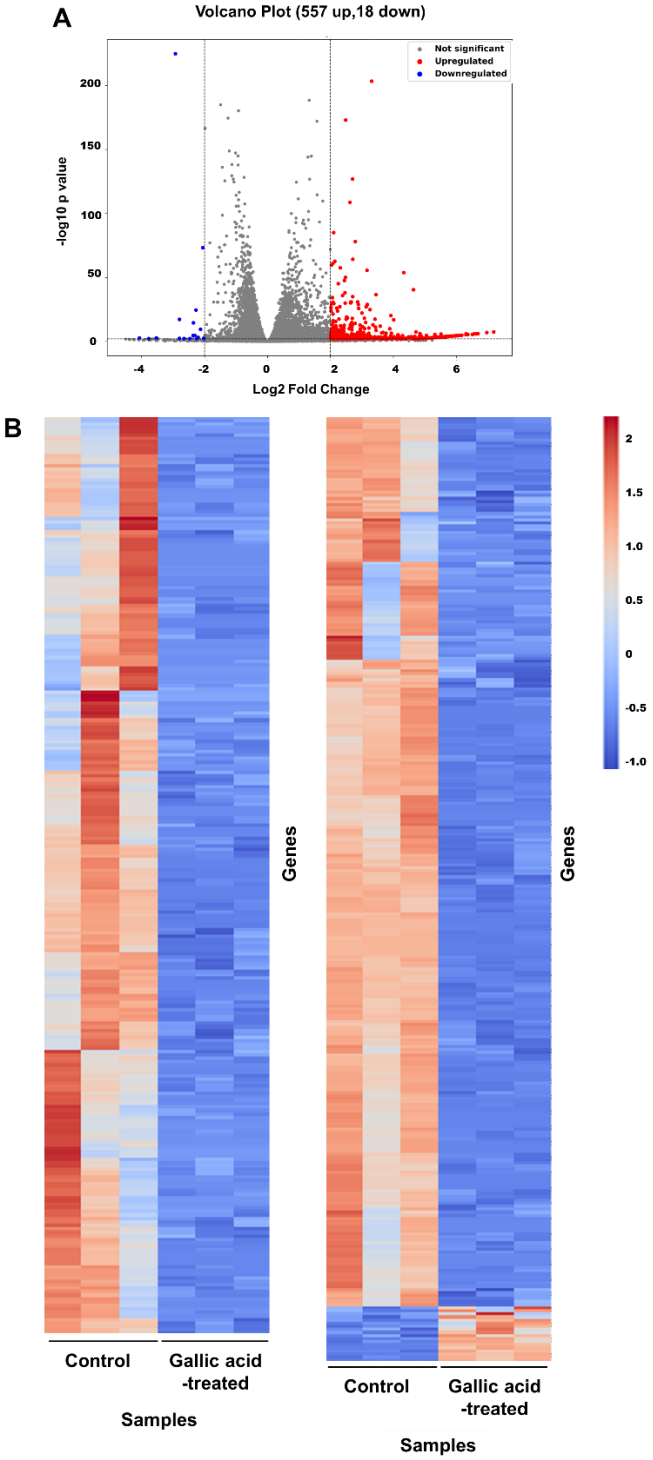

Figure 7.1. Overall changes in the expression of genes upon treatment with Ellagic acid. (A) Volcano plot of gene expression changes between Ellagic acid-treated and control samples. Each dot represents a gene; green dots indicate significant up-regulation and red dots significant down-regulation (adjusted $p < 0.05$, $|\log_2 FC| > 1$), while gray dots are non-

significant. Vertical lines at ±1 $\log_2$ fold-change and a horizontal line at $-\log_{10}$(adjusted p) = 2 denote significance thresholds. (B) Heatmap of z-score–normalized expression for dysregulated genes control and Ellagic acid-treated samples. Red denotes higher expression and blue lower; hierarchical clustering was used to group the genes with similar expression profiles.

