# PROTECTIVE ROLES OF PSILOCYBIN AND EUGENOL IN MITIGATING THE EFFECTS OF HIGH-GLUCOSE AND HIGH-LIPID TREATMENT OF HUMAN SKIN FIBROBLASTS (BJ-5TA)

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## Dedication

I dedicate this thesis to my beloved parents and my dear husband—three pillars of strength in my life.

Their endless patience, unconditional love, and unwavering support have carried me through every challenge of this journey. Without their encouragement, sacrifices, and belief in me, this accomplishment would not have been possible.

## Abstract

Skin aging, particularly under conditions of metabolic stress, is driven by a complex interplay of oxidative stress, inflammation, mitochondrial dysfunction, and extracellular matrix (ECM) degradation. This thesis investigates the protective effects of two natural compounds—eugenol, a phenolic compound derived from clove oil, and psilocybin, a serotonergic tryptamine alkaloid—against premature aging in human dermal fibroblasts (BJ-5Ta) exposed to high-glucose and high-lipid (HGHL) conditions (25 mM glucose and 400 μM palmitic acid).

Both compounds were assessed using co-treatment and post-treatment approaches. A wide range of assays, including MTT, β-galactosidase staining, qPCR for inflammatory and ECM-related genes, flow cytometry for apoptosis and cell cycle, and wound healing assays, were employed to evaluate cellular viability, senescence, inflammation, and ECM remodeling.

Eugenol treatment significantly reduced oxidative stress, inflammation (notably *IL-1β*, *COX-2*, and *TNF-α*), and cellular senescence, while upregulating ECM components such as *COL3A1* and elastin (*ELN*). Psilocybin also preserved cell viability, reduced apoptosis, mitigated senescence markers, downregulated *IL-1β*, *IL-6*, and *COX-2*, and promoted *ELN* expression under HGHL-induced stress.

Together, the findings demonstrate that both eugenol and psilocybin exert anti-inflammatory, antioxidative, and anti-senescent effects, helping to preserve dermal fibroblast integrity and function under metabolically stressful conditions. These results highlight the therapeutic potential of natural bioactive compounds in preventing or delaying skin aging and pave the way for future clinical research on their use in dermatological applications.

## Contributions of Author

**Conceptualization**: The conceptual framework for this research was developed by Igor Kovalchuk and Farzaneh Norouzkhani.

**Methodology**: The research methodology was designed and implemented by Farzaneh Norouzkhani, Esmaeel Ghasemi Gojani, Bo Wang, and Dongping Li.

**Validation**: Ensuring the validity of the research findings was a collaborative effort involving Igor Kovalchuk and Olga Kovalchuk.

**Formal Analysis**: The formal analysis of the data was conducted by Farzaneh Norouzkhani, Esmaeel Ghasemi Gojani, Igor Kovalchuk and Olga Kovalchuk.

**Resources**: The necessary resources for this study were provided by Igor Kovalchuk and Olga Kovalchuk.

**Data Curation**: Farzaneh Norouzkhani and Igor Kovalchuk were responsible for curating and managing the research data.

**Writing—Original Draft Preparation**: The initial drafts of all chapters were prepared by Farzaneh Norouzkhani.

**Writing—Review and Editing**: Farzaneh Norouzkhani, Igor Kovalchuk, and Olga Kovalchuk collaborated on reviewing and editing the manuscript.

**Visualization**: Farzaneh Norouzkhani was responsible for creating visualizations to enhance the presentation of the research.

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**Project Administration**: The administrative aspects of the project were managed by Igor Kovalchuk and Olga Kovalchuk.

**Additional Contributions:** Salma Shujat assisted with the preliminary literature review and reference formatting. Aadarsh Shrestha and Rocio Rodriguez-Juarez supported experimental preparation and data entry during initial phase of the study.

## Uses of Generative AI in this Work

In this thesis, generative artificial intelligence (AI) was used exclusively for the creation of a subset of visual figures. Specifically, Figures 1, 2, 3, 4, 6, and 7 were generated using OpenAI's ChatGPT (Pro version with DALL·E image generation, 2025).

The conceptualization, scientific content, structural composition, and visual layout of these figures were entirely designed and directed by the author, based on the research findings and theoretical frameworks presented in this work. The generative AI tool functioned solely as a graphic rendering assistant to produce visual illustrations aligned with these design specifications.

No generative AI was used in the composition, organization, or editing of the written content of this thesis, nor in the generation of hypotheses or research planning.

Responsibility for the accuracy, integrity, and scientific validity of all material—including AI-generated figures—remains entirely with the author. All sources, citations, and figure legends were verified manually for factual and academic correctness.

This use of generative AI complies with institutional guidelines and is disclosed here in accordance with the University of Lethbridge's policies regarding responsible and transparent academic practice.

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## Full Abreviations List (Alphabetical)

**AGEs** – Advanced Glycation End-products

**AI** – Artificial Intelligence

**AMPK** – Adenosine Monophosphate-Activated Protein Kinase

**AP-1** – Activator Protein 1

**ARDs** – Age-Related Diseases

**ATP** – Adenosine Triphosphate

**BPAG1** – Bullous Pemphigoid Antigen 1

**BPAG2** – Bullous Pemphigoid Antigen 2

**Cat** – Catalase

**CCN1** – Cellular Communication Network Factor 1

**CDKs** – Cyclin-Dependent Kinases

**cDNA** – Complementary DNA

**C/EBPβ** – CCAAT/Enhancer-Binding Protein Beta

**CFD** – Complement Factor D

**cGAS–STING** – Cyclic GMP-AMP Synthase–Stimulator of Interferon Genes

**COL1A1** – Collagen Type III Alpha 1

**COL3A1** – Collagen Type III Alpha 3

**COL17A1** – Collagen Type XVII Alpha 1 Chain

**CoQ10** – Coenzyme Q10

**COX-2** – Cyclooxygenase 2

**CR** – Calorie Restriction

**CREB** – cAMP Response Element-Binding Protein

**CT** – Control **(**Untreated Fibroblasts)

**CT+E15** – Eugenol 15mM Induced on Untreated Fibroblast

**CT+P15** – Psilocybin 15mM Induced on Untreated Fibroblast

**DPPH** – 2,2-Diphenyl-1-picrylhydrazyl

**E15** – Eugenol 15mM

**E15+HGHL** – Eugenol 15mM Co-Treated with High Glucose/ High Lipid

**E2F1** – E2F Transcription Factor 1

**ECM** – Extracellular Matrix

**EGF** – Epidermal Growth Factor

**ER** – Endoplasmic Reticulum

**ERK** – **Extracellular Signal-Regulated Kinase**

**ETC** – Electron Transport Chain

**ELN** – Elastin

**FDG** – Fluorescein Digalactoside

**FOXO** – Forkhead Box O

**FRTA** – Free Radical Theory of Aging

**GSH** – Glutathione

**HA** – Hyaluronic Acid

**HGHL** – Control, High Glucose/ High Lipid

**HGHL/E15** – Eugenol 15mM Post-Treated High Glucose/ High Lipid

**HGHL/M** – Media Post-Treated High Glucose/ High Lipid

**HGHL/P15** – Psilocybin 15mM Post-Treated High Glucose/ High Lipid

**HNE (4-HNE)** – 4-Hydroxynonenal

**HPMCs** – Human Peritoneal Mesothelial Cells

**$H_2O_2$** – Hydrogen Peroxide

**IGF-1** – Insulin-like Growth Factor 1

**IMDM** – Modified Dulbecco's Medium

**IL-1β** – Interleukin 1 Beta

**IL-6** – Interleukin 6

**IL-10** – Interleukin 10

**15-LOX** – 15-Lipoxygenase

**MAPK** – Mitogen-Activated Protein Kinase

**MAMs** – Mitochondria-Associated Membranes

**MiDAS** – Mitochondrial Dysfunction-Associated Senescence

**MMPs** – Matrix Metalloproteinases

**MMP-2** – Matrix Metalloproteinase 2

**MMP-9** – Matrix Metalloproteinase 9

**mtDNA** – Mitochondrial DNA

**mTOR** – Mechanistic Target of Rapamycin

**NF-κB** – Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells

**NRF2** – Nuclear Factor Erythroid 2–Related Factor 2

**NRF2/ARE** – Nuclear Factor Erythroid 2–Related Factor 2 / Antioxidant Response Element

**P10** – Psilocybin 10mM

**P15** – Psilocybin 15mM

**P10+HGHL** – Psilocybin 10mM Co-Treated with High Glucose/ High Lipid

**p16/Rb** – p16^INK4a/ Retinoblastoma Protein Pathway

**p53/p21** – p53/p21 – p53 Tumor Suppressor Protein / p21^CIP1 Cyclin-Dependent Kinase Inhibitor

**PA** – Palmitic Acid

**PBS** – Phosphate-Buffered Saline

**PD** – Population Doubling

**PDLFs** – Periodontal Ligament Fibroblasts

**PI** – Propidium Iodide

**PS** – Phosphatidylserine

**qRT-PCR** – Quantitative Reverse Transcription Polymerase Chain Reaction

**RAGE** – Receptor for Advanced Glycation End-products

**RCS** – Reactive Carbonyl Species

**RF** – Radiofrequency

**RONS** – Reactive Oxygen and Nitrogen Species

**ROS** – Reactive Oxygen Species

**RPE** – Retinal Pigment Epithelial

**SA-B-gal** – Senescence-Associated β-galactosidase

**SASP** – Senescence-Associated Secretory Phenotype

**SIPS** – Stress-Induced Premature Senescence

**SOD** – Superoxide Dismutase

**SQLE** – Squalene Epoxidase

**STAT1/3** – Signal Transducer and Activator of Transcription 1 and 3

**STING** – Stimulator of Interferon Genes

**T2D** – Type 2 Diabetes

**TGEV** – Transmissible Gastroenteritis Virus

**TGF-β** – Transforming Growth Factor Beta

**TNF-α** – Tumor Necrosis Factor Alpha

**TLR4** – Toll-Like Receptor 4

**TRPV1** – Transient Receptor Potential Vanilloid 1

**TSPAN8** – Tetraspanin 8

**TXNIP** – Thioredoxin-Interacting Protein

**UPR** – Unfolded Protein Response

**YAP/TAZ** – Yes-associated Protein / Transcriptional Co-Activator with PDZ-binding Motif

# 1. Chapter 1: General Introduction and Literature Review

## 1.1. Aging

The global population is rapidly growing older, driving major demographic changes worldwide. Nations like South Korea and Japan are projected to have some of the oldest populations (He et al., 2023). As societies adapt to these changes and improve living standards, there is growing interest in understanding aging and its broad implications.

