Thesis Report

on

Mechanistic Insights into Marigold Phytochemicals and Their Therapeutic Potential

by

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under the supervision of

Dr. Jaspreet Kaur Dhanjal

Submitted in partial fulfillment of the requirements for
the degree of Master of Technology
in Computational Biology

Department of Computational Biology,
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Certificate

This is to certify that the thesis titled "Mechanistic Insights into Marigold Phytochemicals and Their Therapeutic Potential" being submitted by Ms. Sarvani Gupta for the partial fulfillment of the requirements for the degree of Masters in Technology in Computational Biology at Indraprastha Institute of Information Technology Delhi (IIIT-Delhi), is an authentic record of work carried out under my supervision. In my opinion, the thesis has reached the standards fulfilling the requirements of the regulations relating to the degree. This work has not been submitted anywhere else for the reward of any other degree.

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Declaration

I submit this project entitled "Mechanistic Insights into Marigold Phytochemicals and their Therapeutic Potential" to the Department of Computational Biology, Indraprastha Institute of Information Technology Delhi (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.

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Abstract

Calendula officinalis (marigold) has long been valued in traditional medicine for its therapeutic effects, yet the molecular mechanisms underlying its action remain largely unexplored. This study investigates the bioactive phytochemicals of marigold and their potential role in treating cancer and non-alcoholic fatty liver disease (NAFLD), using computational analysis of publicly available RNA-sequencing datasets.

Five major compounds - Lutein, and Lycopene, Vitamin E, Fucoxanthin, and Quercetin were selected for detailed analysis. The workflow involved quality control, alignment to species-specific reference genomes, differential gene expression analysis using DESeq2, and functional enrichment via KEGG pathways and Gene Ontology.

Lycopene was found to influence genes involved in lipid metabolism and inflammation, with dysregulation of *SQLE*, *PNPLA3*, *PFKFB3*, *IL10*, *SIRT7*, *COL1A1*, and *ACTA2*, to name a few. Vitamin E affected insulin and MAPK signaling pathways, leading to changes in *GATA3*, *PNPLA3*, *SREBF2*, *IL6*, and *CD36*. Fucoxanthin modulated metabolic and inflammatory processes through genes such as *ISG15*, *FDFT1*, and *PCSK9*. Quercetin demonstrated strong antioxidant and anti-inflammatory potential by altering the expression of *SOD2*, *CAT*, *TNF*, *NOX1*, and *Smad4*. Lutein influenced cancer-associated pathways like PI3K/AKT and apoptosis, with dysregulation of *GATA3*, *ING3*, *TES*, *PCK2*, and *PELP1*.

While many of the gene expression changes suggest therapeutic benefit, certain observations - particularly involving Vitamin E and Lycopene, indicate complex responses that warrant further functional validation.

Overall, this study offers molecular insights into the mechanisms underlying the traditional use of marigold and supports the potential of its phytochemicals as plant-based therapeutics for complex diseases such as cancer and NAFLD.

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Chapter1. Introduction

Herbal remedies have historically served as the foundation for pharmacology, with many modern drugs originating from medicinal plants [1]. For millennia, phytomedicines have been integral to various traditional medical systems (TMS) for health promotion and disease prevention. Because of their increased therapeutic uses, the need for phytochemical resources has increased globally as research on medicinal plants has progressed [2]. The World Health Organization (WHO) defines traditional medicine as the comprehensive body of knowledge, abilities, and practices derived from the cultural beliefs and experiences unique to various societies, regardless of whether they are scientifically explainable. This knowledge is utilized for the maintenance of health, as well as for the prophylaxis, detection, enhancement, or medication of bodily and mental illness (WHO/EDM/TRM/2000.1) [3]. According to the National Medicinal Plants Board (NMPB), India is divided into 15 agroclimatic zones. Of the approximately 17,000 to 18,000 species of flowering plants found in the country, around 7,000 species have documented uses in the traditional medicinal practices across all five systems of Indian traditional medicine [2]. An estimated 3–4 billion people, or around 80% of the world's population, depend on herbal products for at least some of their basic healthcare, demonstrating the critical role that herbal medicine plays in global healthcare [4]. In underdeveloped nations (such as sub-Saharan Africa and some areas of Asia), where up to 90% or more of the population takes traditional treatments, this tradition is particularly widespread [4]**.** The usage of herbal supplements is widespread (tens of percent of people) and increasing even in developed nations [4]. This extensive use is reflected in the commercial value; by 2020, the global market for herbal products was expected to reach over US$115 billion [5]. These figures highlight the significance of medicinal plants on a global scale and the necessity of applying contemporary techniques to research their safety and effectiveness.

Originally from southern Europe, *Calendula officinalis L*., sometimes known as pot marigold, is a vibrantly blooming annual plant in the Asteraceae (Compositae) family that is grown all over the world [6]. This plant produces unique orange-yellow daisy-like flowers that are full of phytochemicals that are biologically active. For thousands of years, marigold has been utilized as an antibacterial, anti-inflammatory, and wound-healing agent in traditional medical systems such as Unani, Ayurveda, and European herbalism. Calendula remedies, for instance, have long been

used internally to treat digestive and menstrual issues as well as topically to treat minor burns, wounds, and skin irritations [6]. Many over-the-counter herbal ointments and beverages contain it because of its reputation for safety and calming skin benefits. In summary, *C. officinalis* is a well-characterized plant with a long history of use as a versatile herbal cure in ethnomedicine.

The unique combination of bioactive components in *C. officinalis* is what gives it its medicinal properties. The flowers are rich in flavonoids (such as isorhamnetin, quercetin glycosides, rutin, and related derivatives), triterpenoid esters and glycosides (such as calendulosides, faradiol mono- and diacetates), saponins, phenolic acids (such as caffeic acid and chlorogenic acid), carotenoid pigments (particularly lutein, zeaxanthin, and β-carotene), sterols, and volatile oils, according to phytochemical analyses [6,7]. Together, these substances have a variety of biological effects. Carotenoids support vitamin A metabolism and antioxidant activity; triterpenoids and saponins frequently have cytotoxic and immunomodulatory effects; flavonoids and phenolics are strong antioxidants and anti-inflammatory agents. In actuality, these components are primarily responsible for calendula's reputation as an anti-inflammatory and wound-healing plant by inhibiting pro-inflammatory mediators [7,8]. Interestingly, of all the herbs, *C. officinalis* has one of the highest levels of carotenoid content. For instance, calendula flower extract was reported to be high in lutein, zeaxanthin, and lycopene, which are likely responsible for its strong hepatoprotective and antioxidant properties [7,9]. These phytochemicals make *C. officinalis* a particularly appealing target for therapeutic development because oxidative stress and inflammation are factors in many chronic illnesses.

Calendula officinalis for the management of Non-Alcoholic Fatty Liver Disease (NAFLD)

Hepatic steatosis, oxidative stress, and progressive liver injury are caused by the pathogenic triad of chronic inflammation, insulin resistance, and lipid excess, which are commonly acknowledged as the primary hallmarks of non-alcoholic fatty liver disease (NAFLD) [10]. The multifactorial nature of NAFLD pathogenesis, including oxidative stress, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, and altered lipid metabolism, is depicted in Figure 1.1. Natural compounds that alter these pathways are becoming more and more popular. Of these, *calendula officinalis*, or simply calendula, has a phytochemical profile that shows promise for hepatoprotection. Its effectiveness in models of fatty liver is supported by preclinical studies. The treatment of *C. officinalis* flower extract considerably improved blood lipid profiles and decreased

hepatic lipid buildup in a rat model of diet-induced dyslipidemia (caused by a "cafeteria" high-fat diet) [11]. Histological evaluations revealed significant decreases in inflammation and hepatic steatosis. Additionally, the extracts have been shown to restore the depleted antioxidant enzymes and reduced oxidative stress, both of which are critical in the development of NAFLD. Calendula's high flavonoid and carotenoid content, which boosts liver antioxidant capacity, is primarily responsible for these effects. Another study found that pretreatment with calendula extract increased glutathione, superoxide dismutase, and catalase levels while decreasing lipid peroxidation and serum transaminases, protecting rats from CCl_4-induced hepatotoxicity [7]. Further, quercetin, a significant calendula flavonoid, reduced inflammation and hepatic oxidative stress in diabetic mice with non-alcoholic fatty liver disease [12]. Similarly, carotenoids in calendula, such as lutein and zeaxanthin, exhibit hepatoprotective effects by acting as antioxidants and regulating genes involved in lipid metabolism. According to reviews, the prevalence of NAFLD and liver fat content are inversely correlated with dietary carotenoid intake [9].

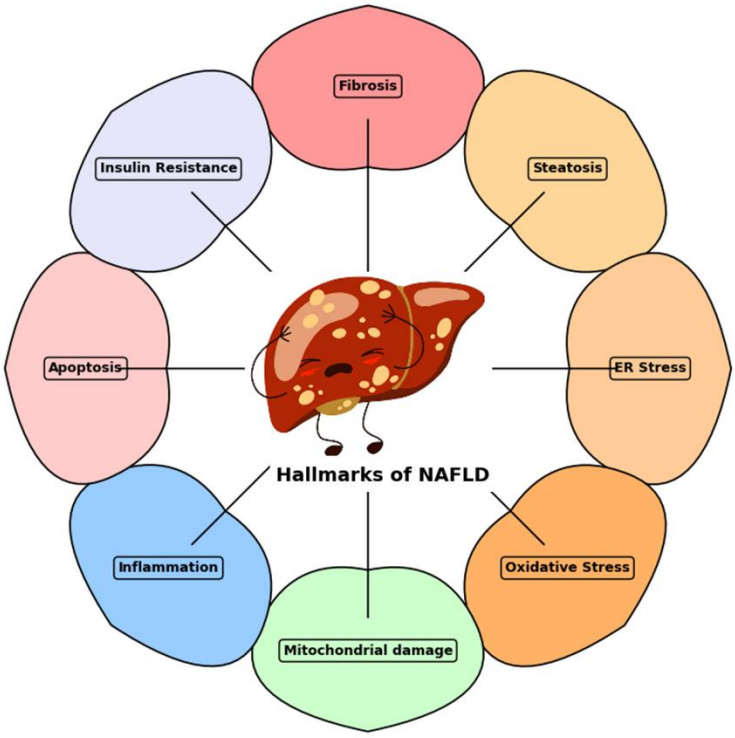

Figure 1.1. Hallmarks of Non-Alcoholic Fatty Liver Disease (NAFLD). An illustration of the key pathogenic processes for NAFLD to progress. Insulin resistance, oxidative stress, lipid buildup, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, fibrogenesis, and chronic inflammation are some of these characteristics. Steatohepatitis, hepatic steatosis, and ultimately

fibrosis is caused by the interaction of these variables. Knowing these characteristics offers a mechanistic framework for assessing natural substances and treatment approaches that address the pathophysiology of NAFLD.

As shown in Figure 1.1, *Calendula officinalis* and its bioactive components directly target a number of NAFLD characteristics, particularly oxidative stress, inflammation, and lipid dysregulation. The combination of results from animal models and established biochemical pathways offers a strong justification for investigating calendula as a hepatoprotective and metabolic adjuvant in NAFLD, even though randomized clinical trials are still missing.

Calendula officinalis for cancer prevention and complementary therapy

Dysregulated cell proliferation and inflammatory microenvironments are common features of the complex disease known as cancer. The characteristics of cancer, as shown in Figure 1.2, include a broad range of biological abilities developed during tumor growth, such as persistent proliferative signaling, growth suppressor evasion, resistance to cell death, making replicative immortality possible, angiogenesis, invasion and metastasis, genome instability, inflammation, and immune evasion [13]. These characteristics offer a foundation for comprehending the ways in which possible anti-cancer drugs work and serve as important targets for therapeutic approaches. *Calendula officinalis* may have multi-targeted anti-cancer capabilities, according to a number of studies. Calendula extract, as previously noted, caused apoptosis in a variety of cancer cell types and reduced tumor growth in mice [14]. Calendula's cytotoxicity against several cancer lines was described in detail in a thorough analysis of its anti-tumor potential, which also highlighted its capacity to upregulate pro-apoptotic signaling and downregulate anti-apoptotic proteins [15]. Furthermore, calendula treatment revealed anti-metastatic action and genoprotective properties in preclinical animal models by inhibiting tumor dissemination and protecting DNA from genotoxic shocks [15]. These effects are caused by a number of calendula phytoconstituents. Notably, flavonoids and triterpene esters, such as farradiol derivatives, have been implicated in suppressing tumor cell proliferation and angiogenesis, key mechanisms associated with many cancer hallmarks. Calendula is commonly utilized to enhance therapeutic results and patient comfort in real-world oncology settings. Calendula ointment, for example, has been demonstrated in clinical studies to lessen the severity of radiation-induced dermatitis in patients with breast cancer [16]. This application demonstrates calendula's capacity to reduce inflammation and support tissue integrity,

although being palliative rather than directly anti-neoplastic. Furthermore, there is increasing interest in calendula extracts' ability to work in concert with chemotherapy by boosting the immune system and directly cytotoxically destroying cancer cells. Although further mechanistic research and clinical trials are required, the information now available suggests that *C. officinalis* and its phytochemicals are attractive candidates for cancer prevention and complementary therapy [14,15].

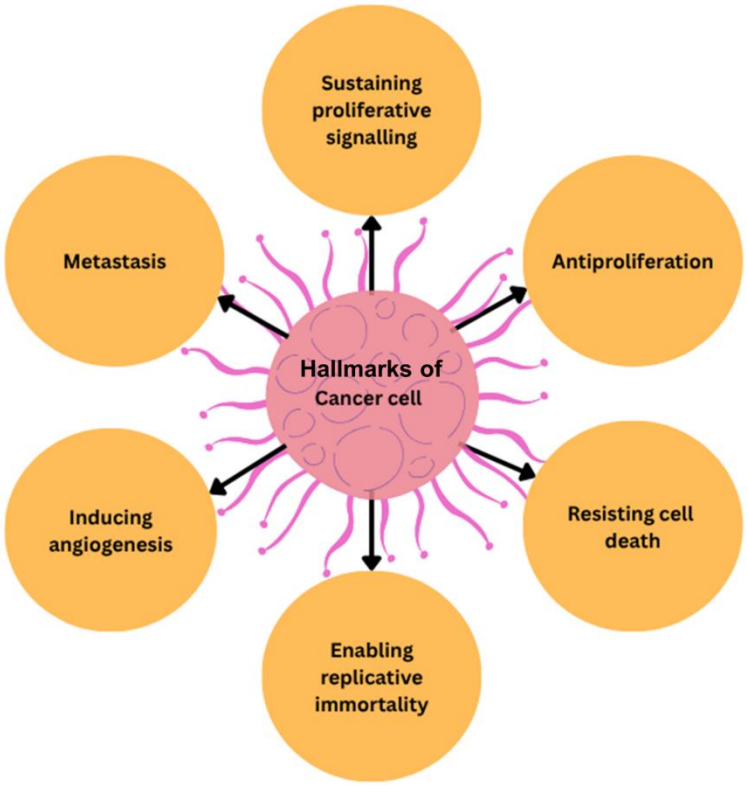

Figure 1.2. Hallmarks of Cancer. Sustained proliferative signaling, growth suppressor evasion, apoptosis resistance, replicative immortality, angiogenesis stimulation, invasion and metastasis activation, inflammation, immunological evasion are different hallmarks of cancer. By focusing on one or more of these characteristics, phytochemicals like those in *Calendula officinalis* exhibit anti-cancer benefits.

In summary, *Calendula officinalis* is a medicinal plant of notable botanical and pharmacological importance, with a diverse phytochemical profile that underlies its broad spectrum of bioactivities. Preclinical evidence demonstrates its potential to modulate key pathological processes in both NAFLD and cancer, primarily through antioxidant, anti-inflammatory, and metabolic regulatory mechanisms. Building on these findings, this thesis employs transcriptomic analyses to elucidate

the molecular actions of specific *C. officinalis*-derived phytochemicals, with the aim of identifying their roles in modulating disease-relevant pathways. This work seeks to position *C. officinalis* as a promising natural candidate for the development of plant-based therapeutics targeting liver disorders and cancer.

Chapter 2. Review of Literature

2.1. Calendula officinalis, an important medicinal plant

The Latin term "calends," which denotes the first day of the month when these flowers usually blossom, is the source of the name Calendula. Because of the way its blossoms open in the morning and wilt in the evening, the plant is often referred to as the "herb of the sun". Calendula has long been used to heal minor burns, wounds, and skin conditions. These days, it is utilized in a variety of products, such as carophyllenic ointment and pot marigold tincture, which are both high in the carotenoids found in the flowers. Furthermore, calendula is a part of the homeopathic treatment Traumeel, which is intended to reduce swelling and pain associated with acute musculoskeletal injuries. Historically, calendula petal powder has been utilized for its tasting and coloring qualities in meals as an affordable substitute for saffron [17]. There are about 25 species in the genus Calendula, but the most common ones are *C. officinalis, C. arvensis, C. tripterocarpa, C. stellata, and C. suffruticosa* [18]. This thesis specifically focuses on *C. officinalis* (commonly known as marigold) and its therapeutic benefits. Here we discussion the pharmacodynamic mechanisms underlying its anti-inflammatory, anti-cancer, anti-diabetes, wound healing, hepatoprotective, and antioxidant activities as well as its role in modulating hepatic lipid metabolism.

2.2. Botanical description of *Calendula officinalis*

The self-seeding annual *Calendula officinalis* grows best in warm, humid climates and usually reaches a height of 12 to 18 inches. It has a composite flower head on top of the stalk that is 5 to 7 cm in diameter. An epicalyx with several tapering lanceolate sepals, thickly covered in glandular hairs on both sides, and yellow-orange tubular florets on the inner side make up this flower head. The yellowish-brown powdered form of *C. officinalis* has a slightly bitter taste and a characteristic pungent smell. Furthermore, it is grown more extensively in China and India [17].

2.3. Economic importance of *Calendula officinalis*

Global marigold production is approximately 600,000 tons, with India contributing around 75-80% of this total. India consumes about 80% of its own marigold production and is recognized for having the best marigolds in the world. Indian marigolds are exported to countries like Japan, Sri Lanka, Iran, North African countries, the US, and the UK. The bulk of marigold production in

India is concentrated in the southern part of the country, particularly in the peninsular region. Andhra Pradesh and Uttar Pradesh are the leading marigold-producing states, with Andhra Pradesh having the largest area dedicated to marigold cultivation. Other states with significant marigold cultivation include Maharashtra, Tamil Nadu, Orissa, Karnataka, Uttar Pradesh, and Kerala [19].