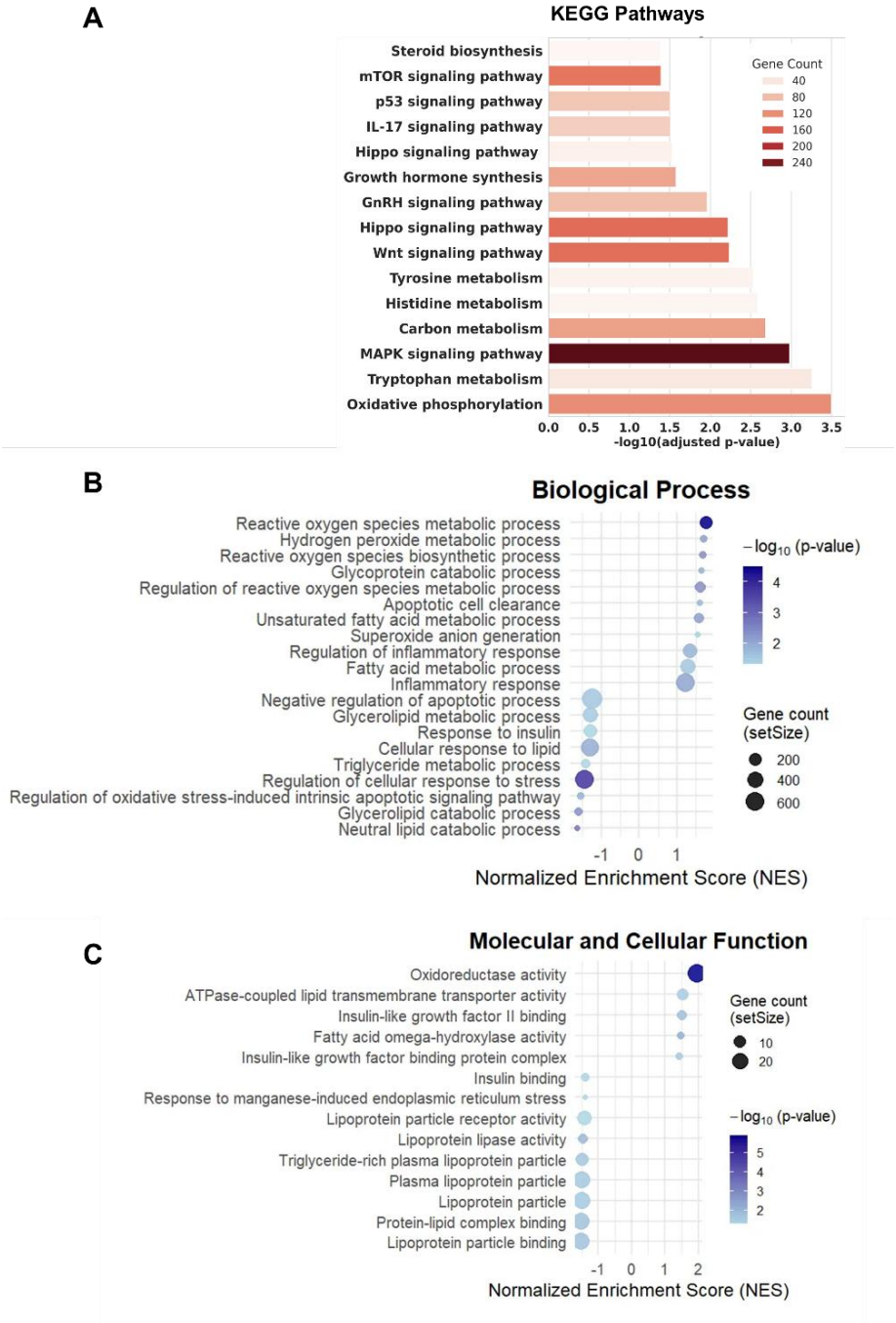

Figure 7.2. Enrichment analysis of differentially expressed genes in Phytosterol-treated samples. (A) KEGG pathway enrichment bar chart for all differentially expressed genes. Bars represent $-\log_{10}$(adjusted p), and darker shades indicate larger gene counts. Prominent

pathways include oxidative phosphorylation, MAPK signaling, tryptophan metabolism, and Wnt signaling. (B) GO Biological Process enrichment bubble plot. The x-axis is the normalized enrichment score (NES), bubble size indicates the number of genes, and color intensity reflects $-\log_{10}$(adjusted p). Enriched processes cover reactive oxygen species metabolism, fatty acid catabolism, apoptotic signaling, and lipid metabolism. **(C)** GO Molecular and Cellular Function bubble plot, ranked by NES. Bubble size corresponds to gene set size, and color depth to $-\log_{10}$(adjusted p). Key functions include oxidoreductase activity, ATP-driven lipid transport, insulin-like growth factor binding, and lipoprotein particle binding.