While increased longevity provides more time for individuals to remain active and engaged, it also raises the burden of age-related diseases (ARDs), including cardiovascular disease, diabetes, cancer, Parkinson's, and Alzheimer's. These chronic conditions often persist for years, resulting in prolonged discomfort and functional decline due to progressive degeneration (López-Otín et al., 2013).

Scientific research has established aging as a major risk factor for chronic inflammatory diseases, such as atherosclerosis, dementia, and cancer. It affects the structure and function of multiple organs and tissues, accelerating disease development (Franceschi et al., 2018). Consequently, aging is no longer viewed as a passive process but as an active biological driver of disease, necessitating a deeper mechanistic understanding.

Efforts to elucidate the molecular basis of aging have gained urgency in light of global longevity trends. Aging involves complex genetic and cellular changes that impair biological function. However, distinguishing between primary aging drivers and downstream effects remains a challenge. This complexity has given rise to geroscience—a field that connects aging biology with disease prevention, aiming to delay the onset of age-related diseases through innovative interventions (Sierra, 2016; Liguori et al., 2018; Li et al., 2024).

Among the organs affected by aging, the skin stands out as one of the most visible indicators. As the body's first barrier against environmental insults, the skin undergoes marked changes with age (Parrado et al., 2019). Skin aging has become a significant social and scientific concern, particularly among women. In China, for instance, nearly 90% of women participate in anti-aging routines, contributing to the rapid growth of the industry—valued at Chinese yuan (CNY) 64.6 billion in 2020 (He et al., 2023). Reflecting its broad relevance, skin aging continues to attract attention from researchers and clinicians alike.

### 1.2. The Difference Between Aging and Senescence

Although often used interchangeably, aging and senescence are distinct but interconnected biological processes. Aging refers to the gradual decline in physiological function across the entire organism over time. It involves a reduced capacity to respond to stress, diminished metabolic efficiency, and impaired tissue recovery (Schmeer et al., 2019). Aging is shaped by both intrinsic factors—such as genetics and epigenetic regulation—and extrinsic factors including lifestyle, diet, and environmental exposures. Many of these are modifiable, offering opportunities to promote healthier aging and extend lifespan (He et al., 2023).

At the cellular level, aging is associated with cumulative molecular damage, mitochondrial dysfunction, and oxidative stress. These hallmarks lead to tissue degeneration and increase susceptibility to ARDs such as Alzheimer's and Parkinson's (Wissler Gerdes et al., 2020; Cai et al., 2022). The physiological consequences manifest both visibly—such as in wrinkles or gray hair—and functionally, in the form of reduced resilience and heightened disease risk (Roger et al., 2021; Amorim et al., 2022).

In contrast, cellular senescence describes a state in which cells irreversibly cease to divide and lose functional capacity. This process is triggered by various stressors, including telomere attrition,

DNA damage, oxidative stress, and disrupted trophic signaling (Schmeer et al., 2019; Leyane et al., 2022). First described by Hayflick in 1974, replicative senescence occurs when normal human cells reach a division limit and enter permanent cell cycle arrest.

Senescent cells remain metabolically active and adopt a distinct phenotype characterized by morphological changes and altered gene expression. A key feature is the senescence-associated secretory phenotype (SASP), a pro-inflammatory cocktail of cytokines, chemokines, and proteases that significantly influences the tissue microenvironment (Roger et al., 2021).

Senescence plays a dual role. Acutely, it acts as a protective mechanism by halting the proliferation of damaged or precancerous cells. Chronically, however, the accumulation of senescent cells contributes to tissue dysfunction, persistent inflammation, and the onset of ARDs—a paradox highlighted by Muñoz-Espín et al. (2014). With age, their buildup impairs regeneration and promotes chronic inflammation (Vizioli et al., 2020; Li et al., 2021).

The SASP is regulated by transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and CCAAT/enhancer-binding protein beta (C/EBPβ), which sustain inflammatory gene expression. Furthermore, extranuclear DNA in senescent cells activates the guanosine monophosphate-adenosine monophosphate (GMP-AMP) synthase-stimulator of interferon genes (cGAS–STING) pathway, amplifying innate immune responses and reinforcing the inflammatory microenvironment. Metabolic reprogramming supports SASP persistence, fostering a pro-inflammatory microenvironment (Li et al., 2021; Vizioli et al., 2020). This process contributes to inflammaging—a state of chronic, low-grade inflammation implicated in many age-related conditions (White et al., 2021; Cai et al., 2022).

While senescence is vital for development and tumor suppression, its long-term accumulation is increasingly recognized as a driver of aging-related pathologies and functional tissue decline.

### 1.3. Integrated Theories of Aging

Aging is a complex, multifactorial process that has been explored through numerous scientific theories. Broadly, these theories fall into three main categories: genetic or programmed theories, stochastic or damage-based theories, and the cross-linking theory. Each of these frameworks offers distinct insights into the biological, molecular, and evolutionary underpinnings of aging, yet they often intersect and complement one another (Gladyshev, 2016).

Genetic theories propose that aging is a biologically regulated process, much like development and maturation, governed by an internal molecular clock. One of the most widely cited examples is the Hayflick limit, which demonstrates that human cells divide a finite number of times before entering senescence, largely due to telomere shortening. In this view, aging is not random but rather a continuation of the developmental program. Supporting this idea, the neuroendocrine theory suggests that hormonal shifts—such as those seen in menopause—play a significant role in the aging process. While interventions like hormone replacement therapy have been explored, their effects remain inconclusive and sometimes controversial (Jin, 2010; Gladyshev, 2016). These programmed theories imply that specific genes and signaling pathways may actively regulate the onset and progression of aging.

In contrast, stochastic or damage-based theories argue that aging is the result of cumulative damage over time, caused by both internal metabolic processes and external environmental factors. Among the most influential is the free radical theory of aging (FRTA), which attributes cellular deterioration to reactive oxygen species (ROS) generated during normal metabolism. Although oxidative damage undoubtedly increases with age, its direct causative role in aging is still debated (Madkour, 2019; Gems, 2022). The DNA damage hypothesis expands on this concept by

proposing that aging stems from the accumulation of unrepaired or misrepaired DNA lesions, which ultimately disrupt gene expression and cellular function. Closely related is the role of mitochondrial dysfunction, as mitochondria are key sources of ROS and regulators of cellular energy and apoptosis. Studies have shown that impaired mitochondrial activity is closely linked to age-related decline (Sreedhar et al., 2020; Walker, 2022). Additionally, alterations in signaling pathways such as insulin-like growth factor-1 (insulin/IGF-1) and reductions in growth hormone levels have been associated with lifespan regulation. Mutations in these pathways have even extended lifespan in model organisms. Another key concept within this group is immunosenescence—the gradual weakening of the immune system—which increases vulnerability to infections, cancer, and chronic inflammation in aging individuals (Anisimov and Bartke, 2013).

Adding another layer to our understanding, the cross-linking theory proposes that aging arises from chemical reactions that create abnormal bonds between molecules, particularly proteins and DNA. These cross-links make tissues more rigid and less functional over time. One notable mechanism is glycation, in which glucose binds to proteins, forming advanced glycation end-products (AGEs) that interfere with normal protein function. This process is particularly accelerated in individuals with diabetes and contributes to the physical and molecular signs of aging, such as skin stiffness, reduced elasticity, and chronic inflammation (Lipsky & King, 2015).

Beyond these mechanistic explanations, evolutionary theories offer insights into why aging occurs at all. Early ideas, such as those of Weisman in the 1890s, proposed that aging benefits the species by removing older individuals and reducing competition for limited resources. In 1952, Peter Medawar introduced the mutation accumulation theory, suggesting that natural selection is

effective in removing harmful mutations early in life but exerts little influence over mutations that appear in later years. George Williams later proposed the antagonistic pleiotropy theory, which posits that certain genes are beneficial in early life (e.g., testosterone, which significantly contributes to promoting fertility and strength) but have negative effects later, such as increasing disease risk. The discovery of the Hayflick limit aligns with this idea, highlighting the trade-off between early-life advantages and late-life costs. In 1972, Thomas Kirkwood proposed the disposable soma theory, which argues that organisms allocate limited energy to reproduction rather than long-term maintenance of somatic cells, leading to gradual deterioration with age. These evolutionary frameworks are not mutually exclusive; together, they provide context for why aging has persisted across species despite its individual drawbacks (Weinert and Timiras, 2003; Lipsky & King, 2015).