2.4. Phytochemicals of Marigold

Several phytochemical investigations have confirmed the presence of various classes of chemical compounds, with the most prominent being terpenoids, flavonoids, coumarins, quinones, volatile oils, carotenoids, and amino acids in *Calendula officinalis* [20]. Table 2.1 shows list of plant parts, bioactivities and associated compounds of *Calendula officinalis.*

Table 2.1. List of Plant parts, Bioactivities and Associated Compounds of *Calendula officinalis.*

Plant Part	Bioactivity	Compounds
flower	Analgesics, Anti-bacterial agents, Anti-infective agents, Anti-infective agents, local, Anti-inflammatory agents, Antineoplastic agents, Antiprotozoal agents, Dysmenorrhea, Estrogen receptor, modulators, General tonic for rejuvenation, Hepatitis, Hypertension, Hypnotics and sedatives, Hypotension, Immunostimulant, Leukorrhea, Menstruation-inducing agents, Wounds and injuries	(+)-alpha-Cadinene, (+)-delta-Cadinene, (+)-gamma-Cadinene, (+)-gamma-Gurjunene, (-)-Bornyl acetate, (-)-Loliolide, (-)-alpha-Cadinol, (-)-beta-Bourbonene, (3S,4aR,6aR,6bR,8S,8aS,12S,12aS,12bR,14bR)-4,4,6a,6b,8a,12,14b-Heptamethyl-11-methylene-docosahydro-picene-3,8-diol, (E)-beta-ocimene, (S)-cis-Verbenol, (S,1Z,6Z)-8-Isopropyl-1-methyl-5-methylenecyclodeca-1,6-diene, 1,10-Di-epcubenol, 1-Isopropyl-4,7-dimethyl-1,3,4,5,6,8a-hexahydro-4a(2H)-naphthalenol, 2,5-Dihydroxybenzoic acid, 2-[(5E)-6,10-dimethylundeca-1,5,9-trien-2-yl]oxirane, 4-Carvomenthenol, 4-Hydroxybenzoic acid, 4-Hydroxycinnamic acid, 4-Methyl-1,3-nonadiene, 5-Methoxy-2,3-dihydro-1-benzofuran, 6,10,14-Trimethylpentadecan-2-one, 6,8-Nonadien-2-one, 6-methyl-5-(1-methylethylidene)-, 6-Methyl-5-hepten-2-one, 6-Methylhept-5-en-2-ol, 7-epi-alpha-Eudesmol, 9,12,15-Octadecatrienal, 9,12-Octadecadienal, 9,12-Octadecadienoic acid, Actinidiolide, dihydro-, Allo-Aromadendrene, Anethole, Aromadendrene, Brein, Bulnesol, Butyl palmitate, Cadalene, Cadina-1,4-diene, Caffeic acid, Calendoflaside, Calendol, Camphene, Carotol, Carvone, Caryophyllene oxide, Caswell No. 264AB, Cedrelanol, Chikusetsusaponin iva, D-

		Limonene, Daucosterol, Dotriacontane, Eicosane, Elemol, Ethyl palmitate, Ethyl tetradecanoate, Geranylacetone, Germacra-1(10),5-dien-4-ol, Guaiol, Heneicosane, Hentriacontane, Heptacosane, Heptadecane, Hexacosane, Hexanoic acid, Humulene, Isoamyl laurate, Isofucosterol glucoside, Isoquercitrin, Isorhamnetin, Isorhamnetin-3-O-neohesperidoside, Lauric acid, Ledol, Levomenol, Linalool, Longifolene, Lup-20(29)-ene-3,16-diol, (3beta,16beta)-, Lupeol, Menthone, Methyl arachidate, Methyl behenate, Methyl dodecanoate, Methyl heptadecanoate, Methyl icosa-11,14,17-trienoate, Methyl linoleate, Methyl linolenate, Methyl palmitate, Methyl pentadecanoate, Methyl salicylate, Methyl stearate, Methyl tetracosanoate, Methyl tetradecanoate, Musk ambrette, Myrcene, Myrtenyl acetate, Narcissin, Nonacosane, Nonadecane, Octacosane, Octadecane, Oleanolic acid, Oplopanone, Palmitic acid, Patchouli alcohol, Pentacosane, Quercetin, Quercetin 3-neohesperidoside, Quercetin-3-glucoside, Sabinene, Santolina triene, Stearic acid, Sterol, Stigmastan-6,22-dien, 3,5-dedihydro-, Stigmasterol, Stigmasterol glucoside, T-Muurolol, Taraxasterol, Tetracosane, Triacontane, Tricosane, Tricyclene, Tritriacontane, Ursadiol, Valencene, Vanillic acid, Viridiflorene, Viridiflorol, alpha-Amyrin, alpha-Calacorene, alpha-Carotene, alpha-Copaene, alpha-Farnesene, alpha-Gurjunene, alpha-Ionone, alpha-Muurolene, alpha-Patchoulene, alpha-Pinene, alpha-Sinensal, alpha-Terpineol, beta-Acorenol, beta-Amyrin, beta-Cadinene, beta-Calacorene, beta-Caryophyllene, beta-Cubebene, beta-Eudesmol, beta-Farnesene, beta-Gurjunene, beta-Ionone, beta-Pinene, beta-Selinene, beta-Sitosterol, cis-Chrysanthenyl acetate, cis-Muurola-4(14),5-diene, cis-Myrtanol, d-Borneol, epi-Cubebol, epi-Cubenol, gamma-Muurolene, gamma-Patchoulene, gamma-Terpinene, isorhamnetin-3-O-glucoside, p-Mentha-2,4-diene, trans-alpha-Bergamotene

leaf	Diaphoretic,Hemostasis, Varicose veins, Anti-bacterial agents	(3S,5S,8S,9S,10S,13R,14S,17R)-10,13-dimethyl-17-[(2R)-6-methylheptan-2-yl]-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol, 24-Methylenecholesterol, Campestanol, Campesterol, Cholesterol, Clerosterol, Episterol, Lathosterol, Schottenol, Sterol, Stigmastenol, Stigmasterol, Vitamin E
root	Anti-inflammatory agents,hypnostics and sedatives, Periodontal diseases,pregnancy complications	Calenduloside A, Calenduloside B, Calenduloside C, Calenduloside D, Calenduloside E, Calenduloside G, Chikusetsusaponin iva, Inulin
seed	Anti-inflammatory agents,wound healing	Quercetin, alpha-Carotene
whole plant	Anti-bacterial agents, Antifungal agents,wound healing,ulcer,sprains and strains, spermatocidal agents,spasm,skin disease.	Oleanolic acid, Salicylic acid

2.4.1. Terpenoids

Various terpenoids have been identified in the petroleum ether extract of *Calendula officinalis* flowers. These include sitosterols, stigmasterols [21], diesters of diols [22], and 3-monoesters of taraxasterol, ψ-taraxasterol and lupeol [23,24]. Additionally, compounds such as erythrodiol, brein [25], ursadiol [26], faradiol-3-O-palmitate, faradiol-3-O-myristate, faradiol-3-O-laurate [27], arnidiol-3-O-palmitate,arnidiol-3-O-myristate, arnidiol-3-O-laurate, calenduladiol-3-O-palmitate, and calenduladiol-3-O-myristate [28,29] have been reported. Furthermore, oleanolic acid saponins, including calenduloside A-H [30–33],and oleanane triterpene glycosides such as calendulaglycoside A, calendulaglycoside 6′−O−n−methylester, calendulaglycosideA6′−O−n−butylester, calendulaglycosideB, calendulaglycosid O − n − butylester, calendulaglycosideC, calendulaglycosideCA6′ − O − n –methylester [29], calendulaglycosideCA6′−O−n−butyleste,calendulosideFA6′−O−n−butylester, and calendulosideGA6′−O−n−methylesterhavebeenisolated. A newly discovered triterpenic ester from the oleanane series, cornulacic acid acetate, has also been isolated from the flowers [34].

2.4.2. Flavonoids

Several flavonoids have been extracted from the ethanol extract of *Calendula officinalis* inflorescence. These include quercetin, isorhamnetin [33], isoquercetin, isorhamnetin-3-O-β-D-glycoside, and narcissin. Additionally, calendoflaside, calendoflavoside, calendoflavobioside [35], rutin, and isoquercitrin neohesperidoside have been identified. Other compounds reported include isorhamnetin-3-O-neohesperidoside, isorhamnetin-3-O-2G-rhamnosyl rutinoside, isorhamnetin-3-O-rutinoside, quercetin-3-O-glucoside, and quercetin-3-O-rutinoside [29].

2.4.3. Coumarins

The ethanol extract of *Calendula officinalis* inflorescence has been found to contain coumarins, including scopoletin, umbelliferone, and esculetin [36].

2.4.4. Quinones

Quinones identified in *Calendula officinalis* include plastoquinone, phylloquinone, and α-tocopherol in the chloroplast, ubiquinone, phylloquinone, and α-tocopherol in mitochondria, as well as phylloquinone in the leaves [37].

2.4.5. Volatile oil

The flowers of *Calendula officinalis* contain the highest concentration of volatile oil at the full flowering stage (0.97%) and the lowest during the pre-flowering stage (0.13%) [38]. The composition of volatile oil varies across different phases of the vegetative cycle. Several monoterpenes and sesquiterpenes have been identified in the oil, including α-thujene, α-pinene, sabinene, β-pinene, limonene, 1,8-cineole, p-cymene, trans-β-ocimene, γ-terpinene, δ-3-carene, nonanal, terpinen-4-ol, 3-cyclohexene-1-ol, α-phellandrene, α-terpineol, geraniol, carvacrol, bornyl4acetate, sabinyl acetate, α-cubebene, α-copaene, α-bourbonene, β-cubebene, α-gurjunene, aromadendrene, β-caryophyllene, α-ylangene, α-humulene, epi-bicyclo-sequiphellandrene, germacrene D, alloaromadendrene, β-saliene, calarene, muurolene, δ-cadinene, cadina-1,4-diene, α-cadinene, nerolidol, palustron, endobourbonene, oplopenone, α-cadinol, and T-muurolol. The essential oil is particularly rich in α-cadinene, α-cadinol, T-muurolol, limonene, and 1,8-cineole, while p-cymene is present at lower levels during the post-flowering stages [38].

2.4.6. Carotenoids

The methanol extract of leaves, petals, and pollens from *Calendula officinalis* flowers has been found to contain various carotenoids. The carotenoids identified in the pollens and petals include neoxanthin, 9Z-neoxanthin, violaxanthin, luteoxanthin, auroxanthin, 9Z-violaxanthin, flavoxanthin, mutatoxanthin, 9Z-anthroxanthin, lutein, 9/9A-lutein, 13/13Z-lutein, α-cryptoxanthin, β-cryptoxanthin, z-cryptoxanthin, lycopene, α-carotene, and β-carotene. The total carotenoid content, measured in mg/g dry weight, was 7.71% for petals and 1.61% for pollens. In the leaves and stems, the reported carotenoids include neoxanthin, 9Z-neoxanthin, violaxanthin, luteoxanthin, 9Z-violaxanthin, 13Z-violaxanthin, antheraxanthin, mutatoxanthin epimer 1, mutatoxanthin epimer 2, lutein, 9/9 2-lutein, α-cryptoxanthin, β-cryptoxanthin, and β-carotene. The total carotenoid content was recorded as 0.85% in leaves and 0.18% in stems [39,40].

2.4.7. Amino acids

The ethanol extract of *Calendula officinalis* flowers has been found to contain 15 free-form amino acids, including alanine, arginine, aspartic acid, asparagine, valine, histidine, glutamic acid, leucine, lysine, proline, serine, tyrosine, threonine, methionine, and phenylalanine. The amino acid content is approximately 5% in leaves, 3.5% in stems, and 4.5% in flowers [41].

2.4.8. Carbohydrates

The ethanol extract of the plant's inflorescence has been found to contain polysaccharides PS-I, PS-II, and PS-III, which possess a (1→3)-β-D-galactan backbone with short side chains at the C-6 position. These side chains consist of α-arabinan-(1→3)-arabinan and α-L-rhamnan-(1→3)-arabinan, along with various monosaccharides [8,42].

2.4.9. Lipids

The lipids present in the petroleum ether extract of *Calendula officinalis* seeds, leaves, and flowers have been analyzed. In the seeds, neutral lipids were found to be 15.7%, phospholipids 0.6%, and glycolipids 0.9%. The fatty acids identified 5 in flowers, including monols, sterol esters, 3-monoesters, and 3-monoester diols, consist of lauric, myristic, palmitic, stearic, oleic, linoleic, and linolenic acids. Marigold seeds contain approximately 59% of an 18:3 conjugated trienoic acid (trans-8, trans-10, cis-12) and around 5% of 9-hydroxy-18:2 (trans-9, cis-11) acid, known as

dimorphecolic acid. Additionally, one oxygenated fatty acid, D-(+)-9-hydroxy-10,12-octadecadienoic acid, has also been reported in the seed oil of *C. officinalis* [43].

2.4.10. Other constituents

Other phytochemicals identified include the bitter compound loliolide (calendin) [44], calendulin [45], and n –paraffins [46].

The phytochemicals, present in *Calendula officinalis,* have been associated with a wide range of therapeutic activities, including hepatoprotective, antioxidant, anti-inflammatory, insulin-sensitizing, and anti-apoptotic effects, which collectively target multiple disease-related pathways. As illustrated in Figure 2.1, these bioactive constituents exhibit diverse pharmacological properties, and their broad spectrum of activity supports the potential of *C. officinalis* as a versatile therapeutic agent.

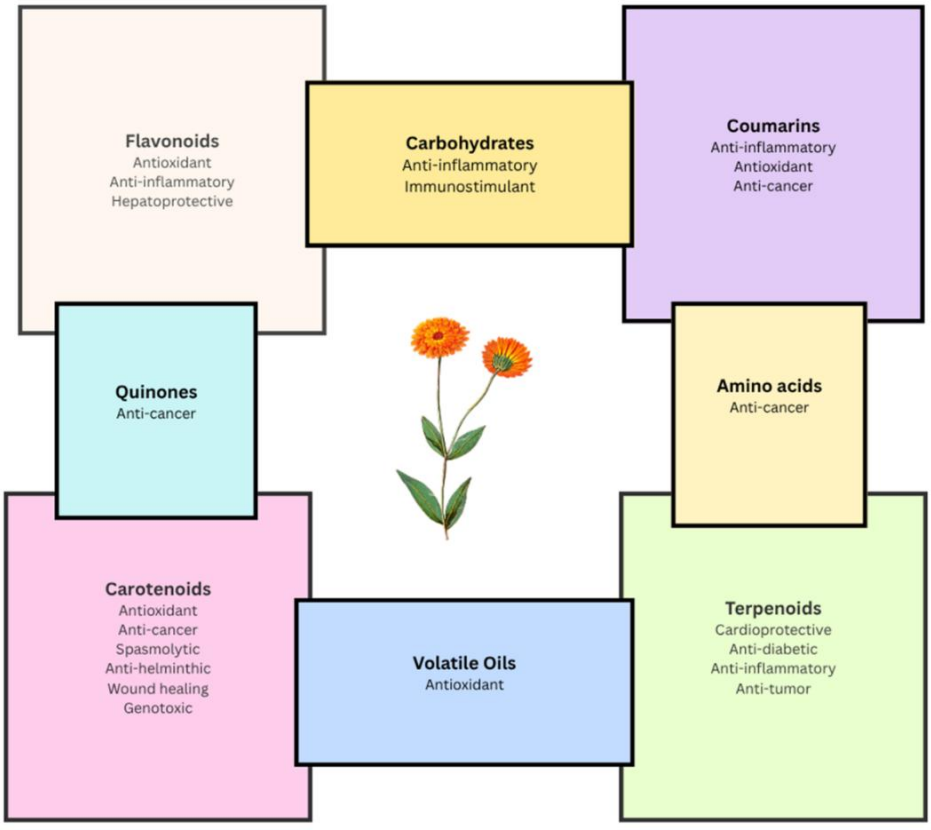

Figure 2.1. Phytochemical constituents of *Calendula officinalis* and their pharmacological properties.

2.5. Therapeutic potential of Marigold

Calendula officinalis L. has been utilized for medicinal purposes since the 12th century. The plant is known for its various biological activities, including promoting angiogenesis, vascular regeneration, pain relief, antimicrobial effects ,antioxidant properties, and immune system modulation [6,47]. In cosmetics, calendula is used in products designed for sensitive skin and soothing applications, such as after-sun products. It is included in various formulations for skin, eye, hair, and bath products and is recognized as safe for cosmetic use [48]. Various Calendula preparations, such as extracts, tinctures, and oils, can be incorporated into topical formulations aimed at promoting wound healing and soothing inflamed and damaged skin [48]. *Calendula officinalis* has been extensively studied for its wide range of pharmacological properties, as demonstrated in both preclinical and clinical research. It shows notable **anti-inflammatory activity**, significantly reducing inflammation and downregulating proinflammatory cytokines and COX-2 in experimental models [8]. The plant also exhibits effective **wound-healing** abilities, enhancing granulation tissue formation and accelerating inflammation resolution, with clinical evidence supporting its use in managing burns and surgical wounds [16]. Additionally, calendula possesses strong **antimicrobial effects**, inhibiting the growth of various bacterial and fungal strains due to its bioactive constituents like essential oils and phenolic compounds [29]. Its **hepatoprotective and antioxidant actions** are evident in liver injury models, where it decreases oxidative stress markers, lowers liver enzyme levels, and boosts endogenous antioxidants such as SOD, catalase, and glutathione [7]. Carotenoids from calendula, such as lutein and zeaxanthin, also contribute to **liver protection**, particularly in fatty liver disease [9]. Toxicological studies suggest that calendula is **safe and well-tolerated**, with minimal side effects even at higher doses [11]. Given its rich phytochemical profile and proven efficacy in multiple biological systems, *C. officinalis* holds promise as a therapeutic agent.

While associations have been reported between marigold (Calendula officinalis) extracts—and their individual phytochemicals—and beneficial effects in metabolic disorders such as NAFLD and in cancer, the underlying molecular mechanisms remain insufficiently understood. This thesis seeks to explore these mechanisms in detail, with a particular focus on the roles of specific bioactive compounds present in this plant. To achieve the same, the following objectives have been formulated:

Objectives

1. To identify the principal phytochemical constituents of Calendula species (marigold) with potential therapeutic value through a systematic review and analysis of existing literature.
2. To investigate the biological activities and molecular mechanisms of marigold-derived phytochemicals using transcriptomic data.