MAPK signalling was found to regulate hepatocyte responses to lipid overload, oxidative stress, and inflammation[169]. One-carbon metabolism influenced methylation, antioxidant defences, and membrane lipid synthesis[170], while growth hormone (GH) signalling deficiencies were associated with liver fat accumulation and insulin resistance[171]. Imbalances in YAP/TAZ contributed to the progression of fatty liver, whereas Hippo signalling regulated hepatocyte proliferation and fibrogenesis[172]. Reproductive hormone pathways, such as GnRH signalling, were also implicated in modulating metabolic traits associated with NAFLD[173].

IL-17 signalling promoted the transition from simple steatosis to inflammatory steatohepatitis, whereas p53 signalling exhibited dual roles, either protecting against or exacerbating steatotic damage depending on the cellular context[174]. Altered amino acid metabolism, including tryptophan, tyrosine, and histidine pathways, reflected gut–liver crosstalk and oxidative stress regulation[175]. Furthermore, defects in steroid biosynthesis aggravated cholesterol toxicity and hepatic fibrosis[176]. Collectively, these dysregulated pathways highlighted the multifactorial pathogenesis of NAFLD. The details KEGG pathway enrichment analysis has been provided in Supplementary file 11.

Gene Expression Alterations Following Gallic Acid Treatment

Differential expression analysis revealed several genes with therapeutic relevance. Notably, IL-33 was upregulated by ~5.37 $\log_2$FC, consistent with previous findings where IL-33 administration reduced ALT levels, hyperglycemia, hepatic triglycerides, and body weight in mice[177]. Adiponectin (ADIPOQ) expression increased by ~2.77 $\log_2$FC, a change associated with reduced hepatic lipid accumulation and improved insulin sensitivity[178].

Similarly, SOD3 overexpression (~2.74 $\log_2$FC) was linked to upregulation of adiponectin, stimulation of energy-burning genes (CPT1α, CPT1β, PGC1α, PGC1β, UCP2), and suppression of inflammatory genes (F4/80, TNFα, CD11c, MCP1, IL6), thereby enhancing

fatty acid oxidation[179]. SIRT2 overexpression (1.77 log_2FC) conferred protection against palmitate-induced lipid accumulation in hepatocytes[180], whereas ATF4 downregulation (–1.02 log_2FC) reduced lipogenic gene activity without compromising β-oxidation or VLDL secretion under nutrient overload[181].

HMGCS2 upregulation (~0.94 log_2FC) promoted ketogenesis, increased ketone body production, and lowered both blood glucose and hepatic fat. ADORA1 upregulation (~1.29 log_2FC) improved lipid regulation under diet-induced steatosis[182]. ABCG5/ABCG8 upregulation (~1.93 log_2FC) enhanced biliary cholesterol efflux and decreased intestinal absorption[183]. Importantly, RNLS expression increased (5.04 log_2FC), activating SIRT1 to protect against mitochondrial and oxidative damage[184]. Finally, MIF knockdown (–0.96 log_2FC) decreased macrophage infiltration and adipose inflammation, thereby enhancing insulin sensitivity in high-fat diet conditions[185].

The results of gene ontology enrichment analysis, in terms of biological processes and molecular functions have been shown in Figure 7.2B and 7.2C, respectively; and the complete data for this analysis has been provided in Supplementary file 12.

These results indicated that gallic acid modulated a multifaceted gene network involving lipid oxidation, suppression of lipogenesis, and attenuation of inflammation, ultimately restoring metabolic homeostasis in NAFLD.