While no single theory fully explains the aging process, and no intervention has yet proven capable of reversing it, these collective insights have shaped a modern understanding of aging as the result of both intrinsic programming and accumulated cellular damage. Mitochondrial dysfunction, genomic instability, and immune decline are now recognized as central contributors to this decline. Accordingly, contemporary anti-aging strategies increasingly focus on preserving mitochondrial integrity, enhancing DNA repair mechanisms, and mitigating oxidative and inflammatory stress—approaches that have shown promise in slowing age-related degeneration and extending health span (Qin et al., 2024).

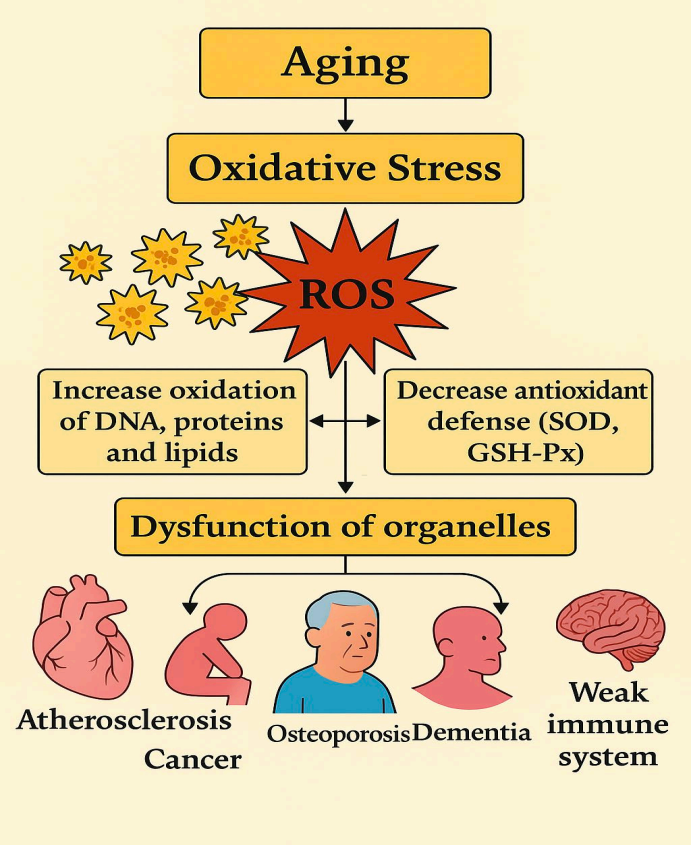


**Figure 1.** Overview of how aging induces oxidative stress and ROS accumulation, leading to increased DNA, protein, and lipid oxidation, and reduced antioxidant defenses (SOD, CAT, GSH-Px). These imbalances contribute to organelle dysfunction and age-related diseases such as cancer, atherosclerosis, osteoporosis, dementia, and neurodegenerative disorders. Figure generated using OpenAI's ChatGPT (Pro version with DALL·E image generation), 2025.

### 1.4. Mitochondrial Dysfunction-Associated Senescence (MiDAS)

Mitochondrial dysfunction is increasingly recognized as a central driver of cellular aging. Mitochondria serve as the primary source of ROS within the cell, and when their function becomes compromised, they produce excessive ROS that can damage cellular components, particularly DNA. This oxidative stress accelerates not only mitochondrial and nuclear DNA damage but also contributes to telomere shortening—the progressive loss of protective sequences (telomerase) at the ends of chromosomes—thereby promoting cellular senescence (Williamson & Davison, 2020). Multiple studies have shown that as organisms age, mitochondrial DNA (mtDNA) sustains more

frequent damage than nuclear DNA due to its proximity to ROS production sites and limited repair mechanisms (Kobayashi & Imanaka, 2024). As aging progresses, the mitochondrial genome becomes a vulnerable target, setting in motion a cascade of functional impairments across the cell. Over recent decades, aging research has increasingly focused on genomic instability, particularly in the context of mitochondrial function. The mitochondrial theory of aging, which evolved from Harman's original FRTA, suggests that aging is largely driven by the cumulative effects of oxidative damage within mitochondria (Lee et al., 2023). ROS produced by the electron transport chain (ETC) disrupt essential cellular processes, contributing to the functional decline characteristic of aging (Long et al., 2024).

This theory highlights the central role of mitochondria in age-related cellular dysfunction (Lefkimmiatis et al., 2021). Beyond ATP production, mitochondria regulate apoptosis, autophagy, and inflammation—processes that deteriorate with age (Son & Lee, 2019). MtDNA's lack of histone protection and limited repair mechanisms compound its susceptibility to oxidative damage, leading to the progressive accumulation of mutations. This fuels respiratory chain inefficiency, further ROS production, and a self-perpetuating cycle of oxidative stress (Nguyen et al., 2023). This vicious cycle is now considered a biochemical hallmark of aging, contributing to cellular energy deficits and impaired resilience.

Although mitochondrial dysfunction is increasingly recognized as a central driver of aging, it alone does not fully explain the complexity of this process. Antioxidant therapies, while theoretically promising in theory, have often failed to yield consistent anti-aging outcomes. In contrast, recent research highlights that mitochondria-targeted antioxidants—unlike general antioxidant strategies—demonstrate higher efficacy by directly reducing mtDNA-specific oxidative stress,

offering more refined therapeutic potential. Moreover, recent findings suggest mtDNA may possess more robust protective mechanisms than previously assumed (Shanaida et al., 2025).

Beyond direct mitochondrial damage, alternative models of aging further highlight how dysregulated signaling pathways, such as mTOR hyperactivation, indirectly contribute to mitochondrial dysfunction and age-related decline. The hyperfunction theory, proposed by Blagosklonny, argues that aging is driven not only by damage accumulation but by the continued activation of growth-promoting pathways, particularly the mechanistic target of rapamycin (mTOR), beyond their developmental roles (Blagosklonny, 2008). This persistent hyperfunction leads to cellular hypertrophy, impaired autophagy, and tissue dysfunction. Supporting this theory, mTOR inhibition with rapamycin has been shown to extend lifespan in multiple organisms. Early-life rapamycin treatment may even reprogram aging trajectories (Davis et al., 1979; Blagosklonny, 2022).

Complementing this, the membrane hypothesis of aging suggests that aging is associated with alterations in cell membrane properties, particularly the mitochondrial membrane. Age-related changes in membrane lipid composition can affect membrane fluidity and permeability, leading to impaired mitochondrial function and increased susceptibility to oxidative stress (Das, 2021). These changes disrupt intracellular signaling and energy balance, contributing to systemic aging.

Together, these frameworks illustrate the multifactorial nature of aging, emphasizing the convergence of mitochondrial dysfunction, cellular signaling dysregulation, and membrane instability. Aging does not result from a single cause, but emerges from the cumulative failure of interconnected biological systems.

Emerging research also challenges the traditional view that ROS is solely deleterious. Moderate levels of ROS may induce adaptive responses, activating protective pathways that enhance cellular

function and longevity (Ristow & Schmeisser, 2014). However, chronic or excessive ROS, particularly from damaged mitochondria, promotes mutations and systemic dysfunction (Shields et al., 2021). This dual role is well-illustrated in exercise physiology: physical activity transiently increases reactive oxygen and nitrogen species (RONS), which at low levels promote adaptive responses but at high levels induce cellular damage.

This adaptive stress model has reshaped how oxidative stress is viewed in aging. While broad antioxidant strategies have shown mixed results, mitochondria-targeted antioxidants have demonstrated more promise by directly reducing mtDNA-specific oxidative stress (Bornstein et al., 2020). These targeted interventions may offer more effective strategies for preserving mitochondrial integrity, slowing cellular aging, and extending health span (Williamson & Davison, 2020).

### 1.5. Dietary Influences on Mitochondrial Function and Aging

Diet is one of the most influential external factors shaping mitochondrial function and aging progressions. Caloric restriction (CR), defined as reduced caloric intake without malnutrition, has been shown to extend lifespan and improve health span across multiple species. These benefits are largely attributed to the modulation of nutrient-sensing pathways, including insulin/IGF-1, sirtuins, and mTOR, all of which regulate mitochondrial function and oxidative stress levels (Green et al., 2022; Weissig and Edeas, 2023). In humans, support is mixed, CR is associated with improved mitochondrial efficiency and reduced oxidative damage.

In contrast, excessive glucose intake—a hallmark of poor dietary habits—has been closely associated with mitochondrial dysfunction and the early onset of cellular aging. Diets high in refined sugars, particularly glucose, are strongly linked to mitochondrial dysfunction and accelerated aging. High glucose intake increases glycolysis and mitochondrial oxidative

phosphorylation, resulting in excessive electron leakage from the ETC. This leak generates large amounts of ROS—especially superoxide radicals—overwhelming the cell's antioxidant defenses (e.g., superoxide dismutase, catalase) and triggering oxidative stress (Aragno and Mastrocola, 2017). This stress damages mitochondrial proteins, lipids, and mtDNA, reduces ATP production efficiency, and accelerates mitochondrial degradation (Pagano et al., 2020; Weissig and Edeas, 2023).

Experimental evidence supports these findings. In one study, human peritoneal mesothelial cells (HPMCs) exposed to 30 mM glucose showed increased double-strand DNA breaks, ROS levels, mitochondrial mass, and membrane potential loss—hallmarks of senescence—compared to cells cultured in 5 mM glucose (Ksiazek et al., 2008). Notably, ROS levels in senescent HPMCs under high-glucose conditions were nearly three times higher than in early-passage cells. Peroxide accumulation was also elevated, highlighting the central role of oxidative stress in glucose-induced premature aging.