Chapter 3: Methodology used

3.1. RNA seq data analysis

Transcriptomic (RNA-seq) data were discovered through a comprehensive literature analysis to understand the state of the field. Upon collecting phytochemical data from publicly available sources, relevant RNA-seq datasets were obtained for further analysis.

3.1.1. Data Availability

There are five phytochemicals, each associated with a different dataset on which RNA-seq data analysis was performed. Below is the list of phytochemicals along with their respective accession numbers:

PHYTOCHEMICAL	ACCESSION NUMBER
Lutein	PRJNA785785
Fucoxanthin	PRJNA598239
Vitamin E	PRJNA450370
Lycopene	PRJNA738497
Quercetin	PRJNA898519

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 gene annotation in respective 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 *p*-value.

3.1.6. Functional Enrichment Analysis

Differentially expressed genes (DEGs) were subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Enriched terms were considered significant at p-value < 0.01.

3.1.7. Visualization

Several visualization techniques were used to efficiently analyze and display the results of differential gene expression. To give a thorough picture of the DEGs, volcano plots were created, emphasizing those with notable shifts in expression according to both p-values and log2 fold change. Additionally, to show the expression patterns of the top DEGs across various sample groups, heatmaps were made using python. By making it easier to find gene clusters with comparable expression patterns, these heatmaps helped explain biological reactions and treatment outcomes.

Chapter 4: Lycopene alleviates NAFLD

4.1. Lycopene as a Phytochemical in Marigold

Carotenoids and other bioactive substances are abundant in marigold. Notably, lycopene is one of the carotenoids found in marigold petals, along with lutein, β-carotene, zeaxanthin, and others [49]. Because of its conjugated double bonds, the lipophilic red-orange pigment lycopene has a potent antioxidant activity [50] which could be useful in treating various diseases such as NAFLD because oxidative stress is one of the various hallmarks of NAFLD. Lycopene is a precursor in the manufacture of other carotenoids and adds to the vivid hue of plants like marigold [49].

4.2. Lycopene and Non-Alcoholic Fatty Liver Disease (NAFLD)

Excessive hepatic fat buildup is a hallmark of non-alcoholic fatty liver disease (NAFLD), which is frequently associated with inflammation and insulin resistance. The development of NAFLD is significantly influenced by oxidative stress and inflammatory pathways, including NF-κB activation [50]. By altering these pathogenic pathways, lycopene has demonstrated hepatoprotective effects in animal models. For instance, lycopene supplementation (20–60 mg/kg/day) dramatically inhibited the development of NAFLD in mice fed a high-fat, high-fructose diet in a 2023 research [51]. Compared to untreated controls, mice treated with lycopene showed better liver enzyme profiles, decreased liver inflammation (lower levels of TNF-α and IL-6), and decreased hepatic triglyceride buildup. Mechanistically, lycopene reduced gut dysbiosis by boosting good bacteria that produce short-chain fatty acids and inhibited the liver's NF-κB/NLRP3 inflammasome pathway, which is a major cause of inflammation [51]. These activities imply that lycopene can stop the "two-hit" (lipid and inflammation) process that drives the development of NAFLD. Lycopene has been shown in preclinical animal research to have positive effects on NAFLD. According to a thorough analysis, lycopene supplementation decreased oxidative stress in the liver and cholesterol levels in mouse models fed a high-fat diet. Lycopene dramatically reduced pro-inflammatory cytokines (TNF-α, IL-6), elevated antioxidant enzyme activity (catalase, SOD) and decreased hepatic and blood triglycerides in obese rats while increasing HDL ("good") cholesterol. In a rat NAFLD model, dose-dependent lycopene therapy also improved lipid profiles (lowering LDL-C and TG) and liver function tests (ALT, AST), as well as increased glutathione and SOD levels, suggesting improved antioxidant defenses. Interestingly, lycopene further validated its anti-

inflammatory mechanism in these mice by downregulating the production of cytokines and inflammatory genes including CYP2E1 [50].

Building on preclinical evidence supporting lycopene's efficacy in mitigating metabolic and inflammatory disturbances associated with NAFLD, I analyzed a relevant RNA-Seq dataset from lycopene-treated mice to examine transcriptomic changes in hepatic tissue.

The analysis utilized six mouse liver RNA-Seq samples (three paired-end controls and three paired-end lycopene-treated) obtained from the NCBI SRA (PRJNA738497). The corresponding mouse GTF annotation file and FASTA genome sequence were retrieved from the genome section of NCBI.

4.3. Results

4.3.1. Identification of Differentially Expressed Genes

Using a volcano plot (Figure 4.1A) with cutoffs of $|\log_2 FC| \geq 2$ and $p < 0.01$ ($-\log_{10}(p) \geq 1.3$), 348 significantly upregulated ($\log_2 FC \geq 2$) genes and 177 significantly downregulated ($\log_2 FC \leq -2$) genes were plotted when comparing experimental to control samples. The data for DSeq file with information of $\log_2 FC$ of all the genes is provided in Supplementary file 1 and an overview has been shown as a heatmap in Figure 4.1B.

4.3.2. Understanding Changes in Gene Expression and Associated Biological Responses

As shown in (Figure 4.2A) the KEGG pathway enrichment analysis highlights several key molecular mechanisms closely linked to the development and progression of NAFLD. The list of pathways are provided in Supplementary file 2. One of the significantly enriched pathways identified was the AMPK signaling pathway, which plays a central role in maintaining cellular energy balance and regulating lipid metabolism. Dysregulation of this pathway is indicative of impaired energy homeostasis and increased lipid accumulation—both hallmark features of NAFLD [52]. Within this pathway, the PFKFB3 gene (6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase 3) is a key regulator. Downregulation of PFKFB3 in hepatocytes can be detrimental, as PFKFB3-driven glycolysis fuels de novo lipogenesis; conversely, global disruption of PFKFB3 has been shown to blunt diet-induced hepatic steatosis, suggesting that active glycolytic flux contributes to fat synthesis and storage [53]. In this study, lycopene treatment resulted

in a $\log_2$ fold change of 2.3 for PFKFB3, indicating upregulation, which could be advantageous in ameliorating NAFLD.

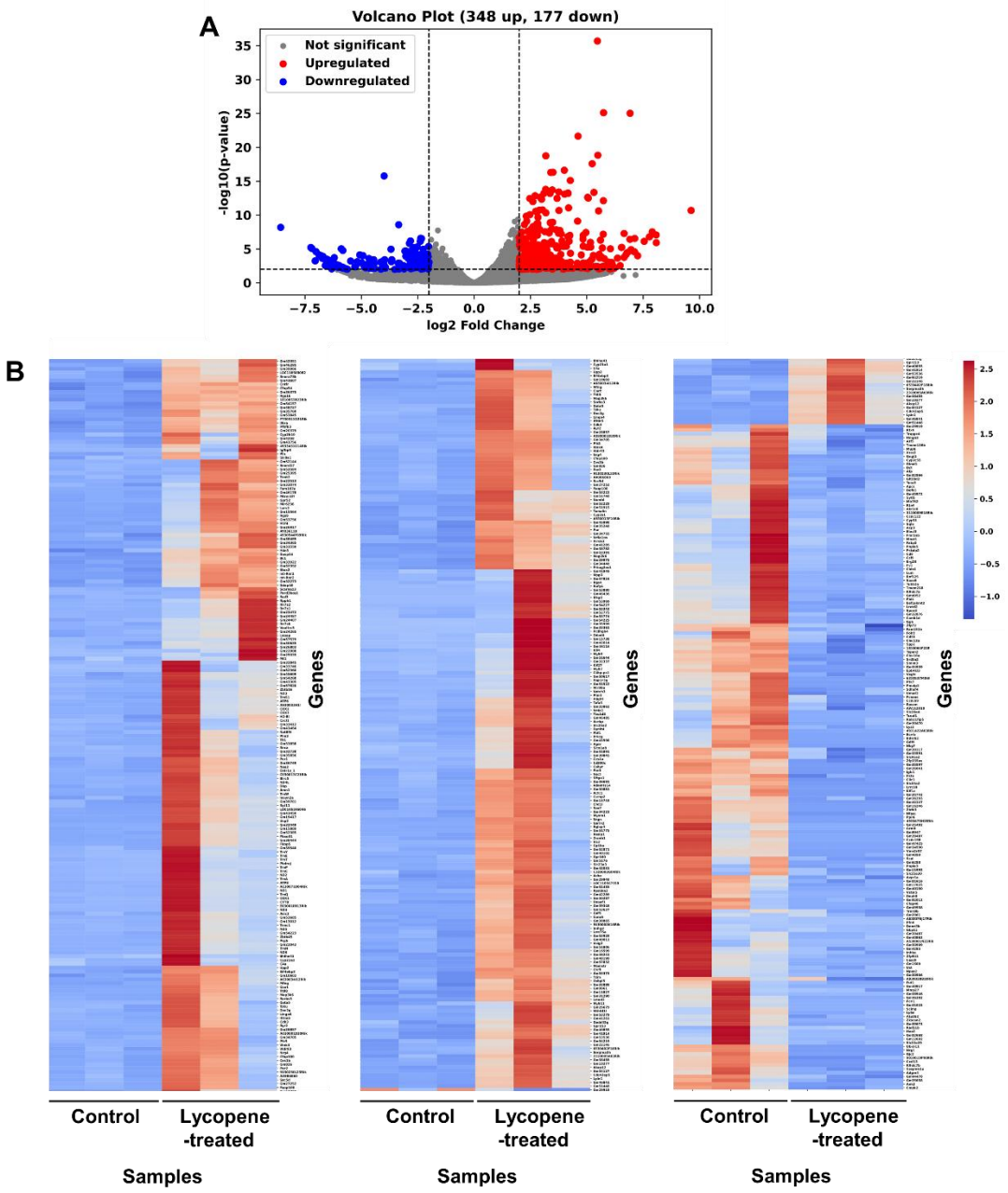

Figure 4.1. (A) Volcano plot illustrating significantly dysregulated genes following lycopene treatment. (B) Heatmap providing an overview of global gene expression changes upon lycopene treatment.

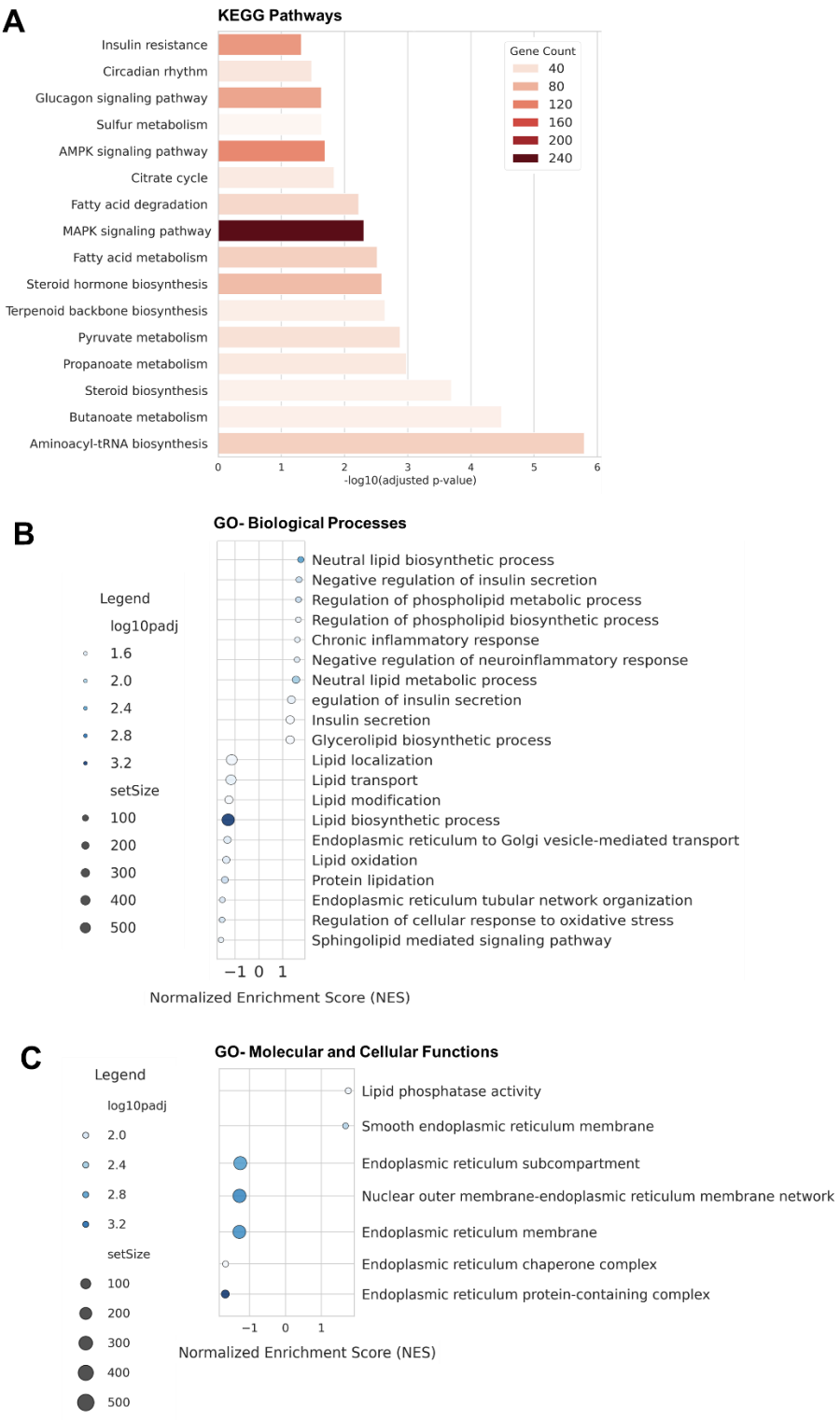

Figure 4.2. Enrichment analysis of differentially expressed genes in lycopene-treated samples, showing (A) KEGG pathways, (B) GO – Biological Processes, and (C) GO – Molecular and Cellular Functions.

Enrichment of the insulin resistance pathway highlights one of NAFLD's primary pathological features - dysregulated insulin signaling, which promotes hepatic glucose overproduction and fat deposition. Similarly, the glucagon signaling pathway reflects disturbances in hepatic glucose and lipid metabolism [54], contributing to the metabolic imbalance characteristic of NAFLD. Enrichment of pathways such as steroid hormone biosynthesis and steroid biosynthesis suggests altered hormonal regulation and cholesterol metabolism, both of which can exacerbate hepatic fat accumulation and inflammation [55].

Several key enzymes in cholesterol biosynthesis were differentially expressed following lycopene treatment. SQLE (Squalene Epoxidase), which converts squalene to squalene epoxide in a late step of cholesterol synthesis, is typically upregulated in advanced NAFLD, driving cholesterol accumulation and worsening liver pathology, including increased risk of progression to NASH and HCC [56, 57]. In this study, lycopene treatment resulted in a $\log_2$FC of −2.4, suggesting that SQLE downregulation may have therapeutic potential. Likewise, FDFT1 (Farnesyl-Diphosphate Farnesyltransferase 1, also known as Squalene Synthase), the first committed enzyme in sterol biosynthesis, is elevated in NAFLD livers, where its overexpression promotes cholesterol and triglyceride accumulation and worsens liver injury [58, 59]. Lycopene treatment reduced FDFT1 expression ($\log_2$FC −1.5), indicating a possible protective effect via suppression of the cholesterol biosynthetic pathway. Similarly, HMGCS1 (3-Hydroxy-3-Methylglutaryl-CoA Synthase 1), another key cholesterol biosynthetic enzyme, is often upregulated in NAFLD, leading to toxic free cholesterol buildup in hepatocytes, with severity correlating to intrahepatic cholesterol content [60]. The observed $\log_2$FC of −1.7 after lycopene treatment suggests that HMGCS1 downregulation may contribute to its hepatoprotective effects.

Enrichment of the fatty acid metabolism and fatty acid degradation pathways indicates an imbalance between lipid synthesis and breakdown, leading to hepatic steatosis—the defining feature of NAFLD [61]. MID1IP1 (Midline-1 Interacting Protein 1, also known as MIG12) acts as a co-factor activating acetyl-CoA carboxylase (ACC), the rate-limiting enzyme in fatty acid synthesis. In NAFLD, MID1IP1 is significantly upregulated as part of the sterol/lipogenesis network; overexpression in mouse liver markedly increases ACC activity, drives fatty acid synthesis, and promotes triglyceride accumulation, while inhibition of MIG12 exerts a protective,

fat-lowering effect [62, 63]. Following lycopene treatment, MID1IP1 showed a $\log_2$ fold change of −1.09, suggesting that its downregulation may have therapeutic potential in NAFLD.

Additional enriched pathways, such as pyruvate metabolism and the citrate cycle (TCA cycle), point to mitochondrial and metabolic dysfunction [64], contributing to oxidative stress and inefficient energy utilization in hepatocytes. The enrichment of the circadian rhythm pathway further suggests that disruptions in the body's internal clock may impair lipid metabolism and inflammatory regulation [65], worsening NAFLD progression.

Other intermediate metabolic pathways—propanoate metabolism [66], butanoate metabolism [67], aminoacyl-tRNA biosynthesis [68], and terpenoid backbone biosynthesis [69]—reflect broader metabolic reprogramming in hepatocytes, affecting protein synthesis, lipid handling, stress adaptation, and cholesterol metabolism. Within this context, ABCG5 (ATP-Binding Cassette G5), together with ABCG8, forms a sterol transporter complex that facilitates biliary cholesterol excretion, thereby preventing hepatic cholesterol accumulation. Loss of ABCG5/ABCG8 function in hyperlipidemic mouse models leads to reduced biliary cholesterol secretion, aggravated fatty liver, worsened insulin resistance, and heightened inflammation; conversely, active ABCG5/ABCG8 protects against cholesterol-induced lipotoxicity [70, 71]. Lycopene treatment increased ABCG5 expression ($\log_2$FC = 1.47), suggesting that its upregulation may help mitigate NAFLD.