Functional Network Analysis of Gallic Acid Targets

Cytoscape network visualization further highlighted the pleiotropic effects of gallic acid (Figure 7.3). Central nodes corresponded to genes associated with ion channel activity and synaptic membrane components (yellow and pink clusters), suggesting neuromodulatory potential. Genes such as GRIA1, CAMK2B, and NTRK2 formed dense red clusters, reflecting roles in neuronal signalling, cell adhesion, and immune regulation.

Blue modules were enriched for pathways involved in photoreceptor cell differentiation and cerebellar Purkinje cell formation, indicating potential roles in neurodevelopment and visual function. Other clusters (purple, navy blue, and green) highlighted gallic acid's regulation of cyclic-GMP phosphodiesterase activity, T cell co-stimulation, interleukin-4 production, and C21-steroid hormone metabolism, thereby demonstrating both immunomodulatory and endocrine effects. Additionally, aldehyde dehydrogenase-associated genes suggested a contribution to detoxification and oxidative stress defence.

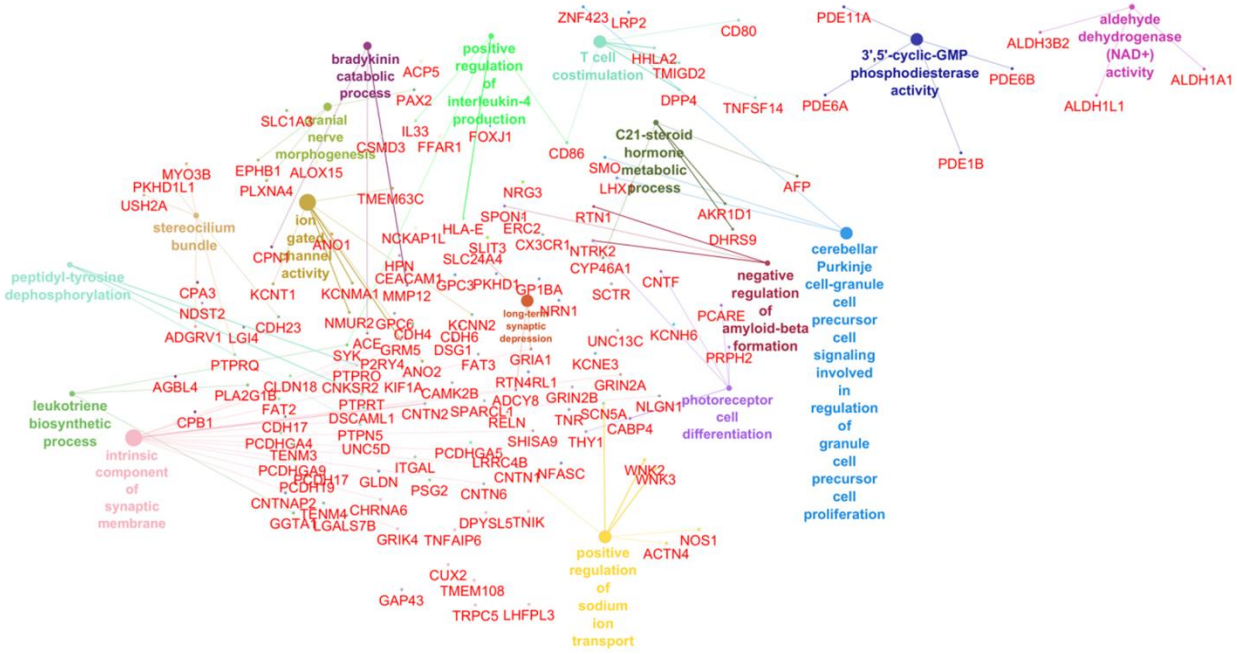

Figure 7.3. Gene ontology network highlighting functional clusters and pathway associations.

Overall, the network confirmed that gallic acid can target interconnected neurological, immunological, metabolic, and detoxification pathways, supporting its therapeutic potential against both metabolic and neuroinflammatory diseases.