Beyond oxidative stress, glucose excess triggers inflammatory pathways and molecular damage through advanced glycation. High glucose levels promote the formation of AGEs—harmful compounds formed when sugars react non-enzymatically with proteins, lipids, or nucleic acids. AGEs accumulate in tissues and impair protein function through cross-linking. Critically, they bind to the receptor for advanced glycation end products (RAGE), activating inflammatory signaling pathways and increasing oxidative stress. This AGE–RAGE interaction further disrupts mitochondrial respiration, promotes mitochondrial dysfunction, and elevates pro-inflammatory cytokine production, thereby contributing to aging and chronic diseases such as cardiovascular and neurodegenerative disorders (Aragno and Mastrocola, 2017).

Similarly, high lipid conditions have been implicated in promoting cellular stress and senescence. High lipid intake is commonly associated with metabolic diseases, and its effects on cellular aging have been explored in several studies. For instance, studies by Romer et al. (2021) and Oberhauser et al. (2021) showed that high lipid-induced lipotoxicity in various cell types, including adipocytes, hepatocytes, and beta-cells, led to cellular inflammation, mitochondrial dysfunction, and premature senescence (Oberhauser et al., 2021; Romer et al., 2021).

High-glucose and high-lipid (HGHL) diets also impair mitochondrial dynamics and biogenesis. Under metabolic stress, mitochondria exhibit disrupted fission–fusion balance, leading to the accumulation of damaged organelles that cannot sustain energy production or regulate ROS. In response, senescent cells may increase mitochondrial mass in a maladaptive attempt to compensate, but these newly synthesized mitochondria are often dysfunctional, exacerbating the problem (Ksiazek et al., 2008; Teodoro et al., 2019).

This progressive mitochondrial dysfunction underlies many ARDs, including cardiovascular disease, type 2 diabetes, and neurodegeneration (Pagano et al., 2020; Zhang et al., 2023). Consequently, dietary interventions that target glucose metabolism and support mitochondrial health are critical for healthy aging. Diets rich in antioxidants and essential nutrients can reduce ROS burden and preserve mitochondrial dynamics.

Additionally, maintaining healthy of glucose and saturated lipid levels in midlife—such as higher HDL cholesterol and lower fasting glucose—is associated with a reduced risk of dementia (Krako Jakovljevic et al., 2021; Zhang et al., 2023). Such diets can also prevent lipid peroxidation and accumulation of cytotoxic byproducts like 4-hydroxynonenal (4-HNE), a marker of oxidative stress in aging tissues including skin (Jaganjac and Zarkovic, 2022).

Ultimately, diet plays a critical role in maintaining mitochondrial integrity and modulating the aging process. By influencing nutrient-sensing pathways, oxidative stress levels, and inflammatory signaling, targeted nutritional strategies can effectively delay cellular aging and enhance longevity. Notably, the skin—owing to its high metabolic demand and continuous exposure to environmental stressors—is among the earliest organs to manifest aging-related changes driven by mitochondrial dysfunction and dietary imbalance (Quan et al., 2025).

### 1.6. Nutritional and Metabolic Triggers of Skin Aging

Diets rich in HGHL impair cellular metabolism and increase the production of ROS, contributing to chronic inflammation and structural degradation in the skin (Naidoo et al., 2018; Papaccio et al., 2022). Due to the skin's high energy demands and continuous environmental exposure, it's particularly susceptible to these metabolic stressors. Mitochondria, as primary sources of ROS during energy production, play a central role in this vulnerability. This connection was first proposed by Denham Harman in 1956 and remains foundational in aging biology. In skin cells, excessive mitochondrial ROS leads to oxidative damage of proteins, lipids, and nucleic acids, accelerating key features of skin aging such as wrinkling, sagging, and pigmentation (Harman, 1992; Harman, 2009).

Beyond superficial changes, poor nutrition-driven oxidative stress also disrupts the deeper dermal architecture, where energy metabolism and ECM maintenance are highly sensitive to mitochondrial dysfunction and inflammatory signaling (Papaccio et al., 2022; He et al., 2023). These imbalances ultimately compromise the structural integrity and regenerative capacity of aging skin. To understand how aging manifests externally, it is essential to consider the structural complexity of the skin.

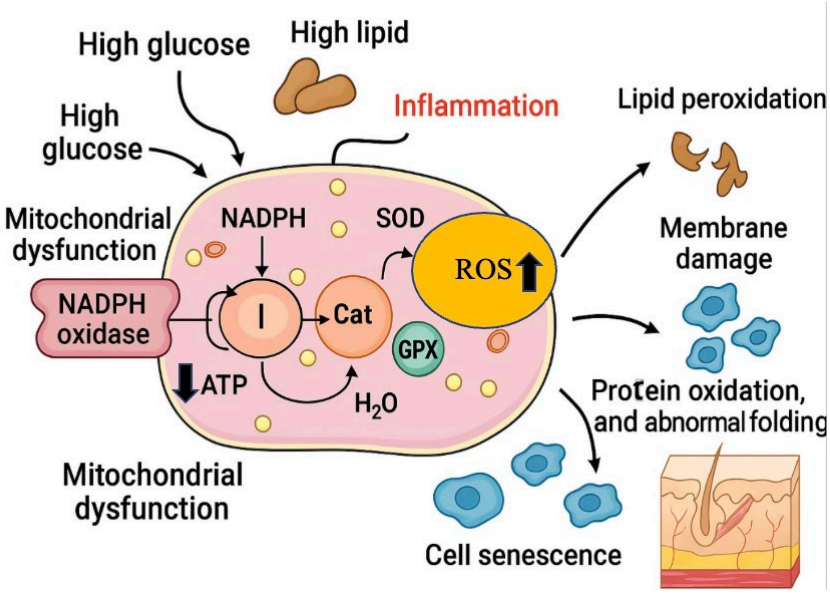


**Figure 2.** HGHL conditions increase cellular fuel, leading to excess electron flow and leakage in the mitochondrial electron transport chain, which elevates ROS production. This results in reduced ATP generation and impaired antioxidant defenses (SOD, Cat, GSH), promoting oxidative damage, lipid peroxidation, protein misfolding, membrane injury, inflammation, and ultimately cell senescence and skin aging. Figure generated using OpenAI's ChatGPT (Pro version with DALL·E image generation), 2025.

### 1.7. Structure of Skin, the Primary Organ that Signifies Aging

As the body's most visible organ, the skin is a primary indicator of aging, reflecting both biological and cosmetic changes. To understand how aging manifests in the skin, it is essential to first examine its layered architecture, cellular diversity, and complex functional roles.

The skin, the body's largest organ, accounts for approximately 16% of total body mass and serves as the primary interface between the internal body and the external environment (McKnight et al., 2022). It plays a crucial role in maintaining homeostasis while providing a physical and immunological barrier against environmental insults. Structurally, human skin comprises three major layers: the epidermis, dermis, and hypodermis. The epidermis itself consists of two components: the *stratum corneum* (non-viable layer) and the viable epidermis, which together form the skin's outermost defense. Beneath lies the dermis, followed by the hypodermis or

subcutaneous tissue, each contributing to the skin's complex functions (Peate et al., 2021; McKnight et al., 2022). The epidermis is primarily composed of keratinocytes and contains structures such as hair follicles, sebaceous glands, and sweat glands. It renews continuously through the activity of basal stem cells. The dermis, populated by fibroblasts, is responsible for producing the ECM—a network rich in collagen and elastin fibers that provides strength, elasticity, and structural support (Walters & Roberts, 2002; Fuchs & Blau, 2020). Together, these layers not only protect the body but also regulate critical functions such as thermoregulation, fluid retention, and the synthesis of hormones and vitamins.

Historically, animal models have been instrumental in studying skin structure, function, and pathology, particularly in wound healing and dermatological disease research. However, the physiological and anatomical differences between species—especially between humans and mice—limit their translational potential. Mouse skin, for example, has a thinner epidermis, more hair follicles, and looser dermal attachment, leading to discrepancies in drug absorption and wound healing outcomes. Although pig skin more closely resembles human skin in terms of thickness, structure, and lipid composition, it still cannot fully replicate human physiological responses (Walters & Roberts, 2002; Kumar, 2024).

To overcome these limitations and adhere to the 3Rs principle (Replacement, Reduction, Refinement), researchers are increasingly developing *in vitro* human skin models (Clark, 2018; Hofmann et al.,2023). This transition has been reinforced by regulatory actions such as the European Union's 2013 ban on animal testing for cosmetic products. These engineered models offer promising alternatives by more accurately mimicking human skin physiology, improving the reliability of toxicity testing, disease modeling, and therapeutic development (Fischer, 2015; Fuchs & Blau, 2020; Hofmann et al., 2023).