Altogether, these pathways illustrate a complex network of metabolic, hormonal, and inflammatory changes that drive NAFLD, offering multiple avenues for therapeutic targeting. Notably, the most significantly enriched pathway was the MAPK signaling pathway, which plays a central role in regulating inflammatory responses [72], insulin signaling [73], and hepatocyte apoptosis [74]—key processes in the progression of NAFLD to more severe stages such as NASH [75]. MAPK also mediates IL-10 anti-inflammatory signaling, while IL-10 itself modulates MAPK pathway activity [76]. IL10 (Interleukin-10) is a potent anti-inflammatory and immunomodulatory cytokine that counteracts pro-inflammatory cytokines and limits immune cell activation. In NAFLD, IL-10 levels decline with disease severity, with the lowest levels observed in obese individuals with NASH, correlating with greater lobular inflammation. This loss of IL-10 removes a critical "anti-inflammatory brake," allowing inflammation to escalate. Animal studies further

demonstrate that IL-10 knockout mice develop more severe steatohepatitis and fibrosis, while IL-10 therapy ameliorates these features [77]. In this study, lycopene treatment led to upregulation of IL10 ($log_2FC = 0.94$), supporting its potential role in attenuating inflammation, reducing fibrosis, and contributing to the resolution of NAFLD pathology [77, 78].

As shown in Figure 4.2B and 4.2C, the biological processes, molecular, and cellular functions enrichment analysis revealed several key molecular functions closely tied to the pathogenesis of non-alcoholic fatty liver disease (NAFLD). A comprehensive list of pathways involved in biological processes, molecular, and cellular functions is provided in Supplementary File 3. A prominent cluster of enriched terms related to lipid metabolism—including neutral lipid biosynthesis, lipid transport, lipid oxidation, and lipid modification—reflects the disrupted balance between lipid synthesis, storage, and degradation, a central feature of hepatic steatosis in NAFLD [79]. Among these, NR4A1 (Nur77) emerged as a key modulator. In macrophages, NR4A1 promotes an anti-inflammatory (M2-like) phenotype and reduces cytokine output, mitigating chronic hepatic inflammation [80]. In hepatocytes, NR4A1 enhances fatty acid oxidation, represses lipogenesis, and alleviates lipid accumulation, partly via GOS2 downregulation. The observed log_2FC of 2.02 following lycopene treatment suggests that NR4A1 upregulation could represent a viable therapeutic approach for NAFLD.

ATG2A (Autophagy-Related 2A), essential for autophagosome membrane expansion and lipid transport during lipophagy, also emerged as a relevant target. Impaired ATG2A function leads to defective autophagy, reduced lipid droplet clearance, and NAFLD exacerbation [81]. The observed log_2FC of 1.3 indicates that lycopene-mediated ATG2A induction may restore autophagic flux, offering hepatoprotective benefits.

Significant enrichment was also observed in insulin-related processes—insulin secretion, regulation of insulin secretion, and negative regulation of insulin secretion—highlighting the central role of insulin resistance in NAFLD pathophysiology [82]. In parallel, enrichment of chronic inflammatory and neuroinflammatory processes underscores the importance of persistent low-grade inflammation in driving progression from steatosis to NASH [83].

Of particular interest, PNPLA3 (Patatin-like Phospholipase Domain-Containing 3)—the major genetic determinant of NAFLD susceptibility—was downregulated (log_2FC –2.2) following

lycopene treatment. The PNPLA3 I148M variant predisposes carriers to hepatic fat accumulation, inflammation, and fibrosis due to impaired lipase activity [84, 85]. Downregulation in this context suggests a therapeutic mechanism for reducing hepatic lipid burden.

Enrichment of endoplasmic reticulum (ER)-related processes, including ER tubular network organization and vesicle-mediated transport, pointed to the involvement of ER stress in NAFLD pathology [86]. Lycopene treatment increased BCL2 expression (log_2FC 1.35), an anti-apoptotic protein known to protect hepatocytes from lipotoxicity-induced apoptosis [89]. BCL2 restoration could therefore attenuate liver injury and fibrosis in NAFLD.

Other ER-associated regulators included SIRT7 (Sirtuin-7), which suppresses ER stress, modulates fibrogenesis by reducing hepatic stellate cell activation, and limits lipogenesis [90, 91]. The observed log_2FC of 0.97 suggests lycopene may enhance SIRT7-mediated protection against both metabolic and fibrotic damage.

Enrichment in lipid phosphatase activity indicates altered phosphoinositide metabolism, crucial for membrane trafficking and lipid homeostasis [92]. Additionally, structural ER terms—smooth ER membrane, ER sub-compartments, and ER chaperone complexes—reflect the organelle's central role in lipid/cholesterol biosynthesis and protein folding, processes frequently disrupted in NAFLD [86].

Fibrosis-related genes also showed significant expression changes. TIMP2 (Tissue Inhibitor of Metalloproteinases-2), typically elevated in NAFLD-associated fibrosis and promoting ECM accumulation [94], was markedly downregulated (log_2FC –1.94), suggesting antifibrotic potential. Conversely, COL1A1 (Collagen Type I Alpha-1) was unexpectedly upregulated (log_2FC 1.79), despite its known role in fibrosis progression [95], raising questions about potential context-specific regulation following lycopene exposure. Similarly, ACTA2 (Alpha-Smooth Muscle Actin), a hallmark of hepatic stellate cell activation and fibrosis [96], was upregulated (log_2FC 1.9), indicating complex effects on fibrogenesis that require further investigation.

As shown in Figure 4.3, Cytoscape-derived interaction networks highlight lycopene-induced reprogramming of cellular processes. Key hubs included DNA damage-response factors (e.g., TP53) linked to extracellular matrix genes (FN1, COL4A1, COL4A2), suggesting concurrent

modulation of tissue remodeling and genomic stability. Mitochondrial NADH dehydrogenase subunits (ND1–ND6, ND4L) formed a distinct cluster, indicating enhanced oxidative phosphorylation. Enrichment in pentose phosphate pathway enzymes suggested altered cellular redox balance, while changes in aminoacyl-tRNA synthetases and ribosomal proteins (RPL7, RPL10, RPL31) pointed to modifications in translational regulation. Immune-related subnetworks encompassed inflammatory and apoptotic signaling components (TLR7, IL1RL1, IRAK1, TNF, CASP8), whereas epigenetic regulators (PHF8, KDM5C, KDM5D) indicated transcriptional reprogramming. Cytoskeletal and adhesion-associated genes (DOCK10, SHROOM2) and growth factor receptor connections (VEGFD, NRP2) further underscored lycopene's broad modulation of intercellular signaling, collectively supporting its multi-layered therapeutic effects in NAFLD.

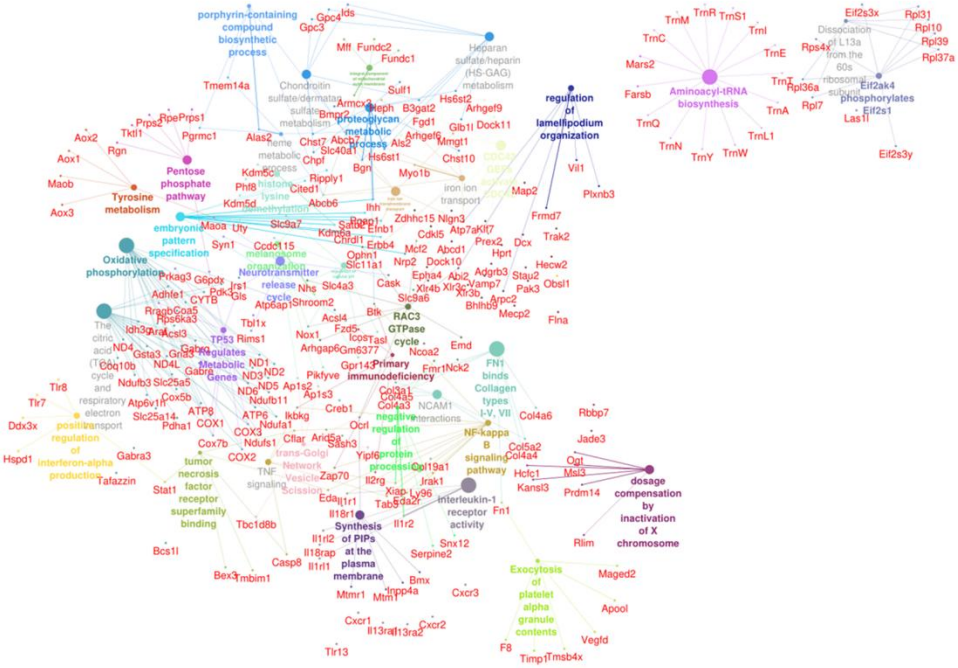

Figure 4.3. Cytoscape-derived interaction network depicting lycopene-induced reprogramming of cellular processes.

This summary highlights the intricate web of genetic and molecular factors that contribute to the pathophysiology of NAFLD and highlights how efficient treatment approaches can be developed through lycopene treatment.

Chapter 5. Antioxidant potential of Vitamin E in combating NAFLD

5.1 Vitamin E as a Phytochemical in Marigold

Tocopherols and tocotrienols, two types of vitamin E molecules, are abundant in marigold (*Calendula officinalis*) flowers. Marigold petals contain four tocopherols and four tocotrienols (α, β, γ, and δ), with α-tocopherol being the most common. Marigold contains tocols, powerful antioxidants that help neutralize free radicals and reduce oxidative stress. These antioxidant properties are key contributors to the plant's liver-protective and therapeutic effects. As such, vitamin E compounds (tocols) derived from marigold may hold significant promise in treating conditions like non-alcoholic fatty liver disease (NAFLD) that are linked to oxidative damage [97].

5.2 Vitamin E and Non-Alcoholic Fatty Liver Disease

Vitamin E comprises lipid-soluble antioxidants, primarily tocopherol and tocotrienol homologues (α, β, γ, and δ). These compounds terminate oxidative chain reactions in cell membranes by donating a hydrogen atom from the chromanol ring to neutralize lipid peroxyl radicals and other reactive species. Beyond direct antioxidant activity, vitamin E downregulates pro-oxidant and fibrogenic pathways (iNOS, NADPH oxidase, TGF-β) and enhances endogenous antioxidant defenses (superoxide dismutase, catalase, glutathione peroxidase), thereby reducing oxidative stress and lipid peroxidation—key drivers of NAFLD progression [98].

Vitamin E also exerts anti-inflammatory effects by inhibiting NF-κB signaling and lowering proinflammatory cytokines (TNF-α, IL-1β, IL-6). It increases adiponectin levels, which suppress lipogenesis in hepatocytes and promote fatty acid oxidation. To preserve hepatocyte integrity, vitamin E modulates apoptosis-related pathways, decreasing pro-apoptotic proteins (p53, caspases, BAX) and increasing the anti-apoptotic protein BCL-2. Its anti-fibrotic action involves inhibition of TGF-β signaling and hepatic stellate cell activation, limiting collagen deposition and fibrosis [98].

Tocotrienols, the unsaturated side-chain isomers of vitamin E, share these hepatoprotective mechanisms and may be even more potent. In NAFLD models, tocotrienol supplementation reduced fibrotic markers (α-SMA, TGF-β), ER stress, inflammation, and steatosis, while improving lipid metabolism and insulin sensitivity [99]. Collectively, these activities—ROS

scavenging, inflammation reduction, lipid homeostasis restoration, and fibrogenesis prevention—underpin vitamin E's protective role in liver health.

Building on evidence of vitamin E's antioxidant and hepatoprotective potential, we performed transcriptome profiling using a publicly available RNA-Seq dataset (NCBI SRA: PRJNA450370). This dataset comprised six paired-end RNA-Seq libraries from chicken abdominal fat tissue—three controls and three vitamin E-treated samples. The *Gallus gallus* reference genome and gene annotation (GTF) files were retrieved from NCBI for sequence alignment and expression analysis.

5.3 Results

5.3.1 Identification of Differentially Expressed Genes

Using a volcano plot (Figure 5.1A) with cutoffs of $|\log_2 FC| \geq 2$ and $p < 0.01 (-\log_{10}(p) \geq 1.3)$, 46 significantly upregulated ($\log_2 FC \geq 2$) genes and 282 significantly downregulated ($\log_2 FC \leq -1$) genes were plotted when comparing experimental to control samples. The data for these genes along with their $\log_2 FC$ value is provided in Supplementary file 4 and an overview of changes in global expression pattern has been shown as a heatmap in Figure 5.1B.

5.3.2 Understanding Changes in Gene Expression and Associated Biological Responses

Comprehensive enrichment analyses identified several key metabolic and signaling pathways strongly associated with altered gene expression in non-alcoholic fatty liver disease (NAFLD). The KEGG pathway bar plot (Figure 5.2A) and Supplementary File 5 list these pathways, many of which are central to lipid metabolism, inflammation, oxidative stress, and fibrosis. Among them, the MAPK signaling pathway, with the highest gene count, is essential for mediating oxidative stress [100], hepatocyte death [101], and inflammatory responses [72]. The MAPK cascade regulates GATA3 stability and Th2 differentiation via the ubiquitin–proteasome pathway [102]. Beyond its established roles in adipogenesis and Th2 signaling, GATA3 is now recognized as a modulator of metabolic dysfunction in fatty liver disease. In obese individuals with metabolic dysfunction–associated fatty liver disease (MAFLD), GATA3 overexpression in adipose tissue promotes pro-inflammatory M1 macrophage infiltration, potentially contributing to insulin resistance and hepatic inflammation [103]. In our dataset, Vitamin E treatment markedly upregulated GATA3 ($\log_2 FC = 3.5$, DESeq2), suggesting therapeutic potential. However, liver transcriptome studies

show GATA3 expression negatively correlates with Th2-cell abundance [104], indicating possible immune dysregulation. Genome-wide association data reveal GATA3 binds the promoter of the lipogenic regulator PPARγ2, reducing its expression and potentially limiting de novo lipogenesis [105]. Collectively, these findings position GATA3 as a promising target for reducing hepatic steatosis and immune-mediated injury.

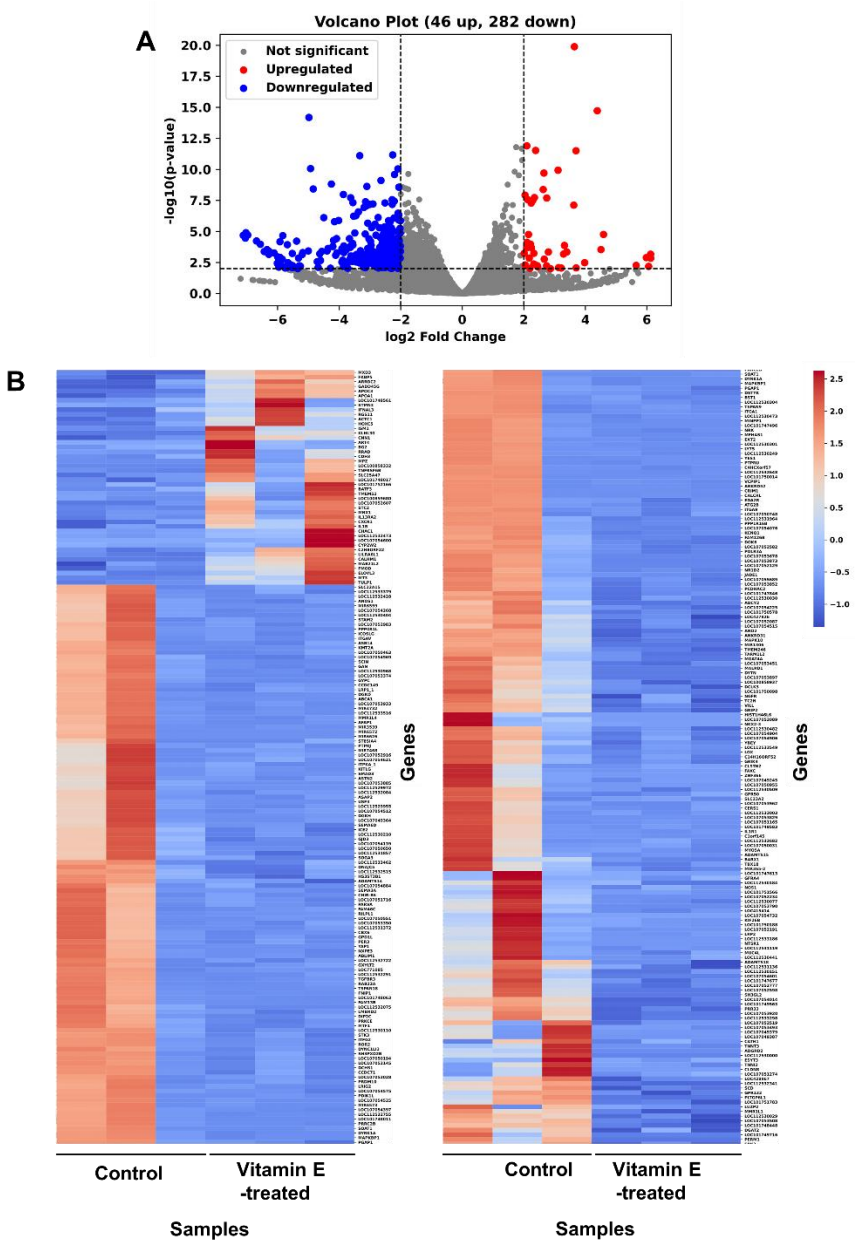

Figure 5.1. (A) Volcano plot illustrating significantly dysregulated genes following Vitamin E treatment. (B) Heatmap providing an overview of global gene expression changes upon Vitamin E treatment.

Other enriched KEGG pathways also map closely to NAFLD pathology. Dysregulation of Wnt signaling—critical for liver regeneration [106] and fibrosis [107]—has been linked to steatohepatitis and cirrhosis. The insulin signaling pathway [108], significantly enriched in our analysis, reflects the central role of insulin resistance in worsening lipid accumulation and metabolic dysfunction. The phosphatidylinositol signaling pathway, involved in metabolic regulation, ER stress [109], and intracellular signal transduction [110], also emerged as significant. Innate immune activation via C-type lectin receptor signaling may drive hepatic inflammation [111] and Kupffer cell activation [112]. Notch signaling, implicated in hepatic stellate cell activation, contributes to fibrogenesis [113]. Enrichment of amino acid biosynthesis pathways may indicate altered hepatic metabolism under stress [114]. Fatty acid metabolism and biosynthesis pathways—directly linked to hepatic lipid accumulation [115]—were also strongly represented.

At the gene level, CREBL2 regulates metabolic processes in liver and muscle. Knockdown studies show increased glucose uptake, glycolysis, and triglyceride biosynthesis in myoblast and hepatoma cells [116]. In our dataset, CREBL2 upregulation (log_2FC of 1.47) post–Vitamin E treatment suggests potential benefits in NAFLD. Collectively, these KEGG-derived pathways and genes underscore the multifactorial basis of NAFLD, involving insulin resistance, oxidative/ER stress, inflammation, apoptosis, and lipid dysregulation.