Chapter 8. Conclusion

The hepatoprotective actions of *Terminalia arjuna* were elucidated through a comprehensive systems biology approach, integrating RNA-seq transcriptomics with pathway enrichment analyses and mechanistic insights from the literature. Four principal phytochemicals—maslinic acid, phytosterols, ellagic acid, and gallic acid—were shown to act in concert against the key pathological drivers of non-alcoholic fatty liver disease (NAFLD).

Maslinic acid activated the SIRT1/AMPK axis, enhancing fatty acid oxidation while reducing lipid synthesis. Phytosterols restored cholesterol homeostasis and attenuated inflammatory signaling by inhibiting NF-κB. Ellagic acid strengthened antioxidant defenses and suppressed inflammation through Nrf2 activation and MAPK inhibition. Gallic acid exerted multi-faceted effects, including modulation of lipid metabolism, reinforcement of antioxidant capacity, and regulation of neuro-immune–metabolic pathways, highlighting its potential role in mitigating systemic complications of NAFLD.

High-resolution RNA-seq profiling revealed broad molecular shifts induced by these bioactives, while enrichment analyses confirmed their regulation of critical metabolic, inflammatory, and stress-response pathways. By marrying omics-driven evidence with ethnopharmacological knowledge, this study not only substantiated the traditional use of T. arjuna but also unveiled detailed, multi-target mechanisms underpinning its hepatoprotective potential.

While these findings are promising, further validation *in vivo* is essential, supported by pharmacokinetic investigations and exploration of phytochemical synergies. Future directions include preclinical animal models, integration of proteomic and metabolomic datasets, and ultimately clinical evaluation to translate these insights into safe, plant-derived therapeutics or nutraceutical formulations for effective NAFLD management.

Chapter 9. Future Direction

Terminalia arjuna harbors a diverse spectrum of health-promoting phytochemicals, including antioxidants, anti-inflammatory agents, lipid-lowering compounds, anti-diabetic molecules, and hepatoprotective constituents, which collectively highlight its potential against complex metabolic disorders such as non-alcoholic fatty liver disease (NAFLD)[209]. While experimental studies have begun to uncover the mechanistic basis of its hepatoprotective effects, research to date has primarily focused on the activity of individual compounds in simplified model systems. Such reductionist approaches, though valuable, provide only a partial understanding of the therapeutic potential of *T. arjuna*.

Looking ahead, future investigations must expand beyond single-compound evaluations to explore the synergistic interactions that are likely to exist among the multiple phytochemicals present in *T. arjuna*. These compounds may act in a complementary or even synergistic manner to modulate lipid metabolism, oxidative stress, and inflammatory signaling in ways that cannot be captured by isolated compound testing. Systems biology frameworks, integrating transcriptomics, proteomics, and metabolomics, will therefore be critical in elucidating the network-level changes induced by *T. arjuna* and in mapping its multi-target effects on hepatic and systemic metabolism.

Equally important is the use of preclinical models that more accurately mimic the complexity of human disease. NAFLD rarely occurs in isolation; it is frequently associated with comorbidities such as obesity, type 2 diabetes, dyslipidemia, and cardiovascular dysfunction. Animal models that incorporate these overlapping metabolic disturbances should be prioritized to better evaluate the translational relevance of *T. arjuna*–derived interventions. Such models would allow researchers to probe not only hepatoprotective effects but also potential benefits on whole-body energy homeostasis, vascular health, and insulin sensitivity.

In addition, pharmacokinetic and pharmacodynamic studies remain largely underexplored for *T. arjuna* phytochemicals. Determining the bioavailability, tissue distribution, metabolism, and elimination of its key bioactive molecules—both alone and in combination—will be essential for identifying therapeutically relevant doses and formulations.

Ultimately, well-designed clinical trials will be indispensable to validate efficacy in humans, determine optimal dosing regimens, and establish safety profiles. Randomized controlled trials in patients with NAFLD, stratified by metabolic status and disease severity, will be particularly

important to assess real-world clinical applicability. Together, such efforts will bridge the gap between traditional ethnopharmacological knowledge and modern evidence-based medicine, paving the way for *T. arjuna*–derived nutraceuticals or adjunct therapies in the effective management of NAFLD and related metabolic disorders.

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