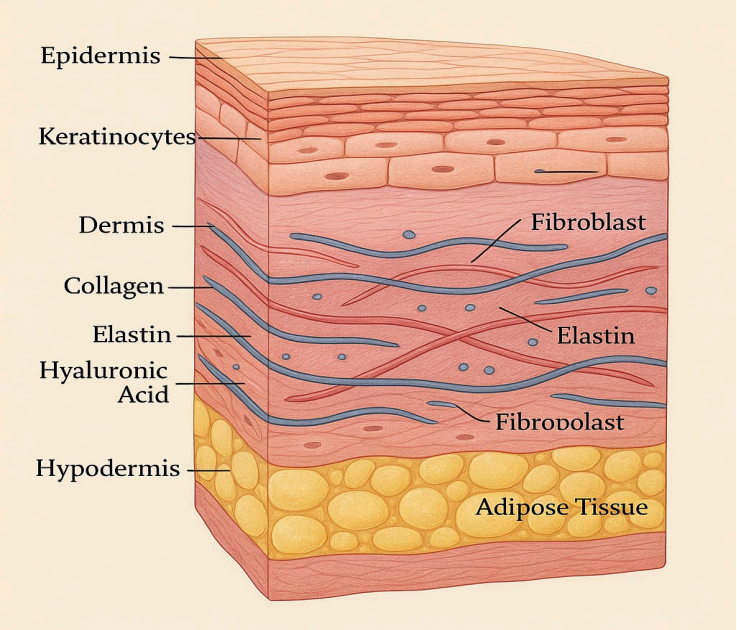


**Figure 3.** Illustration of healthy skin showing a balanced metabolic state in fibroblasts. Mitochondria efficiently produce ATP with minimal ROS generation, supporting collagen synthesis and maintaining epidermal integrity—hallmarks of youthful, well-functioning skin. Figure generated using OpenAI's ChatGPT (Pro version with DALL·E image generation), 2025.

### 1.7.1 Epidermis

The epidermis, the outermost layer of the skin, serves as a dynamic and regenerative barrier that protects the body from environmental insults such as toxins, pathogens, UV radiation, and dehydration (Shin et al., 2023). Composed primarily of keratinocytes, it is organized into five distinct strata that reflect progressive stages of cellular differentiation. These keratinocytes originate from epidermal stem cells located in the *stratum basale***,** where they continuously give rise to transit-amplifying daughter cells that rapidly proliferate before differentiating and migrating upwards through the epidermis (Fuchs & Blau, 2020).

As these cells ascend, they undergo morphological and biochemical transformations, eventually forming the outermost *stratum corneum*. The differentiation process involves a precise switch in keratin expression—from keratins 5 and 14 in basal keratinocytes to keratins 1 and 10 in suprabasal layers—alongside the synthesis of structural proteins such as involucrin, profilaggrin, loricrin, and

trichohyalin (Shin et al., 2023). These proteins contribute to the formation of the cornified envelope, which—together with a lipid-rich matrix composed of ceramides and omega-hydroxy fatty acids—forms a robust hydrophobic barrier that limits water loss and blocks microbial invasion (Menon, 2014; Has & Nyström, 2015).

The *stratum corneum*, approximately 10–20 μm thick and consisting of 15–25 layers of anucleate corneocytes, plays a critical role in minimizing transepidermal water loss, which averages around 250 mL/day in adults. Epidermal thickness varies regionally, from about 30 μm in the eyelids to up to 600 μm in the palms and soles (Shin et al., 2023). Despite lacking vascular and lymphatic networks, the avascular epidermis depends entirely on diffusion from the underlying dermis for oxygen, nutrients, and waste exchange—underscoring the importance of the epidermal-dermal interface in skin homeostasis (Menon, 2014).

The epidermis is anchored to the dermis by the basal lamina, a specialized ECM consisting of the *lamina lucida* and *lamina densa.* These layers are enriched with structural proteins including type IV collagen, laminins, and fibronectin, which facilitate adhesion and signaling between basal keratinocytes and the dermal compartment (Rashmi, 2020). Hemidesmosomes—complexes composed of bullous pemphigoid antigens (BPAG1 and BPAG2) and integrin α6β4—secure this connection by linking keratin intermediate filaments to laminin-5 and collagen VII anchoring fibrils (Menon, 2014; Has & Nyström, 2015).

Beyond keratinocytes, the epidermis contains specialized cell types that contribute to pigmentation, sensation, and immune defense. Melanocytes, located in the *stratum basale,* synthesize melanin, which protects against UV radiation. The balance of eumelanin and pheomelanin production, shaped by genetic and environmental factors, determines skin pigmentation (Shin et al., 2023). Merkel cells—mechanoreceptors found near the basal layer—

facilitate tactile sensation and may influence keratinocyte behavior. Langerhans cells, predominantly found in the *stratum spinosum,* function as antigen-presenting cells that link the skin to systemic immune responses by activating cytotoxic T cells after migrating to lymph nodes (Menon, 2014).

Cornification, the terminal differentiation process that converts keratinocytes into corneocytes, is tightly regulated by calcium gradients across the epidermal layers. This process is essential for barrier integrity and is susceptible to dysregulation with aging. Disruption of calcium signaling impairs the formation of the cornified envelope, increases oxidative stress, and weakens the barrier function, accelerating visible signs of skin aging (Shin et al., 2023).

With advancing age, the epidermis undergoes structural and functional decline. The basal layer's ability to renew keratinocytes diminishes, leading to thinning of the epidermis, delayed wound healing, and reduced barrier regeneration (Menon, 2014; Shin et al., 2023). Aging also affects the composition and integrity of the basement membrane, compromising its role in anchorage and signaling between the epidermis and dermis. The result is a more fragile epidermal layer, increased susceptibility to environmental damage, and a reduction in skin elasticity and hydration, which are hallmark features of aged skin.

Through its stratified architecture, continual renewal, and specialized cellular composition, the epidermis plays a central role in protecting the body, maintaining hydration, and organizing local immune responses. Its gradual deterioration with age marks one of the earliest and most visible aspects of cutaneous aging (Menon, 2014).

### 1.7.2. Dermis

Beneath the epidermis lies the dermis—a structurally and functionally dynamic layer that provides mechanical strength, vascular supply, sensory input, and immunological defense essential for skin

health and homeostasis. It plays critical roles in thermoregulation, tissue repair, immune surveillance, and environmental sensing (Fuchs & Blau, 2020). Anatomically, the dermis is divided into two layers: the superficial papillary layer and the deeper reticular layer. The papillary layer lies directly beneath the epidermis and contains finger-like projections (dermal papillae) that enhance nutrient diffusion and strengthen epidermal–dermal connections. The reticular layer, which accounts for most of the dermal thickness, provides tensile strength and elasticity through densely interwoven collagen and elastin fibers (Menon, 2014; Has & Nyström, 2015).

Collagen—comprising roughly 70% of the dermis—imparts structural integrity, while elastin enables skin stretch and recoil. These ECM proteins are primarily synthesized by dermal fibroblasts, which also produce other matrix components such as laminin, fibronectin, and vitronectin. Among these, fibronectin is critical for cellular adhesion, migration, and differentiation, particularly during wound healing (Menon, 2014). The ground substance, rich in mucopolysaccharides, contributes to dermal hydration and mechanical resistance.

The dermis is highly vascularized, supporting both epidermal and dermal layers with oxygen, nutrients, and waste removal. It also contains specialized arteriovenous anastomoses that allow blood to bypass capillary networks, a feature important for thermoregulation (Kumar, 2024). Alongside the blood vessels, the dermal lymphatic system regulates interstitial fluid balance, supports immune cell trafficking, and aids in clearing metabolic waste (Fuchs & Blau, 2020).

Numerous sensory nerve fibers embedded within the dermis enable the perception of touch, pressure, temperature, and pain, playing an essential role in environmental responsiveness. The papillary layer, especially in areas like palms and soles, houses rich nerve endings responsible for detecting fine tactile stimuli (Menon, 2014; Kumar, 2024).

The reticular layer also houses vital dermal appendages, including hair follicles, sebaceous glands, sweat glands, and additional sensory and autonomic nerve endings. Sweat glands regulate body temperature, while sebaceous glands secrete sebum that maintains skin hydration and barrier integrity (Kumar, 2024).

As the skin ages, the dermis undergoes pronounced structural and functional changes. These include thinning of the dermal matrix, fragmentation of collagen and elastin fibers, and reduced fibroblast activity. Oxidative stress plays a key role, with elevated levels of ROS activating pro-aging pathways such as NF-κB and AP-1, which accelerate ECM degradation and fibroblast senescence (Zou et al., 2021). These changes lead to dermal sagging, wrinkle formation, and impaired mechanical resilience.

The dermis also serves as a reservoir of immune cells, including dendritic cells, macrophages, mast cells, eosinophils, neutrophils, B cells, and subsets of T lymphocytes such as γδ-T, αβ-T, and natural killer T cells (Kumar, 2024). These immune elements play crucial roles in immunosurveillance, inflammation regulation, and tissue repair, ensuring the skin's first-line defense against pathogens and injury (Matsumura et al., 2016; Victorelli et al., 2019; Levine, 2020; Rahmouni et al., 2022).

Connecting the dermis to the overlying epidermis is the basement membrane—a specialized extracellular matrix essential for skin architecture and function**.** This semipermeable interface allows for the bidirectional exchange of nutrients, oxygen, and metabolic signals. It is composed of two primary zones: the *lamina lucida*, where keratinocytes adhere to laminins via hemidesmosomes, and the *lamina densa*, made predominantly of collagen IV and laminins, stabilized by perlecan and nidogens. Anchoring fibrils of collagen VII bind the *lamina densa* to the underlying dermis. Both keratinocytes and fibroblasts contribute to the synthesis of basement

membrane proteins, although nidogens are exclusively secreted by fibroblasts (Menon, 2014; Hofmann et al., 2023).

### 1.7.3. Hypodermis

The hypodermis, also known as the subcutis or subcutaneous tissue, is the deepest layer of the skin, providing essential support for structural integrity, energy storage, thermoregulation, and immune function. Though often underappreciated, this layer plays a pivotal role in maintaining skin architecture and systemic homeostasis, particularly as it relates to aging.