The biological process enrichment plot (Figure 5.2B; Supplementary File 6) further highlights dysregulated lipid transport and storage through enrichment of lipoprotein biosynthesis and metabolic processes [117]. Chronic hepatic inflammation—a hallmark of NASH—is supported by enrichment of inflammatory response and lipopolysaccharide response pathways [118]. The fatty acid translocase CD36 plays a pivotal role in hepatic uptake of long-chain fatty acids. Elevated CD36 expression correlates with steatosis severity in NAFLD patients [119], while hepatocyte-specific deletion in mice improves lipid profiles, reduces inflammation, and increases insulin sensitivity [120]. In our dataset, Vitamin E treatment upregulated CD36 ($log_2FC = 3.0$). Mechanistically, CD36 knockdown restores autophagy and reduces lipid droplet accumulation, while overexpression inhibits AMPK-mediated lipophagy [121]. CD36 palmitoylation enhances plasma membrane localization and fatty acid uptake; inhibition of this modification protects against NASH [119]. These data position CD36 as both a key driver and potential therapeutic target in NAFLD.

Additional enriched processes include amide biosynthesis and metabolism—suggestive of urea cycle disturbances and mitochondrial impairment [122]—and membrane lipid biosynthesis pathways such as glycerophospholipid [86], glycerolipid, and phosphatidylcholine metabolism [123], all of which can exacerbate ER stress and hepatocyte injury. Although enrichment of alcohol biosynthesis is not directly tied to ethanol metabolism in NAFLD, it may reflect altered metabolic flux [124]. Insulin response pathways, critical in NAFLD pathogenesis [125], were also enriched, supporting the link to de novo lipogenesis and impaired β-oxidation. Growth factor–related transmembrane protein tyrosine kinase signaling may influence hepatocyte survival [126], proliferation, and fibrosis [127].

The molecular function enrichment plot (Figure 5.2C) reinforces these findings. Enrichment of lipid transporter and transfer activities, cholesterol binding, and sterol/cholesterol transport underscores dysregulated lipid homeostasis [128], a driver of lipotoxicity [129], oxidative stress [88], and ER stress [130]. Apolipoprotein binding implicates altered lipoprotein assembly and secretion, potentially promoting hepatic lipid retention. GTPase activity enrichment points to impaired vesicle transport and lipid trafficking [132], both relevant to insulin resistance. Conversely, protein tyrosine kinase activity [133], transmembrane receptor signaling, and molecular transducer activity show negative enrichment, indicating possible reversal of NAFLD when these are suppressed.

IL6 illustrates this duality: while elevated IL-6 promotes hepatic inflammation and insulin resistance, Mendelian randomization suggests classical IL-6 signaling may protect against hepatic fat accumulation [134]. In our dataset, IL6 downregulation post–Vitamin E treatment ($\log_2FC = -0.9$) indicates potential therapeutic benefit via immune modulation.

Gene-specific analyses further refine these insights. SREBF-2 integrates cholesterol metabolism and autophagy, with variants such as rs133291 C/T linked to insulin resistance, dyslipidemia, and NAFLD progression [136]. Mechanistic studies show SREBF-2 promotes autophagy gene expression, enhancing lipid droplet clearance [137], while pharmacologic activation induces PNPLA8, reducing hepatic lipid content [138]. In our data, SREBF-2 was upregulated post–Vitamin E ($\log_2FC = 1.97$), supporting its therapeutic relevance. GCK, regulated by GCKR, influences hepatic glucose flux; GCKR variants (e.g., rs780094, rs1260326) increase triglyceride synthesis and fibrosis risk [139]. GCK downregulation in our dataset ($\log_2FC = -0.95$) suggests benefit by limiting lipogenesis.

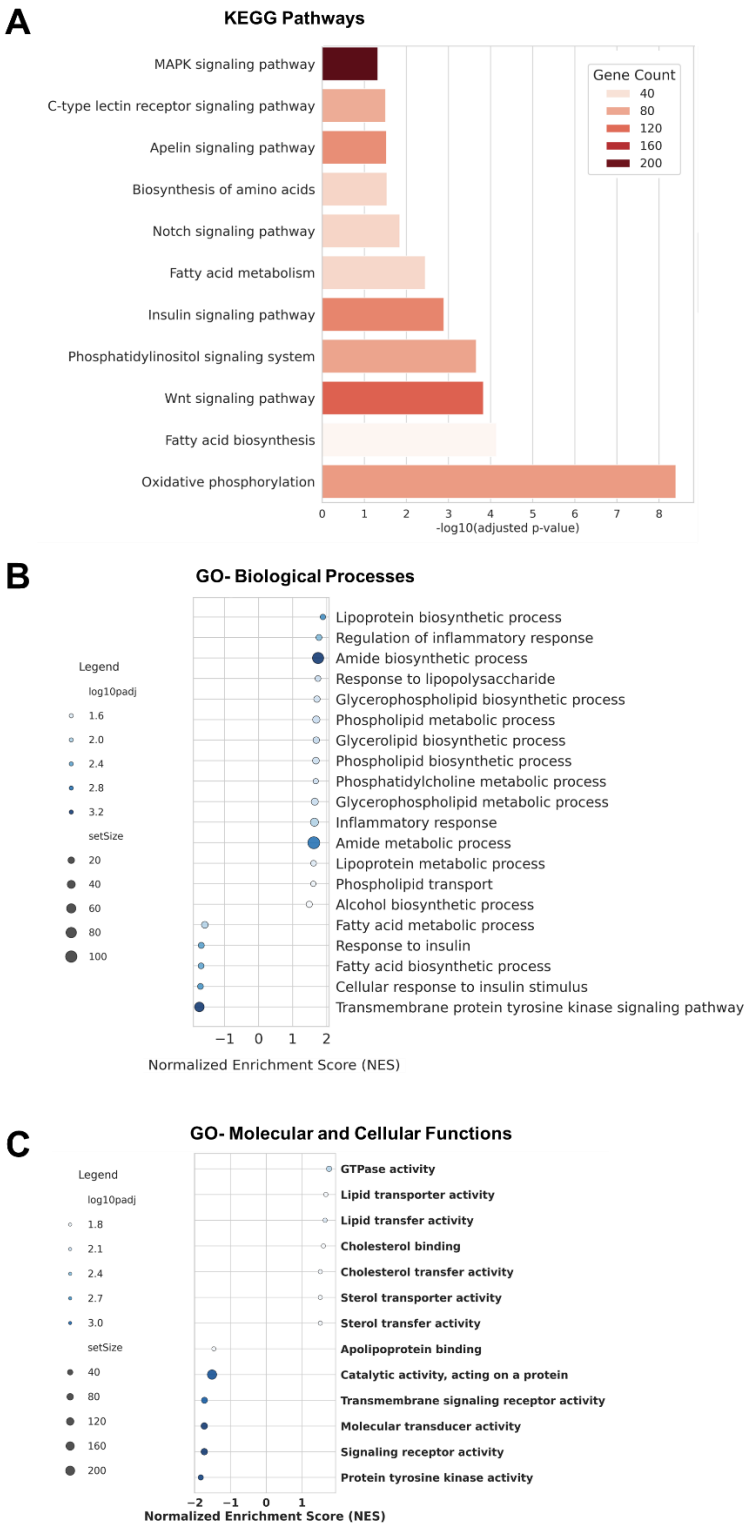

Figure 5.2. Enrichment analysis of differentially expressed genes in Vitamin E-treated samples, showing (A) KEGG pathways, (B) GO – Biological Processes, and (C) GO – Molecular and Cellular Functions.

The PNPLA3 I148M variant is the strongest genetic predictor of NAFLD severity, impairing lipid droplet hydrolysis and increasing oxidative stress [140,141]. It modulates treatment responses—G allele carriers respond better to weight loss and DPP-4 inhibitors, while C allele carriers benefit more from statins and omega-3 fatty acids [142]. In our data, PNPLA3 was upregulated post–Vitamin E treatment ($log_2FC = 1.53$), underscoring its role as a precision medicine target. Finally, IL1B (interleukin-1β) drives inflammation, stellate cell activation, and lipid accumulation [143]. While inhibition reduces fibrotic gene expression, it has limited effect on established fibrosis [144]. Downregulation in our data ($log_2FC = -0.95$) suggests anti-inflammatory benefits.

The pleiotropic activities of vitamin E were revealed by distinct yet highly interconnected modules in the Cytoscape-derived vitamin E interactome (Figure 5.3). Anchored by RELA, ANXA1, TLN1, and CD63, a central signaling nexus known as "positive regulation of endocytosis" directly interacted with G-protein-coupled receptors (GPR37, DCN, LRRK2) and growth-factor-receptor clusters (e.g., PDGFB, FGF10, NTRK2), suggesting coordinated modulation of cellular uptake and downstream proliferative/survival pathways. The cAMP–PKA axis (PCK2, PRKAR2B) and solute carriers (SLC26A1, SLC13A1, APBA1) formed a metabolic subnetwork to the left, while a distinct hub of enzymes involved in carbohydrate metabolism (FBP1, ALDOB, PFKM, PDE6B) highlighted changes in the flux of glycolysis and gluconeogenic processes. Vitamin E's impact on innate-immune surveillance was reflected in an immune-signalling module that included TBK1, IFN-W1, and the cytosolic DNA-sensing machinery (POLR3G, NFKBIB, TBK1). On the periphery, semaphorin family members (SEMA4D, SEMA3C/E) and their receptor NRP1 created a neurovascular cluster associated with "axon guidance," while epigenetic and vesicle-trafficking factors (EP300, HCFC2, TAF6L) linked transcriptional regulation to organelle dynamics. Lastly, smaller modules linked to branched-chain–amino-acid catabolism (HMGCS1, BCAT2) and one-carbon metabolism (GCAT, PSAT1) emphasized vitamin E's ability to modify redox and nutrient-sensing pathways. This topological landscape collectively showed that vitamin E coordinates a multi-layered response that may be responsible for its hepatoprotective effectiveness in non-alcoholic fatty liver disease. This reaction spans membrane trafficking, signal transduction, energy metabolism, and immune modulation.

By outlining the intricate web of genetic and molecular processes that underlie the etiology of NAFLD, this summary emphasizes the possibility of mechanism-based treatment approaches.

Taking vitamin E supplements has been shown to be effective in regulating several important pathogenic processes, including inflammation, oxidative stress, and dysregulated lipid metabolism. In NAFLD patients, vitamin E provides a promising adjuvant method for reducing disease progression and enhancing hepatic function by focusing on these important pathways.

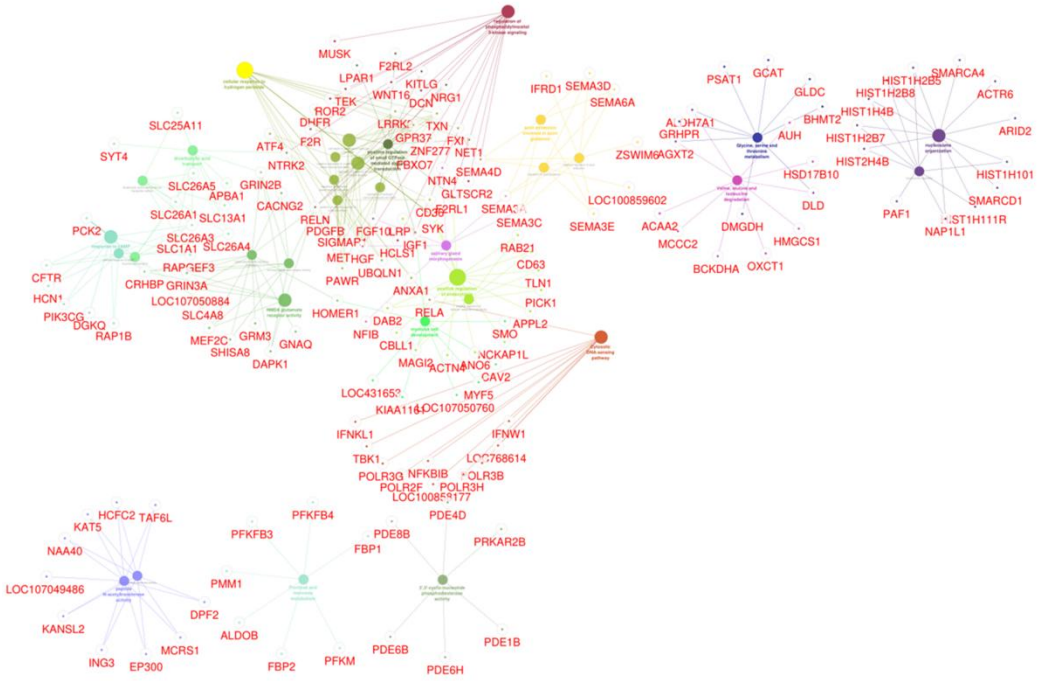

Figure 5.3. Cytoscape-derived interaction network depicting Vitamin E-induced reprogramming of cellular processes.

In summary, integration of pathway, process, and gene-level analyses reveals a complex molecular network underlying NAFLD, involving lipid metabolism, immune regulation, insulin sensitivity, and stress responses. Vitamin E supplementation modulates several critical nodes—GATA3, CD36, CREBL2, SREBF-2, GCK, PNPLA3, IL6, and IL1B—providing a multi-targeted therapeutic strategy to mitigate disease progression and enhance hepatic function.

Chapter 6. Fucoxanthin, a potent phytochemical for the treatment of NAFLD

6.1. Fucoxanthin as a Phytochemical in Marigold

Interestingly, marigold flower extracts have been found to contain trace amounts of fucoxanthin, a xanthophyll carotenoid that is usually linked to brown seaweed, along with more prevalent carotenoids like lutein and zeaxanthin. In a recent phytochemical profiling investigation, fucoxanthin was detected using HPLC-DAD in several solvent extracts of marigold petals such as ultrasound-acetone extract [145]. Fucoxanthin gives marigold extracts possible antioxidant, anti-inflammatory, and anti-obesity qualities, although being present in less concentrations than lutein or zeaxanthin. When marigold extracts are utilized in functional foods or nutraceuticals, they may provide further health benefits.

6.2. Fucoxanthin for Managing NAFLD

Targeting important pathogenic pathways such as lipid buildup, oxidative stress, inflammation, and fibrosis, fucoxanthin, a xanthophyll carotenoid, has demonstrated encouraging multimodal therapeutic effects in the treatment of non-alcoholic fatty liver disease (NAFLD). It modulates lipid metabolism in free fatty acid induced HepG2 models. Fucoxanthin reduces the accumulation of triglycerides and cholesterol by activating AMPK, suppressing lipogenic factors (SREBP-1c, FAS), and increasing PPARα and CPT-1. It enhances antioxidant defenses (↑ SOD, GSH-Px, CAT, HO-1, NQO1) and lowers lipid peroxidation (↓ MDA) by activating Nrf2 and its downstream effectors. It inhibits fatty acid-stressed hepatocytes' production of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), TLR4, MyD88, phosphorylated IκBα, and NF-κB p65 [PMID: 35447899]. Fucoxanthin dramatically reduced oxidative and inflammatory damage as well as early-stage fibrosis in mouse models of diet-induced NASH [146]. Fucoxanthin's effectiveness in lowering hepatic steatosis, insulin resistance, hyperlipidemia, and liver inflammation is demonstrated by preclinical and small-scale human trials [147].

To explore the transcriptional impact of fucoxanthin, RNA-Seq data from the NCBI Sequence Read Archive (SRA: PRJNA598239) were analyzed. The dataset comprised 18 human lung fibroblast samples, including six untreated controls and two treatment groups exposed to 5 μM

and 1 µM fucoxanthin (six replicates each). Reads were aligned to the human reference genome using its corresponding GTF annotation file obtained from NCBI's genome repository, followed by downstream expression analysis.

6.3. Results

6.3.1. Identification of Differentially Expressed Genes

Using a volcano plot (Figure 6.1A) with cutoffs of $|\log_2FC| \geq 2$ and $p < 0.05$ ($-\log_{10}(p) \geq 1.3$), 25 significantly upregulated ($\log_2FC \geq 2$) genes and 9 significantly downregulated ($\log_2FC \leq -1$) genes were plotted when comparing experimental to control samples. The data for DSeq file along with dysregulated genes is provided in Supplementary File 7 and an overview of changes in global expression pattern has been shown as a heatmap in Figure 6.1B.

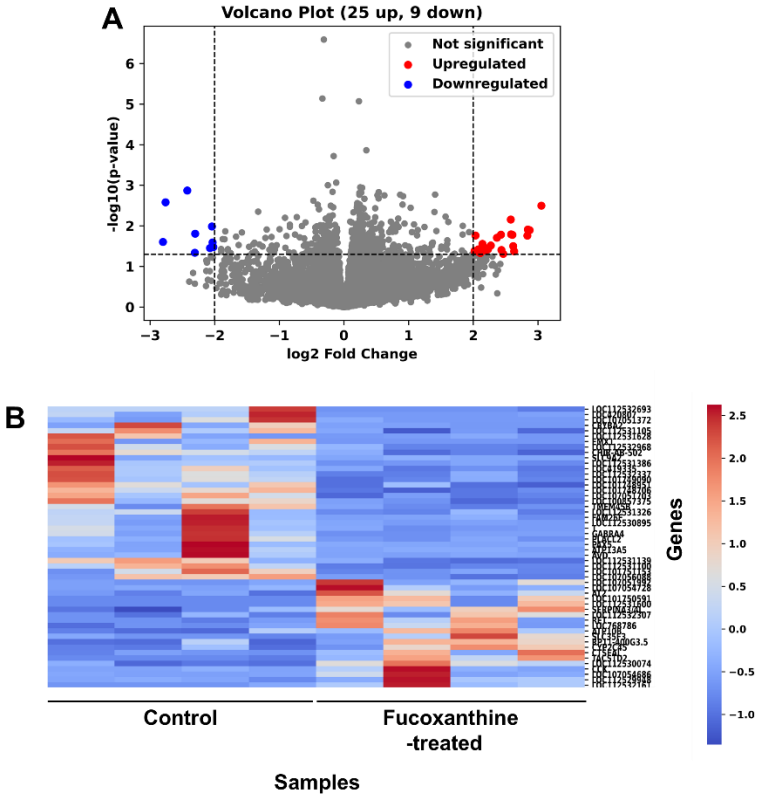

Figure 6.1. (A) Volcano plot illustrating significantly dysregulated genes following Fucoxanthin treatment. (B) Heatmap providing an overview of global gene expression changes upon Fucoxanthin treatment.