Structurally, the hypodermis is composed primarily of adipocytes—cells that store energy in the form of lipid droplets. These adipocytes account for nearly 50% of total body fat and are organized into lobules separated by septa of connective tissue. This adipose matrix is tethered to the dermis through a scaffold of collagen and elastin fibers, providing both flexibility and mechanical stability. Beyond adipocytes, the hypodermis contains fibroblasts, macrophages, and a stromal-vascular fraction consisting of preadipocytes, endothelial cells, pericytes, and T cells, highlighting its complex cellular heterogeneity (Hofmann et al., 2023).

Functionally, the hypodermis is richly vascularized**,** containing large blood vessels and nerve trunks that supply the dermis and epidermis. This vascular bed is critical not only for nutrient and oxygen delivery but also for thermoregulation and immune cell trafficking. Moreover, the hypodermis performs important endocrine functions by secreting adipokines and other bioactive molecules that influence appetite regulation, glucose metabolism, inflammatory responses, angiogenesis, and hair growth. It also acts as an anchoring platform, attaching the skin to underlying muscles and bones while maintaining tissue flexibility and mobility (Menon, 2014; Kumar, 2024).

From a research and biomedical standpoint, accurate *in vitro* modeling of the skin must include a functional hypodermal layer. This involves replicating its vasculature, diverse cellular populations, and immunological responsiveness to enable sustained viability and realistic tissue responses under experimental conditions (Hofmann et al., 2023). Failure to incorporate the hypodermis in skin models can lead to oversimplification, particularly in studies investigating wound healing, drug delivery, or age-related changes.

Together with the epidermis and dermis, the hypodermis completes the skin's integrated trilaminar structure. These three layers work synergistically to create a dynamic barrier that defends against environmental threats, regulates internal homeostasis, and adapts to physiological demands.

However, the hypodermis itself is not immune to aging. As the body ages, adipose tissue in the hypodermis diminishes in both volume and function. This atrophy reduces the layer's ability to cushion mechanical impact and retain skin volume, contributing to visible thinning, fragility, and structural collapse of aging skin. The loss of subcutaneous fat is particularly pronounced in the face and extremities, where it accelerates the formation of fine lines, hollowness, and sagging (Kumar, 2024). These changes not only alter physical appearance but may also impair thermoregulation, wound healing, and mechanical protection, further underscoring the hypodermis's critical role in skin aging.

### 1.7.4. Comparative Impact of Aging Across Layers

Among all skin layers, the dermis is most profoundly affected by the aging process due to its central role in maintaining structural integrity. The decline in fibroblast function and ECM protein synthesis significantly compromises the skin's ability to retain elasticity and resilience, leading to visible signs of aging such as wrinkles, sagging, and loss of firmness. Dermal fibroblasts, in particular, are the most commonly studied cells in early aging research, as they play a pivotal role

in ECM maintenance, collagen production, and inflammatory regulation. This central role makes the dermis a primary target in anti-aging interventions and regenerative dermatology (Menon, 2014; Shin et al., 2023).

### 1.8. Molecular Mechanisms of Skin Aging

Skin aging is a multifactorial process driven by both intrinsic (biological) and extrinsic (environmental) factors that converge on oxidative stress, inflammation, cellular senescence, and metabolic decline. At the molecular level, this aging process disrupts the balance between collagen synthesis and degradation, impairs antioxidant capacity, and alters cellular signaling (He et al., 2023; Jin et al., 2023).

Intrinsic aging is governed by genetic programming and internal metabolic processes. ROS accumulation, primarily of mitochondrial origin, causes oxidative damage to proteins, lipids, and DNA. Simultaneously, age-related reductions in enzymatic antioxidants such as superoxide dismutase **(**SOD), glutathione (GSH), and catalase (Cat) weaken the skin's defense system, facilitating cellular dysfunction (Gniadecka et al., 1998; Gu et al., 2022). Extrinsic aging**,** or photoaging, is influenced by chronic environmental exposure—UV radiation, air pollution, smoking, and diet—all of which amplify ROS generation. These external insults activate MMPs, degrading ECM proteins such as collagen and elastin and producing coarse wrinkles, uneven pigmentation, and skin sagging (Chung et al., 2001; Lyu et al., 2022).

While intrinsic aging is inevitable, extrinsic aging is largely modifiable and can be mitigated through sun protection, antioxidant-rich diets, and other healthy lifestyle interventions.

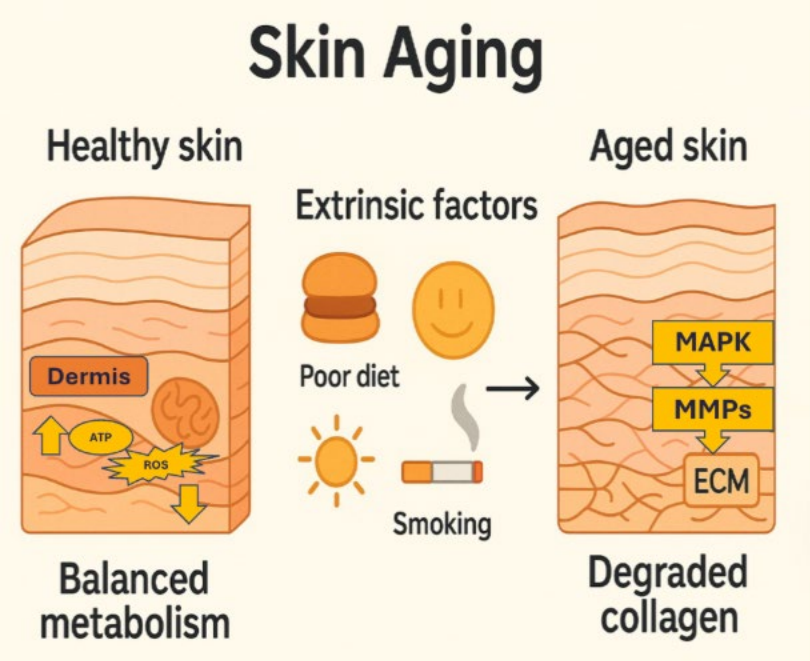


**Figure 4.** Diagram showing the impact of extrinsic factors on skin aging. (Healthy skin with balanced metabolism and sufficient ATP production is disrupted by different factors, leading to mitochondrial dysfunction. This results in increased MMP activity, ECM degradation, and collagen breakdown—hallmarks of aged skin). Figure generated using OpenAI's ChatGPT (Pro version with DALL·E image generation), 2025 and PowerPoint software.

### 1.8.1. Metabolic Reprogramming in Aging Skin

Skin aging involves not only structural deterioration but also profound metabolic reprogramming across multiple cell types throughout the different layers of the skin. As aging progresses, disruptions in glucose, protein, and lipid metabolism impair the skin's ability to maintain cohesion, hydration, and barrier integrity (Mohiuddin, 2019; Alyoussef, 2021). These shifts in cellular energy pathways contribute directly to the functional decline and visible signs of aging.

In keratinocytes, age-associated reductions in glucose uptake and glycolytic activity compromise energy production and epidermal renewal. This metabolic impairment weakens the skin's barrier function and increases transepidermal water loss, ultimately leading to dryness and heightened vulnerability to external insults (Ottum & Mistry, 2015; Choi et al., 2025).

Melanocytes also experience metabolic shifts that affect melanin biosynthesis and distribution, contributing to pigmentation abnormalities such as melasma, solar lentigines, and uneven tone commonly observed in aged skin (He et al., 2023).

Fibroblasts—key regulators of ECM dynamics—exhibit reduced collagen production, elevated MMP activity, and increased oxidative sensitivity during aging. These cells also display dysregulated signaling in the TGF-β/Smad pathway and heightened expression of senescence-associated proteins like cellular communication network factor 1 (CCN1) and complement factor D (CFD), further driving dermal deterioration (Shin et al., 2023; Luo et al., 2024).

At the structural level, the aging epidermis becomes thinner and flatter due to reduced keratinocyte renewal and a decline in critical adhesion proteins such as COL17A1 and laminin 332, weakening epidermal-dermal cohesion and impairing skin resilience (Roig-Rosello & Rousselle, 2020; Wang et al., 2020).

In the dermis, collagen fibers become fragmented and disorganized, elastin is progressively degraded, and fibroblast senescence accelerates. Reduced IGF-1 secretion disrupts keratinocyte proliferation and DNA repair, while extracellular matrix stiffening is exacerbated by chronic inflammation and the activation of mechanosensitive pathways such as cGAS–STING and Yes-associated protein/transcriptional co-activator with PDZ-binding motif (YAP/TAZ) signaling pathway (Sladitschek-Martens et al., 2022). These changes compromise skin elasticity, tensile strength, and wound healing capacity.

The hypodermis also undergoes functional decline, characterized by a progressive loss of subcutaneous adipose tissue, reduced adipokine signaling, and impaired thermoregulation. This fat

atrophy is especially prominent in the face and extremities and contributes to the visible thinning, sagging, and volume loss typical of aged skin (Dyer & Miller, 2018; Ittycheri, 2024).

As previously emphasized, among these structural layers, the dermis is particularly vulnerable to aging due to its reliance on mitochondrial integrity for ECM protein synthesis. As fibroblasts in this layer regulate the production of collagen, elastin, and other matrix components essential for skin elasticity and strength, they are especially sensitive to metabolic disruptions—making them a central focus in understanding diet-induced aging at the cellular level (Sladitschek-Martens et al., 2022).

### 1.9. Dermal Fibroblast Aging Under Metabolic Stress

This metabolic vulnerability impairs fibroblast function at multiple levels—compromising collagen synthesis, accelerating matrix degradation, and amplifying inflammatory signaling pathways that collectively drive dermal deterioration.

In HGHL (25 mM glucose and 400 μM palmitic acid) environments, excessive intake of sugars and fats results in metabolic overload, generating surplus reducing equivalents (NADH, $FADH_2$) that fuel the mitochondrial. This overload increases ROS production—particularly at complexes I and III—causing electron leakage, impaired ATP synthesis, and a self-perpetuating cycle of oxidative stress and mitochondrial dysfunction (Distelmaier et al., 2015; Pinho et al., 2022). These mitochondrial impairments initiate a cascade of cellular responses, including the activation of MAPK/AP-1 signaling and the suppression of TGF-β/Smad pathways, both of which impair collagen synthesis and promote ECM breakdown (Quan et al., 2009; He et al., 2017).

Moreover, nonenzymatic glycation represents a key mechanism of fibroblast aging under metabolic stress. In HGHL conditions, AGEs accumulate and crosslink structural proteins such as collagen and elastin, leading to ECM stiffening and compromised skin elasticity. Through binding to the RAGE, these molecules also trigger NF-κB–mediated inflammatory signaling and enhance oxidative stress (Pageon, 2010; Zgutka et al., 2023). Glycation of nuclear proteins and DNA (DNA-AGEs) further impairs nuclear integrity and activates immune pathways**,** contributing to dermal degeneration (Uceda et al., 2024; Wang et al., 2024).

Fibroblasts under metabolic stress also experience endoplasmic reticulum (ER) dysfunction, characterized by the accumulation of misfolded proteins and lipid-induced ER stress. This activates the unfolded protein response (UPR), which contributes to cell senescence and inflammation (Jiang et al., 2015). Importantly, ER and mitochondria communicate through mitochondria-associated membranes (MAMs)—specialized structures that regulate calcium homeostasis, lipid metabolism, and apoptosis. Disruption of MAM integrity under metabolic stress further amplifies mitochondrial damage and inflammatory signaling, exacerbating fibroblast aging (Raturi and Simmen, 2013).

Chronic low-grade inflammation caused by poor nutrition further disrupts mitochondrial integrity and skin homeostasis. Elevated cytokines such as IL-6 and TNF-α contribute to keratinocyte dysfunction, collagen degradation, and delayed wound healing (Cao et al., 2020; Sharma et al., 2020; Wlaschek et al., 2023). In response to this inflammatory environment, fibroblasts adopt a SASP, characterized by the release of inflammatory mediators including IL-6, IL-8, MMP-2, and MMP-9, which further promote ECM breakdown, tissue remodeling, and immune activation (Coppe et al., 2010; Wang & Dreesen, 2018; Wlaschek et al., 2021).

These inflammatory and oxidative signals also weaken antioxidant defense mechanisms—including Cat, SOD, and GSH— thereby exacerbating damage to proteins, lipids, and nuclear DNA and accelerating mitochondrial dysfunction and skin aging (Juan et al., 2021; He et al., 2023). Together, these interconnected mechanisms highlight the vulnerability of dermal fibroblasts to metabolic stress and their central role in driving the structural and functional decline associated with skin aging, while also illustrating how poor dietary habits can accelerate the aging process.

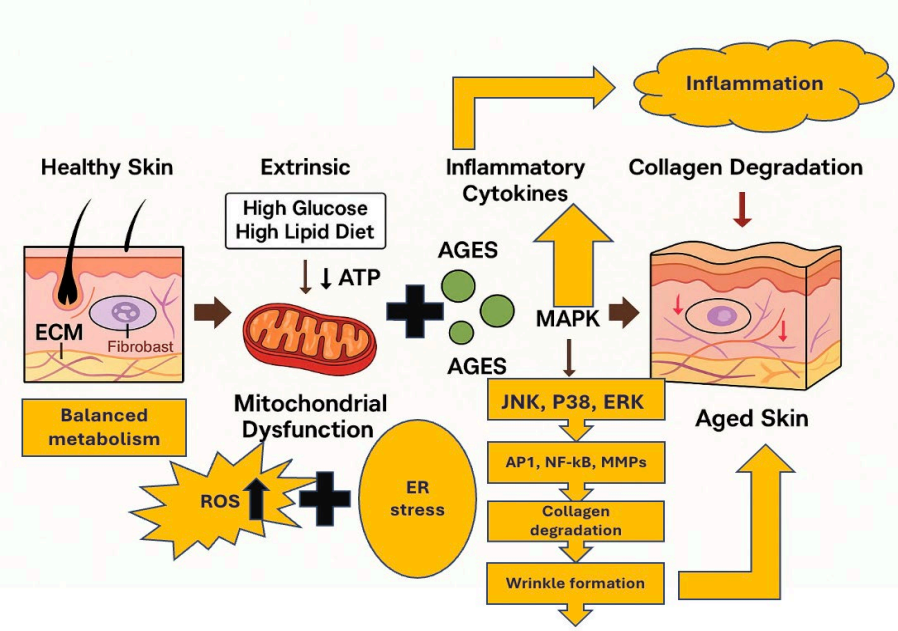


**Figure 5.** Mechanism of skin aging induced by extrinsic metabolic stress. A HGHL diet leads to mitochondrial dysfunction in dermal fibroblasts, reduced ATP production, and accumulation of ROS and AGEs. These AGEs activate inflammatory cytokines and MAPK signaling, promoting collagen degradation and ECM breakdown—resulting in aged skin phenotype. Figure generated using PowerPoint software.

### 1.10. Skin Anti-Aging Strategies

To effectively counteract skin aging, dermatological strategies must target specific molecular pathways implicated in skin deterioration (Dyer & Miller, 2018; Ittycheri, 2024).

One critical contributor to skin aging is the formation of AGEs, which arise from nonenzymatic glycosylation of proteins and disrupt collagen integrity and elasticity. Inhibiting glycation using agents like aminoguanidine has shown promise in reducing AGE accumulation and slowing the aging process (He et al., 2023). Additionally, strategies to preserve and boost collagen levels are central to maintaining dermal structure. Inhibitors of MMPs, such as resveratrol, β-glycyrrhetinic acid, and hydrolyzed collagen, reduce collagen degradation, while agents like ascorbic acid and chondroitin sulfate promote collagen synthesis (Ittycheri, 2024).

Moreover, targeting lipid metabolism is also essential, as lipids support the skin barrier and hydration. Retinol and hyaluronic acid not only improve lipid retention but also stimulate skin renewal and reduce transepidermal water loss, improving elasticity and texture. In parallel, chemicals that restore mitochondrial function—such as metformin, nicotinamide, and vitamin D—have been explored for their ability to enhance cellular energy metabolism, reduce oxidative stress, and delay aging at the cellular level (He et al., 2023).

Antioxidant defense systems also play a protective role in aging skin. Non-enzymatic antioxidants like vitamins C and E, beta-carotene, and CoQ10, along with enzymatic antioxidants such as SOD and Cat, neutralize ROS and mitigate oxidative damage. However, aging is associated with reduced antioxidant capacity and changes in the structure of the cornified envelope, which can heighten susceptibility to oxidative stress. Thus, while antioxidant supplementation may be beneficial, excessive or unregulated use can interfere with normal redox signaling, highlighting the need for balanced antioxidant therapy (Mohiuddin, 2019).

Modern anti-aging dermatology employs both topical and procedural interventions. Topical agents, particularly retinoids, remain the gold standard due to their efficacy in increasing collagen

production, improving dermal architecture, and reducing pigmentation and wrinkles (Milosheska & Roskar, 2022). Vitamin C (ascorbic acid) supports collagen synthesis and reduces oxidative stress, though its application is limited by instability and poor skin penetration. Other topicals, such as α-hydroxy acids (e.g., glycolic and lactic acid), stimulate epidermal turnover and enhance skin texture, while trichloroacetic acid peels have demonstrated success in treating pigmentation and promoting neocollagenesis (Bernstein et al., 2001). Importantly, sunscreens are indispensable for preventing UV-induced photoaging, while newer agents like β-glucans, CoQ10, and alpha-lipoic acid offer additional antioxidant and regenerative effects (Campiche et al., 2019; Fuller, 2022).

Among injectables, hyaluronic acid (HA) is widely used for its hydrating and volumizing effects, as well as its ability to activate fibroblasts through CD44 and CD168 receptors and stimulate collagen production via the TGF-β pathway (Wang et al., 2007; Ayatollahi et al., 2020). Emerging HA formulations, such as those containing epidermal growth factor (EGF), show promise in both enhancing collagen and inhibiting MMP expression (Shin et al., 2023). Furthermore, other fillers, like poly-L-lactic acid and polynucleotides, promote long-term neocollagenesis. Botulinum toxin not only smooths dynamic wrinkles, but also improves elasticity and hydration by modulating neurogenic inflammation (Tam et al., 2024).

Minimally invasive techniques such as microneedling and microdermabrasion stimulate dermal remodeling by inducing controlled micro-injuries, promoting collagen synthesis and enhancing transdermal drug delivery. Recently, interest in stem cell-derived exosomes and stromal vascular fractions from adipose tissue highlights their regenerative potential in reversing photoaging and restoring ECM protein expression (Xiong et al., 2020).