6.3.2. Understanding Changes in Gene Expression and Associated Biological Responses

As shown in Figure 6.2 A, KEGG pathway enrichment analysis reveals numerous signaling and metabolic pathways closely associated with the studied gene set, many of which are highly relevant to the pathophysiology of non-alcoholic fatty liver disease (NAFLD). The complete pathway list is provided in Supplementary File 8. Notably, the NOD-like receptor signaling [148], cytokine–cytokine receptor interaction [149], and cellular senescence [150] pathways indicate a central role for immunological and inflammatory responses in NAFLD progression. Enrichment of the cAMP signaling pathway suggests dysregulated metabolic signaling linked to insulin sensitivity [151] and lipid metabolism [152]. Importantly, the insulin resistance pathway—a hallmark of NAFLD—is significantly enriched, indicating that many implicated genes are tied to metabolic dysregulation. Altered hepatic lipid handling is further supported by enrichment of the ether lipid metabolism [153] and glycerophospholipid pathways. Together, these pathways underscore the multifactorial nature of NAFLD, involving metabolic failure, lipotoxicity, and immune activation.

As shown in Figure 6.2B, biological process enrichment analysis identifies numerous inflammation- and immunity-related pathways, many integral to NAFLD pathology. Enhanced terms such as cytokine production, acute inflammatory response, and modulation of inflammatory response [154] underscore chronic inflammation as a driver of disease progression. COX enzymes are central to inflammatory signaling, with COX-1 (PTGS1) having a constitutive, hepatoprotective role, in contrast to inducible COX-2. COX-1 expression rises in response to growth factors and pro-inflammatory cytokines [155]. Hepatocyte-specific COX-1 deletion worsens NAFLD in mice, with increased inflammation, fibrosis, steatosis, lipid accumulation, cytokine release (TNF-α, IL-6, IL-1β), and macrophage infiltration. Loss of COX-1 impairs autophagy and exacerbates lipid accumulation, suggesting its preservation may mitigate NAFLD, while inhibition (e.g., by NSAIDs) is likely detrimental [156]. Fucoxanthin treatment upregulated COX-1 (log2 fold change = 0.93), indicating a possible therapeutic avenue. Immune dysregulation in hepatic injury is also reflected in humoral immune response, adaptive immunity, and immune system development. Lipid transport and localization terms highlight altered lipid metabolism underlying hepatic steatosis. Key metabolic disturbances, including insulin resistance and mitochondrial dysfunction [157], are supported by enrichment of oxidative phosphorylation and insulin receptor signaling. ANGPTL3, a liver-secreted inhibitor of lipoprotein lipase, increases LDL and triglycerides; elevated levels promote insulin resistance and dyslipidemia in NASH. Inhibition of

ANGPTL3 in animals reduces hepatic steatosis and improves insulin sensitivity, while human loss-of-function variants lower TG/LDL without increasing hepatic fat. Pharmacological ANGPTL3 suppression (e.g., evinacumab) is under investigation [158]. Interestingly, fucoxanthin upregulated ANGPTL3 (log_2 fold change = 1.06), raising questions about its regulatory role in NAFLD. Oxidative phosphorylation disruption is closely linked to insulin resistance and mitochondrial dysfunction in NAFLD [157]. While essential for ATP production, electron leakage at complexes I and III generates ROS; excessive ROS drives oxidative stress and mitochondrial injury [159]. ISG15, an interferon-inducible ubiquitin-like modifier, is upregulated in human and diet-induced NASH. By binding the GCL complex, ISG15 boosts glutathione synthesis, reducing ROS and lipotoxic apoptosis. Overexpression protects against oxidative damage, whereas loss increases stress and cell death [160]. Fucoxanthin increased ISG15 (log_2 fold change = 0.97), suggesting a potential antioxidant mechanism.

As shown in Figure 6.2C, molecular and cellular function enrichment identifies key processes in NAFLD onset and progression. The full list is in Supplementary File 9. Dysregulated lipid handling, a defining feature of hepatic steatosis, is reflected in increased lipid transporter activity [161], including ATPase-coupled intramembrane lipid transport and sphingolipid floppase [162]. Enhanced calcium-dependent phospholipid binding suggests altered membrane signaling and stress responses [163]. Enrichment of IPAF and classical inflammasomes indicates inflammasome-mediated inflammation and innate immunity in NASH progression. Conversely, negative enrichment of lipid kinase activity may reflect impaired phosphoinositide-mediated insulin signaling and lipid metabolism [164,165].

From these hallmarks, FDFT1 and PCSK9 emerge as therapeutic targets. Elevated FDFT1 promotes cholesterol overproduction, lipotoxicity, oxidative stress, and inflammation; inhibition reduces lipid accumulation and protects hepatocytes. Flavonoids like bavachinin downregulate FDFT1 via AKT/mTOR/SREBP-2 signaling, mitigating steatosis [166]. Fucoxanthin decreased FDFT1 (log_2 fold change = −0.94), supporting its therapeutic relevance. PCSK9 promotes LDL receptor degradation, increasing circulating LDL and worsening NAFLD. Overexpression exacerbates steatosis, inflammation, and fibrosis; loss-of-function variants are protective. PCSK9 inhibitors (e.g., alirocumab, evolocumab) reduce LDL-C and improve hepatic steatosis [167]. Fucoxanthin downregulated PCSK9 (log2 fold change = −0.94), aligning with a protective role.

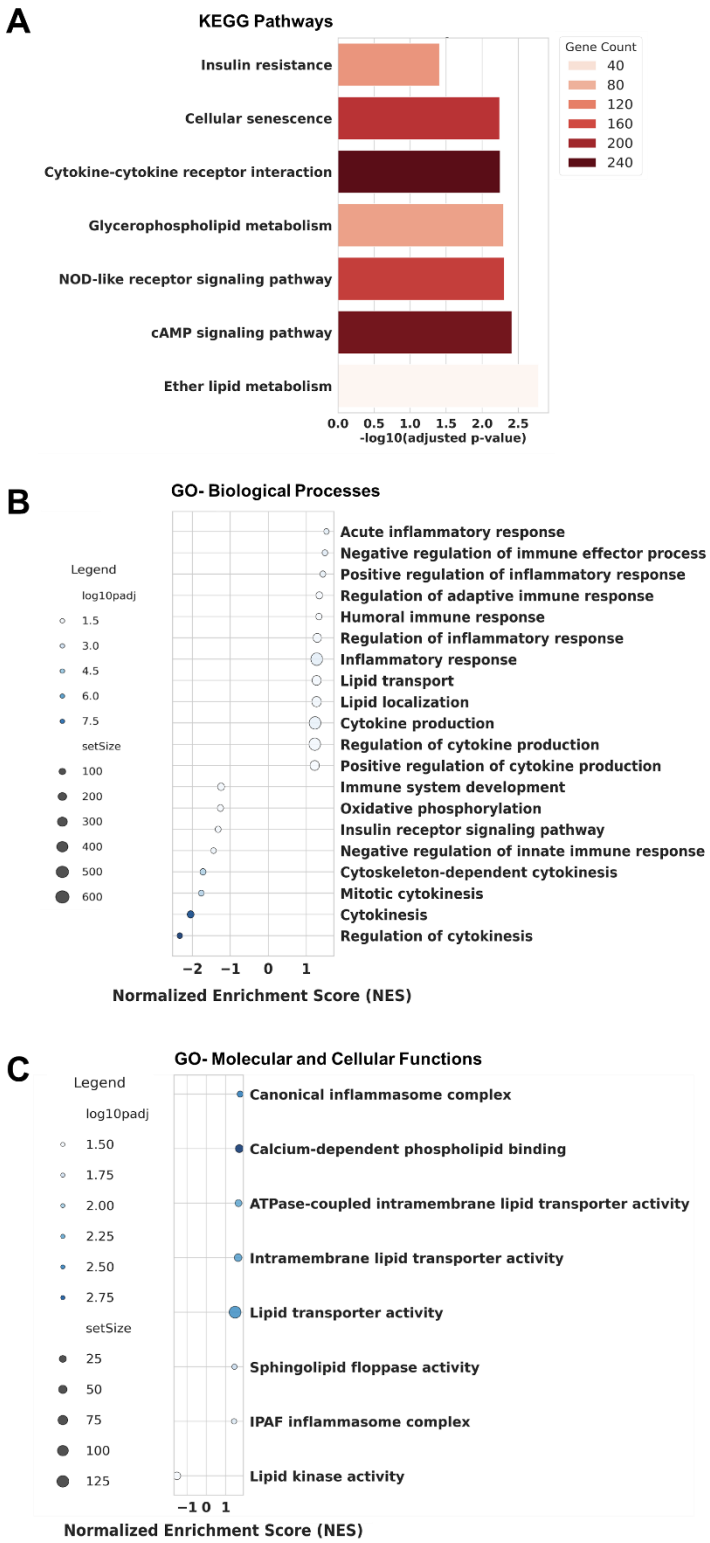

Figure 6.2. Enrichment analysis of differentially expressed genes in Fucoxanthin-treated samples, showing (A) KEGG pathways, (B) GO – Biological Processes, and (C) GO – Molecular and Cellular Functions.

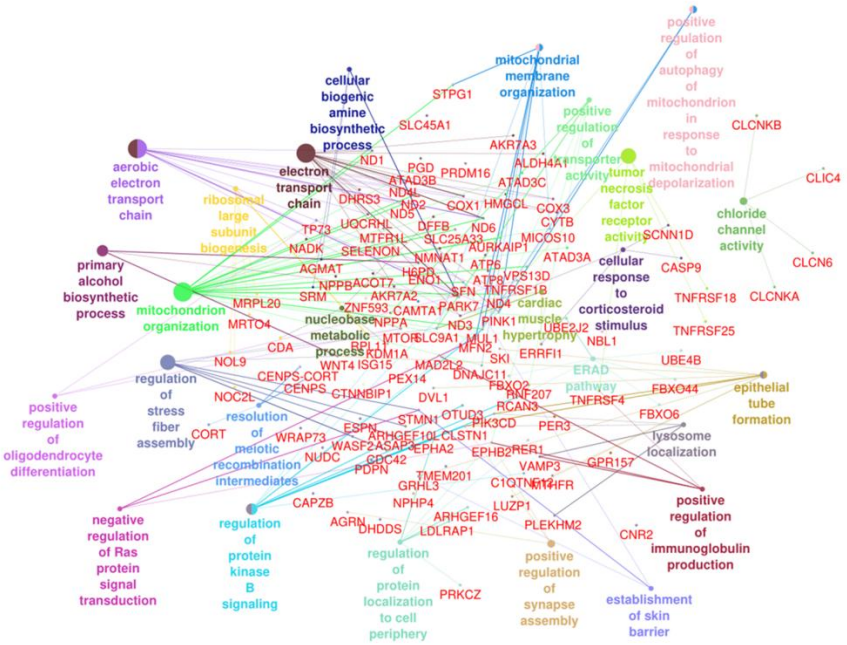

Figure 6.3. Cytoscape based network of Fucoxanthin responsive genes.

The Cytoscape-derived fucoxanthin-responsive interactome (Figure 6.3) illustrates its multifaceted cellular impact. Central modules of respiratory and mitochondrial genes (ND1, NDUFS3, COX3, CYTB) are linked to aerobic electron transport, oxidative phosphorylation, and mitochondrial organization, indicating enhanced biogenesis. Peripheral subnetworks reflect metabolic rewiring (primary alcohol/biogenic amine biosynthesis, ribosomal biogenesis, pentose phosphate pathway), autophagy regulation, Ras and AKT signaling, and morphogenetic programs. Immune-related modules (immunoglobulin production, lysosome localization) suggest modulation of both innate and adaptive immunity. These interconnected changes demonstrate how fucoxanthin coordinates protein synthesis, energy metabolism, signaling, organelle quality control, and immune regulation.

In summary, the integration of oxidative, inflammatory, and metabolic pathways in NAFLD underscores its complexity. Fucoxanthin's broad molecular impact positions it as a promising multimodal therapeutic candidate warranting further preclinical and clinical investigation.

Chapter 7. Quercetin improves liver function

7.1. Quercetin in marigold

Calendula officinalis, or marigold, contains a flavonoid called quercetin, which has potent hepatoprotective, anti-inflammatory, and antioxidant qualities. It has an impact on lipid metabolism and oxidative stress regulation, both of which are essential for treating diseases like non-alcoholic fatty liver disease (NAFLD). Research has verified quercetin's existence in marigold extracts and its role in the pharmacological actions of the plant [168].

7.2. Quercetin in treating NAFLD

Quercetin exerts potent hepatoprotective effects against non-alcoholic fatty liver disease (NAFLD) through multiple pathways. It alleviates hepatic steatosis by restoring mitochondrial function, enhancing autophagy (lipophagy), and improving fatty acid metabolism. Quercetin also suppresses inflammation by inhibiting NF-κB, NLRP3 inflammasome, and TGF-β/SMAD signaling pathways, while reducing oxidative stress via upregulation of antioxidant enzymes such as SOD and GSH. In addition, it modulates gut microbiota composition and regulates bile acid metabolism through activation of FXR/TGR5, further contributing to liver protection [169].

Based on strong preclinical evidence for its antioxidant and anti-inflammatory activities, transcriptomic analysis was performed using an RNA-Seq dataset comprising six rat bladder tissue samples—three paired-end controls and three paired-end quercetin-treated—downloaded from the NCBI SRA (PRJNA898519). The rat reference genome and corresponding GTF annotation file were retrieved from NCBI's genome repository for sequence alignment and downstream expression analysis.

7.3. Results

7.3.1. Identification of Differentially Expressed Genes

Using a volcano plot (Figure 7.1A) with cutoffs of $|\log_2 FC| \geq 2$ and $p < 0.01$ ($-\log_{10}(p) \geq 1.3$), 1035 significantly upregulated ($\log_2 FC \geq 1$) genes and 476 significantly downregulated ($\log_2 FC \leq -2$) genes were plotted when comparing experimental to control samples. The data for DSeq file along

with log_2 fold change value of dysregulated genes is provided in Supplementary File 10 and an overview of changes in global expression pattern has been shown as a heatmap in Figure 7.1B.

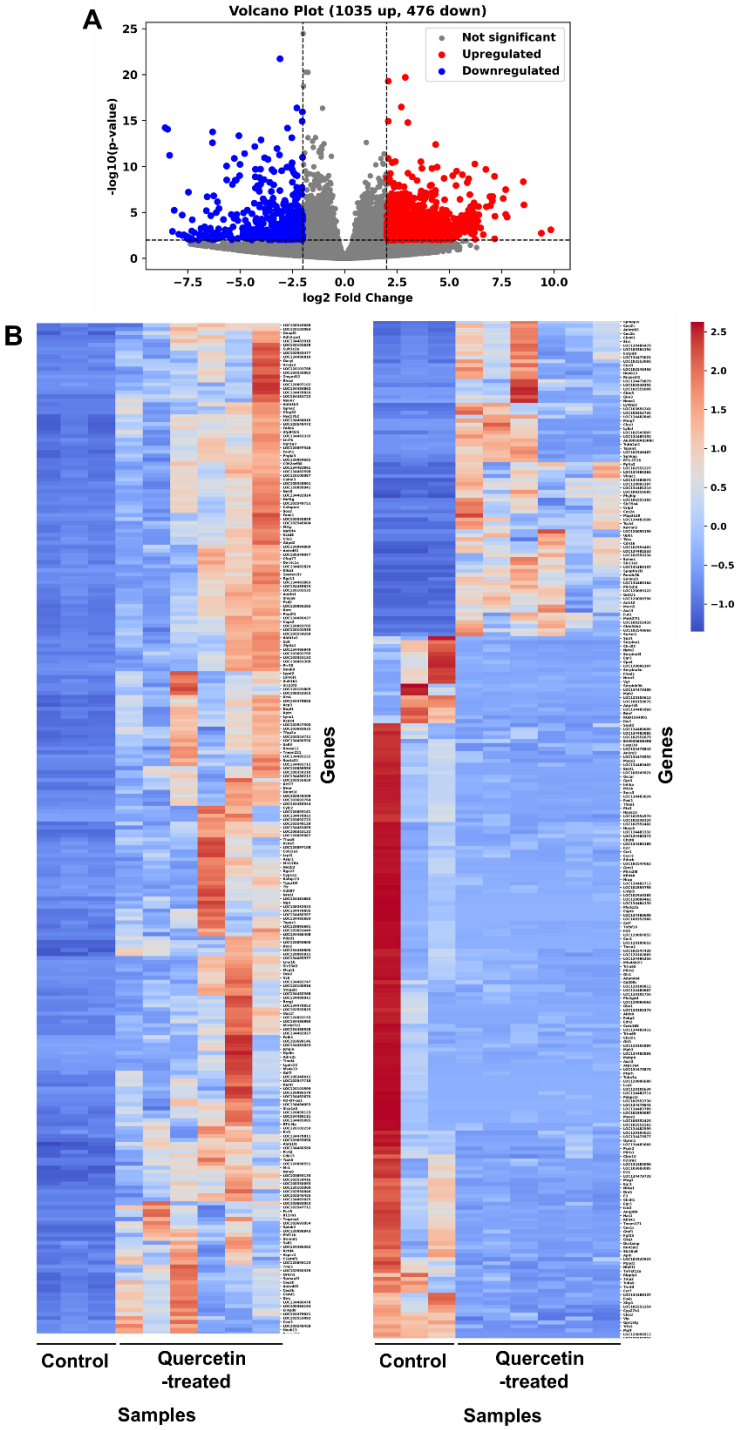

Figure 7.1. (A) Volcano plot illustrating significantly dysregulated genes following Quercetin treatment. (B) Heatmap providing an overview of global gene expression changes upon Fucoxanthin treatment.

7.3.2. Understanding Changes in Gene Expression and Associated Biological Responses

As shown in Figure 7.2A, the barplot of KEGG pathway enrichment analysis highlights several key biological mechanisms and signaling pathways strongly linked to non-alcoholic fatty liver disease (NAFLD). The complete list of KEGG pathways is provided in Supplementary File 11. The analysis identified multiple pathways closely associated with the pathophysiology of NAFLD. The most significantly enriched pathway—lipid and atherosclerosis—reflects the strong correlation between hepatic lipid accumulation and systemic cardiovascular risks frequently observed in NAFLD patients. Abnormalities in hepatocyte lipid handling, a hallmark of NAFLD progression [170], are evidenced by the enrichment of fatty acid metabolism and fatty acid degradation pathways [171].

A key enzyme, ACSL4, facilitates the intracellular conversion of coenzyme A and long-chain fatty acids into fatty acyl–CoA, a process essential for lipid metabolism, energy production, and maintaining cell membrane integrity [172]. Suppressing ACSL4 expression enhances mitochondrial respiration by upregulating PGC1α, promotes fatty acid β-oxidation, and reduces lipid accumulation. In human NAFLD liver tissue, ACSL4 expression is markedly elevated—especially in hepatocytes—and is even higher in NASH. Elevated hepatic ACSL4 levels are strongly correlated with NAFLD progression. Liver-specific ACSL4 deletion has been shown to improve steatosis induced by high-fat diets and reduce hepatic fibrosis in models fed high-fat, high-cholesterol, high-fructose (HCF) or methionine-choline–deficient (MCD) diets. These findings suggest that hepatocyte-specific ACSL4 deletion can prevent NASH progression. Importantly, ACSL4 knockdown also prevents lipid peroxidation, reduces lipid accumulation, and avoids oxidative stress–related cytotoxicity [173]. The observed $\log_2$FC of –2.2 following quercetin treatment further supports ACSL4 as a promising therapeutic target for NAFLD, particularly for alleviating hepatocellular damage, fibrosis, and steatosis. The enrichment of sphingolipid metabolism points to potential changes in insulin sensitivity and lipid signaling [174]—two processes that contribute to inflammation and hepatic steatosis. The unsaturated fatty acid biosynthesis pathway suggests alterations in lipid composition that could exacerbate oxidative stress, a pivotal driver of NAFLD pathology. After quercetin treatment, increased expression of SOD2 ($\log_2$FC = 1.3) confirms enhanced superoxide detoxification, protecting mitochondria from oxidative injury and inflammation (88). Similarly, higher catalase (CAT) activity ($\log_2$FC = 0.9) indicates efficient

hydrogen peroxide breakdown, thereby reducing ROS-induced lipid peroxidation and tissue damage [175].

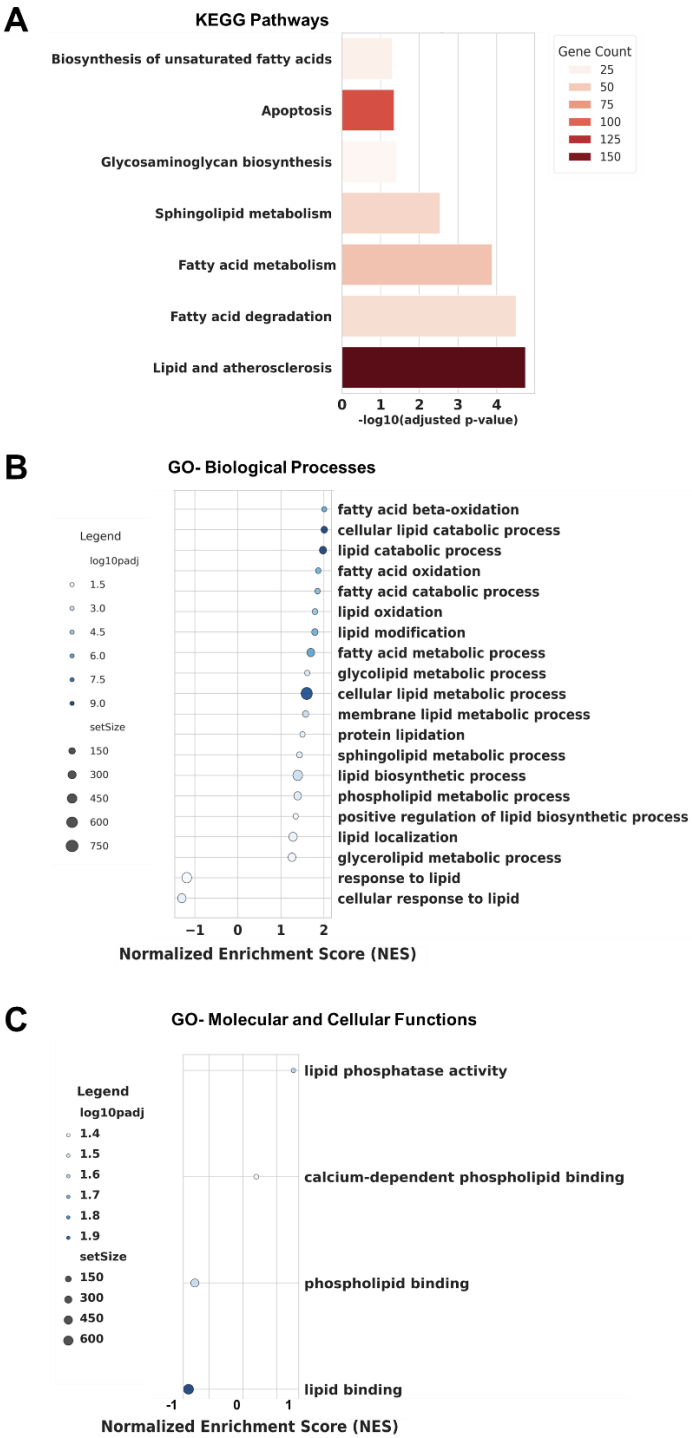

Figure 7.2. Enrichment analysis of differentially expressed genes in Quercetin-treated samples, showing (A) KEGG pathways, (B) GO – Biological Processes, and (C) GO – Molecular and Cellular Functions.

Another therapeutic avenue is the deletion of NOX1, which markedly decreases hepatic oxidative stress and steatosis. In db/db mice, NOX1 deletion completely prevented hepatic steatosis, reduced triglyceride accumulation by approximately 60%, and downregulated key lipogenic genes (Srebp1, Scd1, Fasn) [176]. The observed $\log_2$FC of –1.4 after quercetin treatment underscores NOX1's role in hepatic steatosis and its potential as a treatment target for NAFLD. The enrichment of the apoptosis pathway supports the role of programmed cell death in liver injury and the progression from simple steatosis to NASH [87]. Enrichment in glycosaminoglycan biosynthesis pathways, although less directly related, may point toward mechanisms of fibrogenesis and extracellular matrix remodeling [177].

As shown in Figure 7.2B, biological process enrichment analysis reveals several lipid-associated processes crucial to NAFLD development. Key processes—cellular lipid metabolism, fatty acid metabolism, fatty acid oxidation, and lipid catabolism [170]—reflect enhanced lipid breakdown and utilization, potentially mitigating hepatic steatosis. Quercetin appears to promote mitochondrial β-oxidation and reduce hepatic lipid accumulation, as evidenced by the enrichment of fatty acid catabolic and lipid oxidation processes. Pathways involving phospholipid, glycerolipid, and sphingolipid metabolism suggest modulation of complex lipid signaling networks that influence insulin resistance and inflammation [174]. Increases in positive regulation of lipid biosynthesis and lipid modification may indicate a rebalancing of lipid synthesis and remodeling. Furthermore, processes such as cellular response to lipid and lipid sensitivity suggest that quercetin influences hepatocyte adaptation to lipid stress. Collectively, these enriched processes support quercetin's therapeutic potential in correcting lipid dysregulation—a defining feature of NAFLD pathophysiology.

As shown in Figure 7.2C, molecular function enrichment analysis identifies several enzymatic and receptor-binding activities central to NAFLD progression. A complete list of pathways involved in biological processes, molecular functions, and cellular functions is provided in Supplementary File 12. Enriched functions such as phospholipid binding and lipid binding suggest improved interactions with membrane lipids, potentially influencing lipoprotein transport, lipid signaling, and membrane fluidity—critical aspects of hepatic lipid metabolism. The enrichment of lipid phosphatase activity points to modulation of phosphatidylinositol dephosphorylation, a regulatory step in insulin signaling and metabolic control, both frequently disrupted in NAFLD. Calcium-

dependent phospholipid binding may also play a role in vesicle trafficking and membrane repair during lipid-induced stress and inflammation. Together, these functions highlight molecular mechanisms by which quercetin exerts hepatoprotective effects, particularly through targeting lipid-handling proteins and pathways linked to NAFLD onset and progression.

Several genes targeted by quercetin are directly involved in NAFLD hallmarks and progression. Smad4 expression is upregulated in NASH, and hepatocyte-specific deletion has been shown to slow NAFLD progression by preventing lipogenesis and macrophage polarization [178]. Quercetin-mediated downregulation ($log_2FC = –0.96$) may therefore prevent lipid accumulation, inflammation, and fibrosis. TNF, a major driver of inflammation and metabolic dysfunction, can be therapeutically targeted through suppression, which restores insulin signaling, reduces lipid accumulation, improves hepatic protein synthesis, and decreases inflammatory damage [179]. The observed quercetin-mediated reduction in TNF ($log_2FC = –0.9$) highlights its therapeutic potential. Akt2, essential for fatty liver development in insulin-resistant states, promotes hepatic triglyceride accumulation through increased de novo lipogenesis [180]. Quercetin downregulation of Akt2 ($log_2FC = –0.9$) may thus limit lipid synthesis. PDK4 deficiency enhances AMPK/PPARα-mediated β-oxidation, reduces hepatic triglycerides, and improves insulin sensitivity [181], and quercetin decreases PDK4 expression ($log_2FC = –0.96$). FGF21 mimetics have been shown to reduce inflammation and steatosis [182], and quercetin downregulates FGF21 ($log_2FC = –0.9$). Finally, CYBA, which promotes oxidative stress via NADPH oxidase and contributes to NAFLD progression, is reduced by quercetin ($log_2FC = –0.9$), potentially alleviating oxidative damage and lipid accumulation [183].

As illustrated in Figure 7.3, the Cytoscape-derived protein–protein interaction network shows that quercetin targets (red nodes) are part of several interconnected functional clusters. These include the TNF-signaling module, linking targets such as TAB2, XIAP, and MED14 to apoptosis regulation and oxidative stress response; a neurotransmitter release and synaptic protein–protein interaction cluster involving SYN1, VAMP2, and DLG3, suggesting potential neuromodulatory or neuroprotective functions; enzymes such as NDST1 and CHST7 involved in glycosaminoglycan metabolism; metabolic enzymes such as GCK and ALDH5A1 connected to glucokinase activity and amino acid transport; elements of phospholipase C–mediated G-protein signaling such as

PLCG2 and VIPR1; and components of the renin–angiotensin system including AGTR2 and CDC42.

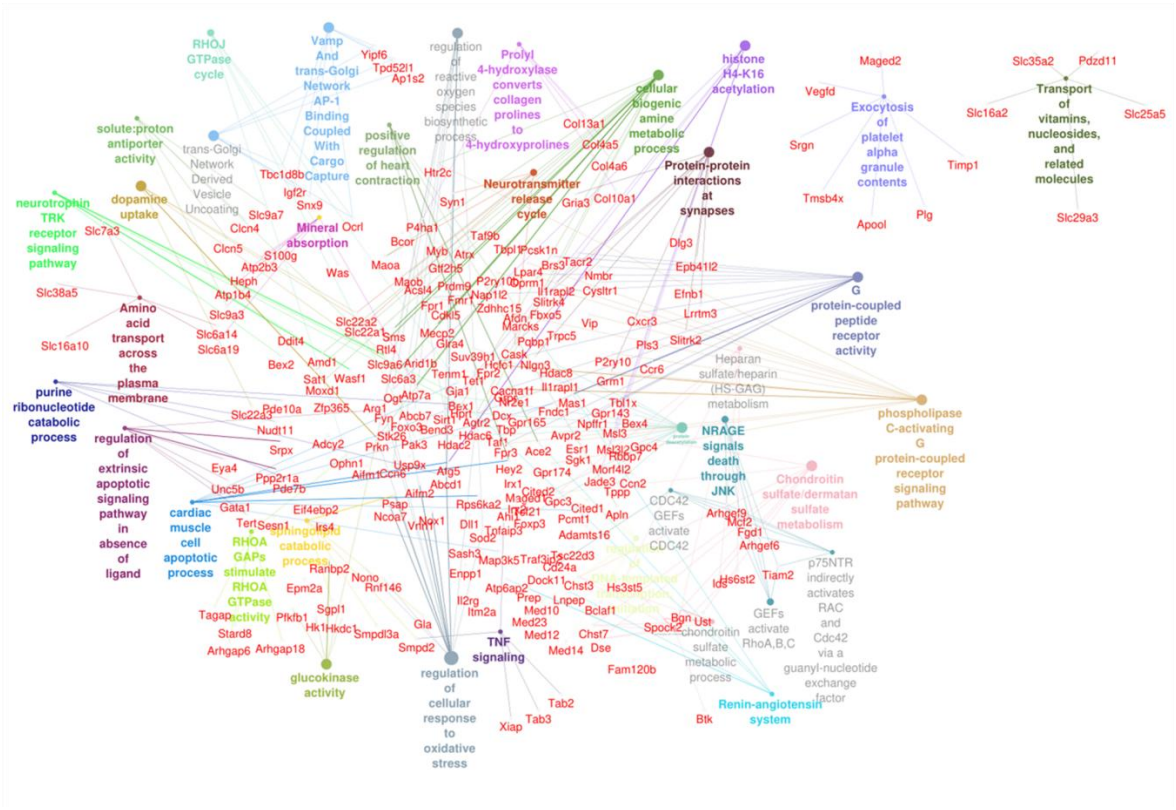

Figure 7.3. Cytoscape based network interaction of quercetin reveals key hub in TNF signalling, neurotransmission and metabolism.

Taken together, this integrative network underscores quercetin's pleiotropic effects, simultaneously modulating metabolic, inflammatory, and signaling pathways relevant to systemic and hepatoprotective outcomes.

Chapter 8. Lutein as a bioactive compound for Cancer Therapy

8.1 Lutein in marigold

Lutein is a yellow pigment that is widely used in dietary supplements and has health advantages. It is a member of the carotenoid family's xanthophyll subclass and is in high demand worldwide. Especially rich in carotenoids, including lutein, the marigold flower is the main commercial source of lutein. 90–99% of the lutein content in marigold petals is found in the form of lutein esters, which are effectively transformed into free lutein for human consumption. Marigold has become the primary raw material for lutein extraction due to its high lutein concentration, making it an essential crop for the functional food and nutraceutical industries [184,185]. A growing body of research supports the potential role of lutein supplementation in cancer prevention and as an adjunct to conventional cancer treatments.

8.2 Lutein in curing cancer

Green vegetables are a good source of lutein, a dietary xanthophyll carotenoid that has shown a variety of anticancer properties. In healthy cells, it can function as an antioxidant; yet, in tumor cells, it paradoxically increases reactive oxygen species (ROS), which leads to oxidative stress-mediated cell death [185,186]. NADPH oxidase in stomach cancer AGS cells is activated by lutein, which raises ROS and NF-κB signaling. This causes DNA fragmentation and apoptosis by upregulating the pro-apoptotic proteins Bax and caspase-3 and downregulating the anti-apoptotic Bcl-2 [187]. In non-small-cell lung cancer (NSCLC) cells, lutein damages DNA and triggers the ATR/Chk1/p53 pathway, which results in G_0/G_1 cell-cycle arrest and apoptosis in vitro and inhibits tumor growth in vivo. In breast cancer models, lutein was demonstrated to increase caspase-3 activity, decrease Bcl-2 levels, and suppress antioxidant-defense proteins (Nrf2, SOD2, HO-1) and cell-survival kinases (phospho-Akt, NF-κB). This combined suppression of survival signaling and activation of apoptotic pathways resulted in decreased proliferation of MCF-7 and MDA-MB-231 cells [186]. Crucially, lutein also affects metastatic behaviors. It has been shown to prevent cancer cells from migrating and invading by altering proteins linked to metastasis, like HES1. These mechanistic investigations, which cover apoptosis induction, cell-cycle arrest, antioxidant/pro-

oxidant effects, and anti-metastatic action, highlight lutein's therapeutic promise in the prevention and treatment of cancer [188].

Given the increasing body of preclinical evidence supporting lutein's role in cancer, a transcriptome-wide analysis was conducted using publicly available RNA-Seq data from chicken liver tissue samples. The dataset comprised eight samples - four paired-end controls and four paired-end lutein-treated—retrieved from the NCBI SRA under accession number PRJNA785785. The *Gallus gallus* reference genome and corresponding GTF annotation files were downloaded from the genome section of NCBI for alignment.

8.3 Results

8.3.1 Identification of Differentially Expressed Genes

Using a volcano plot (Figure 8.1A) with cutoffs of $|\log_2 FC| \geq 2$ and $p < 0.01$ ($-\log_{10}(p) \geq 1.3$), 20 significantly upregulated ($\log_2 FC \geq 2$) genes and 32 significantly downregulated ($\log_2 FC \leq -1$) genes were plotted when comparing experimental to control samples. The data for DSeq file along with list of dysregulated genes is provided in Supplementary File 13 and an overview of changes in global expression pattern has been shown as a heatmap in Figure 8.1B.

8.3.2 Understanding Changes in Gene Expression and Associated Biological Responses

As shown in Figure 8.2A, the KEGG pathway enrichment study revealed numerous pathways intimately linked to cancer biology. The complete list of KEGG pathways is provided in Supplementary File 14. Prominently enriched pathways included the spliceosome, proteasome, apoptosis, and cell cycle. One of the hallmarks of cancer is cell cycle dysregulation [189], which often leads to uncontrolled cell proliferation. Cancer cells also frequently inhibit apoptosis [190]—programmed cell death—to evade elimination. For example, PCK2 (PEPCK-M) protects cancer cells from apoptosis induced by ER stress and nutrient deprivation; inhibiting PCK2 exacerbates apoptotic cell death under glutamine limitation or thapsigargin treatment [191]. PCK2 is elevated in some tumor types, and its inhibition has been shown to slow tumor growth [192]. The observed $\log_2 FC$ of –3.12 following lutein treatment suggests that PCK2 downregulation may have therapeutic potential in cancer. It is increasingly recognized that the spliceosome pathway, which regulates mRNA processing, contributes to the generation of transcript variants associated with cancer. Furthermore, cancer cells often exploit the proteasome pathway [193]—critical for protein

degradation—to target tumor suppressor proteins. Taken together, these enriched pathways indicate that the analyzed gene set may be involved in key carcinogenic processes.

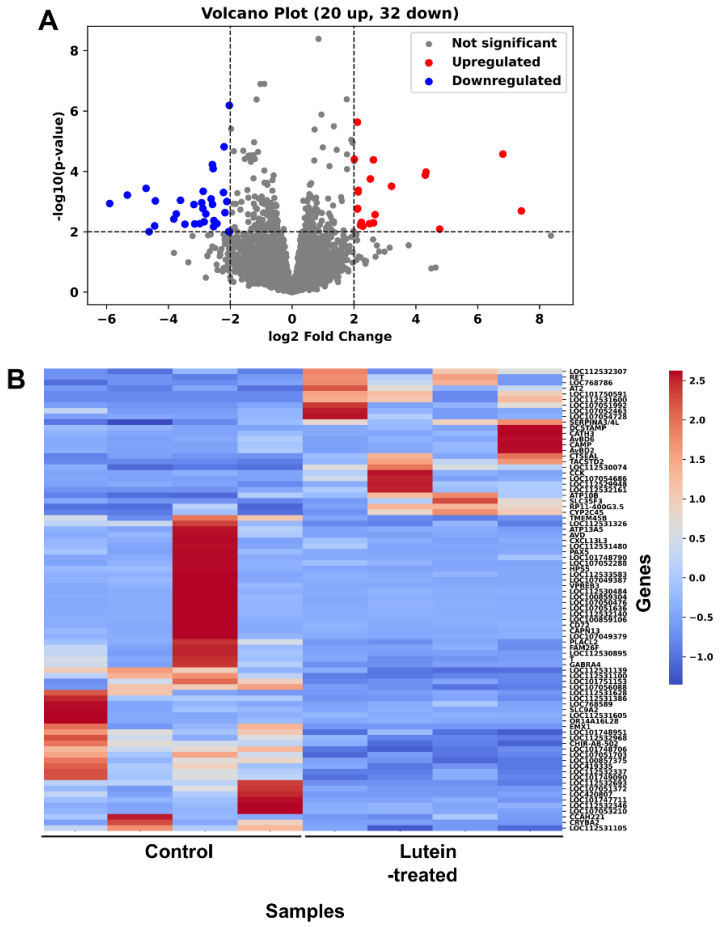

Figure 8.1. (A) Volcano plot illustrating significantly dysregulated genes following Quercetin treatment. (B) Heatmap providing an overview of global gene expression changes upon Fucoxanthin treatment.

As shown in Figure 8.2B, biological process enrichment analysis highlighted several pathways functionally linked to the initiation and progression of cancer. Notably, the meiotic cell cycle and cell cycle checkpoint signaling are essential for maintaining genomic integrity, and their disruption can promote tumorigenesis [194]. The organization of the actin cytoskeleton is vital for cancer cell invasion and migration, driving metastasis [195]. Tissue architecture and integrity depend on adherens junction organization and cadherin-mediated cell–cell adhesion—processes frequently dysregulated during the epithelial-to-mesenchymal transition (EMT), a critical step in metastasis [196]. Additionally, carcinogenic signaling pathway activation often involves changes such as

peptidyl-serine phosphorylation [197]. Collectively, these enriched biological processes suggest a molecular profile consistent with cancer-associated alterations, which may be modulated by lutein's biological effects.

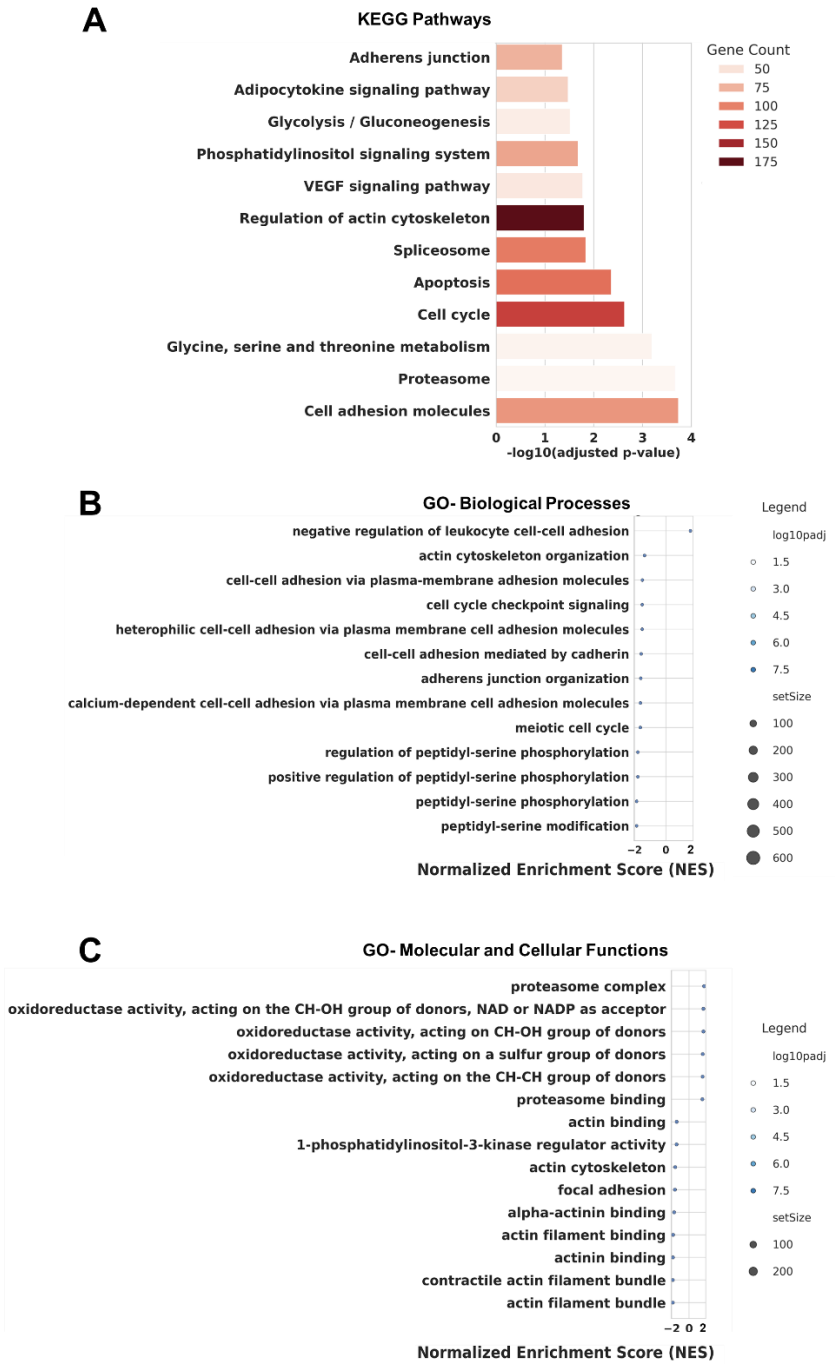

Figure 8.2. Enrichment analysis of differentially expressed genes in Quercetin-treated samples, showing (A) KEGG pathways, (B) GO – Biological Processes, and (C) GO – Molecular and Cellular Functions.

As shown in Figure 8.2C, molecular function and cellular component analyses also revealed pathways closely associated with cancer biology. The full list is provided in Supplementary File 15. The ubiquitin–proteasome system, critical for regulating protein turnover, including degradation of tumor suppressors and cell cycle regulators, is particularly notable [198]. Processes such as actin cytoskeleton organization, actin binding, and focal adhesion are essential for maintaining cell shape and promoting migration and invasion, which cancer cells exploit during metastasis [199]. The PI3K/AKT signaling pathway, one of the most frequently dysregulated in cancer, promotes cell survival, proliferation, and metabolic reprogramming, making enrichment of 1-phosphatidylinositol-3-kinase (PI3K) regulator activity significant [200]. PELP1 acts as a scaffold linking the p85 regulatory subunit of PI3K to membrane-associated estrogen receptor (ERα), enhancing PI3K–Akt signaling and promoting cell survival and proliferation in hormone-responsive cancers [201, 202]. PELP1 overexpression has been linked to breast, ovarian, and prostate cancer, where it increases ERα activity and other signaling cascades, thereby fostering tumor growth and metastasis [203]. Its downregulation following lutein treatment (log_2FC of –2.3) suggests therapeutic potential. Similarly, MEST regulates the PI3K/Akt/mTOR pathway, and loss of MEST imprinting has been linked to ovarian, lung, and breast cancers. With a log_2FC of 1.74 after lutein treatment, MEST emerges as another potential therapeutic target. Although MEST is typically downregulated in cancer and its overexpression can inhibit tumor growth and induce apoptosis, no drugs currently target it directly. Instead, researchers have explored modulation of downstream pathways or epigenetic regulation via histone deacetylase inhibitors and DNA methyltransferase inhibitors [204].

GATA3 is frequently downregulated in breast cancer, promoting tumor growth and metastasis [103]. A log_2 fold change of 1.5 suggests that upregulating GATA3 could have therapeutic benefits, with drugs such as fulvestrant and tamoxifen known to activate its expression. ING3 downregulation has been observed in lung, breast, and head-and-neck cancers; overexpression can suppress tumor growth and induce apoptosis [205], making the observed log_2FC of 1.1 relevant for cancer therapy. TES is another tumor suppressor downregulated in multiple cancer types, including prostate, lung, and breast. Compounds like 4-phenylbutyrate can restore TES expression and inhibit cancer cell growth [206], and the observed log_2FC of 1.07 supports its therapeutic relevance.

Figure 8.3 illustrates functionally distinct yet interconnected modules within the Cytoscape network of lutein's predicted protein interactors, pointing to a multifaceted mechanism of action. These modules include gluconeogenic enzymes (ALDOB, PCK2, DHFR), lysine-degradation and electron-transport components (NDUFA5, DLD, ALDH7A1), and regulators of oxidative stress and apoptosis (NRF1, TXN, CAV2) linked to "cellular response to antibiotic" and "response to purine-containing compound." Another major cluster relates to lipid handling, comprising protein-lipidation machinery (ATG10, ATG12, UGCQ) and cholesterol transporters (ABCA1, STARD4, PMPCB, CAV1). A distinct peroxisomal module (HSD17B4, ABCD2, PNPLA8) underscores lutein's potential to modulate fatty acid oxidation and reactive oxygen species balance. Beyond metabolism, lutein targets are enriched in RNA and protein quality control pathways, including mRNA-processing proteins (CELF2, PTBP3, NCBP1) and tRNA-modification enzymes (TRMT10B, PUS7, DUS4L), implicating roles in translational fidelity and stress granule dynamics. Lutein is also associated with neuroprotective and cell migration–related signaling, such as semaphorin-mediated neurite outgrowth, PI3K signaling, and CAMK-dependent pathways. This integrative network supports a model in which lutein concurrently modulates metabolic, proteostatic, and signaling systems in hepatocytes, thereby exerting antioxidant, lipid-regulatory, and neurotrophic effects.

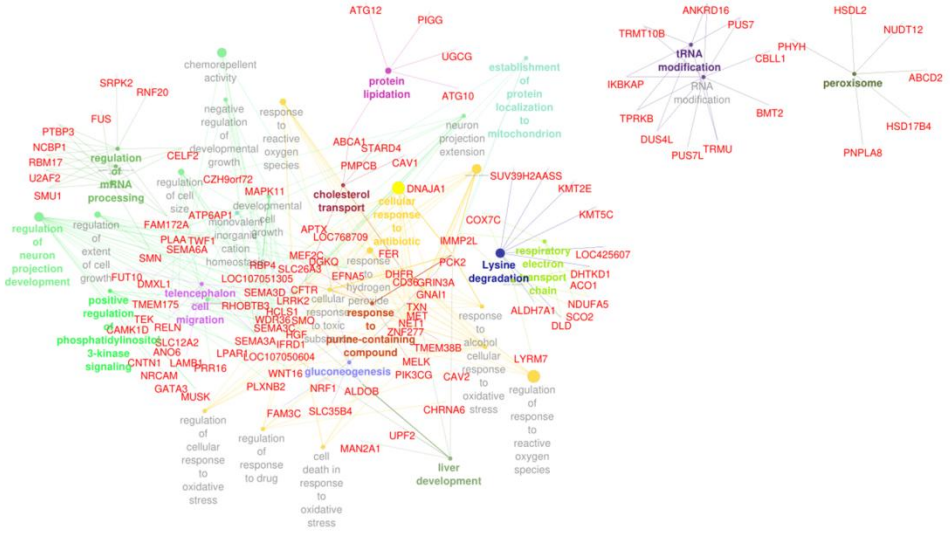

Figure 8.3. Cytoscape based network of Lutein responsive genes.

Overall, these findings suggest that lutein influences multiple tumor-related pathways, and that the molecular functions and cellular components identified are integral to critical oncogenic processes.

Chapter 9: Conclusion

The molecular processes by which bioactive chemicals derived from *Calendula officinalis* (marigold) exert their therapeutic effects were thoroughly examined in this thesis, with a particular focus on cancer therapy and non-alcoholic fatty liver disease (NAFLD). Through the use of a strong computational biology framework and thorough RNA-sequencing data analysis, the study was able to clarify the complex interactions between important biological processes and marigold phytochemicals.

Lycopene, Vitamin E, Fucoxanthin, Quercetin, and Lutein are among the substances obtained from marigolds that have been shown to consistently affect a wide range of genes and pathways that are important to the pathophysiology of cancer and non-alcoholic fatty liver disease. According to the analysis, these substances affect oxidative stress, insulin signaling, lipid metabolism, and inflammatory responses in NAFLD. Quercetin demonstrated a notably consistent and generally advantageous modulation of lipid breakdown, antioxidant defenses, and anti-inflammatory processes, whereas lycopene was demonstrated to affect cholesterol production and inflammatory pathways. Lutein's impacts on cancer cells further demonstrated its ability to control cell cycle progression, trigger apoptosis, and affect pro-survival signaling pathways. The capacity of this work to connect traditional knowledge of herbal medicines with contemporary mechanistic understanding is one of its major contributions. The argument not only supports the long-standing traditional usage of marigold but also lays a scientific basis for the logical creation of novel plant-based medications by offering molecular evidence for the reported therapeutic effects. Specific molecular targets for upcoming drug discovery initiatives are provided by the thorough identification of enriched pathways and differentially expressed genes.

But the analysis also brought to light several complications that require more research. Although it offers wide insights, using multiple species and tissue types for different phytochemical investigations makes it difficult to directly compare and evaluate all of the substances for a single disease. Additionally, certain identified gene regulations seem to conflict with established biological roles in the evolution of disease, such as the upregulation of COL1A1 and ACTA2 by lycopene or CD36 and PNPLA3 by vitamin E. These examples highlight that although transcriptomic data is a great tool for developing hypotheses, determining therapeutic relevance

based only on changes in gene expression necessitates thorough functional and *in vivo* validation to determine the exact biological effects and overall therapeutic direction.

In conclusion, this thesis supports marigold's promise as a natural option for plant-based medications by offering a thorough molecular topography of its therapeutic activities. By opening the door for focused translational research to create innovative treatments for chronic illnesses including cancer and non-alcoholic fatty liver disease, the findings provide a substantial contribution to the domains of computational biology and phytomedicine.

Chapter 10: Future Perspectives

The therapeutic potential of marigold in treating non-alcoholic fatty liver disease (NAFLD) and cancer represents a promising avenue of research, supported by several key factors.

First, marigold is rich in diverse phytochemical compounds, including terpenoids, saponins, sterols, phenolic acids, coumarins, quinones, amino acids, flavonoids, carotenoids, and essential oils. These bioactive substances exhibit anti-inflammatory, antioxidant, and hepatoprotective properties that can mitigate liver inflammation, oxidative stress, and lipid accumulation—hallmarks of NAFLD progression. Moreover, marigold's anticancer potential is largely attributed to phytochemicals such as flavonoids and triterpene esters, which inhibit tumor cell proliferation and angiogenesis. These compounds induce apoptosis, slow tumor growth, and demonstrate genoprotective and anti-metastatic effects, highlighting their promise as cancer therapeutics.

Second, preclinical studies provide encouraging evidence for marigold's efficacy. Calendula contains numerous active compounds that confer cytotoxic, hepatoprotective, and spasmogenic effects. Animal and human studies have shown that marigold extracts reduce C-reactive protein and cytokine levels while protecting cells from free radical–induced damage. In animal models of liver disease, marigold extracts have lowered elevated liver enzymes, improved histological features, and reduced lipid peroxidation. These promising findings justify further investigation in advanced preclinical models and clinical trials. Additionally, preclinical cancer research indicates that marigold extract effectively slows tumor growth in mouse models and induces apoptosis across various cancer cell types. Its genoprotective properties safeguard DNA from genotoxic insults, and its anti-metastatic effects inhibit tumor dissemination.

Third, synergistic effects could enhance marigold's therapeutic efficacy when combined with other natural or synthetic compounds. For example, quercetin, a phytochemical present in marigold, has demonstrated synergy with resveratrol in mitigating circulating inflammatory markers such as TNF-α, IL-6, and monocyte chemoattractant protein-1 (MCP-1) in high-fat diet–induced mice [74]. Similarly, two studies have shown that combining quercetin with ω-3 polyunsaturated fatty acids (PUFAs) sourced from grape seed imparts synergistic anti-inflammatory and antioxidant benefits

in rats [206]. Such combinations targeting multiple facets of NAFLD—including insulin resistance, lipid metabolism, and inflammation—offer promising multi-modal intervention strategies.

Fourth, marigold has a well-established safety profile, having been used traditionally for various ailments. Its historical and continued use suggests it may be suitable for long-term treatment, a critical consideration for chronic diseases such as NAFLD and cancer.

Fifth, advances in genomics and metabolomics could enable personalized treatment approaches in which marigold's efficacy is tailored to individual patients based on their unique genetic and metabolic profiles. Such precision medicine strategies may optimize therapeutic outcomes by aligning treatment with patient-specific disease characteristics.

Sixth, marigold could be integrated into holistic treatment regimens alongside lifestyle interventions such as dietary modification and physical activity to manage NAFLD and cancer more effectively. Investigating its role as a functional food ingredient or dietary supplement may further enhance patient compliance and health outcomes through an integrative approach.

Finally, before marigold can be widely adopted in clinical practice, extensive clinical trials are needed to validate its safety and efficacy. Standardization of marigold extracts is essential to ensure consistent therapeutic results. Regulatory approval by health authorities will be critical for inclusion in clinical treatment guidelines for NAFLD and cancer, paving the way for its acceptance within mainstream medicine.

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