Energy-based devices have also gained traction for their ability to induce dermal remodeling without significant downtime. Fractional lasers, such as $CO_2$, Er:YAG, and picosecond lasers, stimulate fibroblast proliferation, enhance collagen remodeling, and reduce pigmentation (Haykal et al., 2024). Additionally, high-intensity focused ultrasound and radiofrequency deliver thermal energy to the dermis and subcutis, promoting nucleogenesis and tissue tightening. Bipolar RF has also been shown to reduce senescent fibroblasts and induce heat shock proteins, aiding skin repair (Rangarajan et al., 2013). In addition, LED therapy is being explored for its ability to stimulate collagen and mitigate UV-induced damage (Song et al., 2023).

For postmenopausal women, hormone replacement therapy can restore skin elasticity and reduce thinning by replenishing estrogen levels. However, the associated risks require careful evaluation and individualized treatment plans (Geetha, 2025). On a systemic level, compounds such as rapamycin, senolytics, and resveratrol are under investigation for their potential to target fundamental aging mechanisms, including cellular senescence, telomere shortening, and genomic instability—shifting the focus from symptom management to health span extension (Mohiuddin, 2019).

In parallel with clinical advancements, artificial intelligence (AI) is revolutionizing anti-aging research by enhancing molecular data analysis, visualizing skin aging patterns, and tailoring personalized treatment strategies. These innovations accelerate drug discovery and make customized skincare more accessible (Zhavoronkov et al., 2019).

While many advanced interventions exist, topical therapies remain the most accessible and widely used, particularly among broader populations. However, due to the potential side effects of synthetic agents—such as irritation from retinoids—there is growing interest in natural compounds

with anti-inflammatory and antioxidant properties. Recent research highlights promising agents such as psilocybin and eugenol, which show strong anti-aging potential with lower irritation risks (Sgorbini et al., 2023; Husen et al., 2024). This movement toward biocompatible, plant-derived therapies reflect a broader shift in dermatology towards safer, more holistic approaches to skin rejuvenation.

From ancient beauty rituals to today's AI-guided interventions, anti-aging dermatology has advanced tremendously. As research into natural products and personalized technologies continues, the future of anti-aging therapy promises to be not only more effective but also safer and more aligned with the skin's intrinsic biology.

### 1.11. Psilocybin: Evolution, Mechanisms, and Therapeutic Potential for Inflammation and Skin Aging

Psilocybin is a naturally occurring tryptamine compound found in several species of fungi, particularly within the phylum Basidiomycota, including genera such as *Psilocybe*, *Panaeolus*, *Gymnopilus*, and *Conocybe*. Over millions of years, these fungi have evolved under various ecological pressures, developing psilocybin as a bioactive defense mechanism against herbivores and microbial threats. Its production—derived from tryptophan through enzymatic steps involving tryptophan decarboxylase—demonstrates convergent evolution across different fungal taxa, highlighting its evolutionary advantage (Meyer & Slot, 2023).

In addition to its defensive role, psilocybin-producing fungi participate in symbiotic relationships, such as mycorrhizal associations, enhancing plant nutrient uptake in exchange for carbohydrates (Ling et al., 2022; Meyer & Slot, 2023). The compound's interaction with serotonin receptors,

especially 5-HT2A, plays a central role in its psychoactive and physiological effects. Psilocybin is a partial agonist at 5-HT2A, 5-HT2C, and 5-HT1A receptors, influencing perception, emotion, and downstream gene expression (Nkadimeng et al., 2020; Ling et al., 2022). This receptor activity also suggests a co-evolutionary adaptation, possibly influencing animal behavior and contributing to fungal fitness (Nkadimeng et al., 2021; Caspani et al., 2024).

Psilocybin metabolism begins with oral ingestion, followed by absorption through the gastrointestinal tract and conversion to psilocin via hepatic dephosphorylation (Meshkat et al., 2025). Psilocin crosses the blood-brain barrier and circulates through peripheral tissues, where it binds serotonin receptors like 5-HT2A, initiating intracellular signaling cascades that regulate inflammation, oxidative stress, and gene expression (Thomann et al., 2024). Although psilocin is mostly excreted within 24 hours, its downstream cellular effects, including cytokine modulation and antioxidant protection, may persist longer (Zhou et al., 2021).

Historically, psilocybin has been used in indigenous spiritual practices and reemerged in modern science as a potential treatment for various psychiatric conditions. Clinical studies have demonstrated its efficacy in alleviating depression, anxiety, and addiction, particularly in treatment-resistant cases and end-of-life psychological distress (Carhart-Harris et al., 2021). Its low toxicity and minimal abuse potential have further reinforced its attractiveness as a therapeutic agent (Rossi et al., 2022).

Modern trials highlight psilocybin's ability to reduce neuroticism and enhance openness in individuals with depression (Erritzoe et al., 2018). In one phase 2 trial comparing psilocybin with escitalopram, psilocybin demonstrated higher response and remission rates despite similar primary outcome scores (Carhart-Harris et al., 2021). Lifetime use of classic psychedelics, including

psilocybin, has also been associated with lower rates of psychological distress and suicidality (Hendricks et al., 2015).

Beyond neuropsychiatry, psilocybin's systemic effects—including its modulation of inflammation and oxidative stress—are drawing attention for peripheral tissue applications such as dermatology.

Psilocin—the active metabolite of psilocybin—modulates inflammatory pathways by downregulating pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6, while upregulating anti-inflammatory mediators such as IL-10 (Lowe et al., 2021; Nkadimeng et al., 2021). These immune-modulatory effects have been demonstrated across models of neuroinflammation, metabolic disorders, and tissue injury (Ghasemi Gojani, 2024; Laabi et al., 2024).

Studies also suggest that psilocybin acts through suppression of transcription factors such as NF-κB, STAT1, and STAT3, which play key roles in chronic inflammation and aging-related diseases (Ghasemi Gojani et al., 2024; Wiens et al., 2024). In THP-1 macrophages, psilocybin produced a dose-dependent reduction in IL-1β, IL-6, and COX-2, with higher doses offering significant inflammation mitigation (Ghasemi Gojani et al., 2024). Notably, psilocybin's selective activation of 5-HT2A receptors allows for anti-inflammatory effects without the broad immunosuppression typical of corticosteroids (Flanagan & Nichols, 2018).

Recent studies have extended these findings to dermal biology, where psilocybin appears to influence fibroblast viability, collagen production, and ECM remodeling—key factors in skin aging. Psilocybin's capacity to modulate inflammation and oxidative stress suggests potential utility in managing age-related dermal changes. Its effects on fibroblasts may enhance collagen synthesis, ECM remodeling, and wound healing—core components in anti-aging dermatology

(Lowe et al., 2021; Sgorbini et al., 2023). By improving fibroblast viability and reducing chronic inflammation, psilocybin could counteract the degradation of dermal structure often seen in skin aging.

Research in diabetic models has shown psilocybin to protect pancreatic β-cells from damage induced by high glucose and lipid environments—mechanisms relevant to skin aging where similar metabolic stressors contribute to dermal degradation (Ghasemi Gojani, 2024). *Psilocybe natalensis*, in particular, has demonstrated superior antioxidant capacity among psilocybin-containing fungi, further supporting its use in mitigating oxidative skin damage (Nkadimeng et al., 2020).

Psilocybin may also influence regenerative pathways by enhancing serotonin-mediated growth factor expression, potentially promoting neuroplasticity and tissue repair. These actions could further support skin resilience, enhance fibroblast proliferation, and accelerate wound closure (Lowe et al., 2021; Vargas et al., 2023).

When combined with compounds like eugenol, psilocybin's effects may be synergistically amplified, offering new avenues for combination therapies targeting skin aging and inflammation (Gojani et al., 2024).

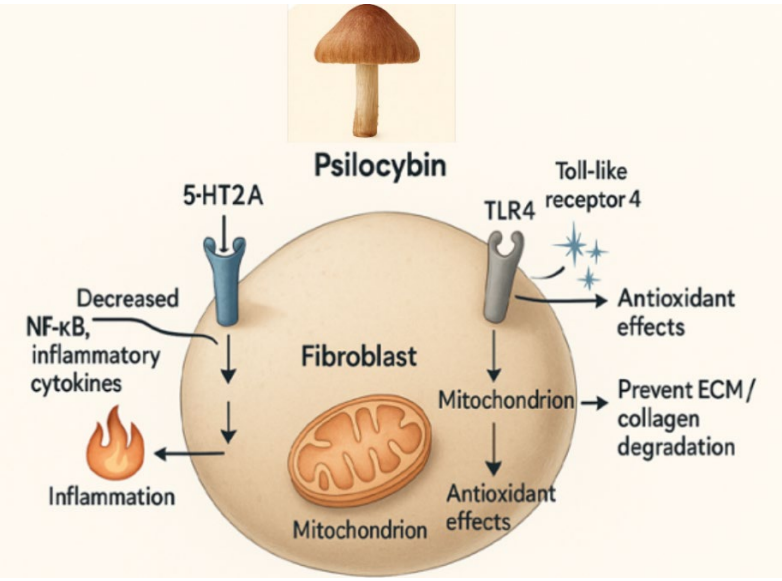


**Figure 6.** Psilocybin's protective effects on fibroblasts. Psilocybin activates 5-HT$_2$A and modulates TLR4, reducing NF-κB and inflammation, enhancing NRF2 antioxidant activity, and preventing ECM/collagen degradation. Figure generated using OpenAI's ChatGPT (Pro version with DALL·E image generation), 2025.

### 1.12. Eugenol: Biosynthesis, Therapeutic Potential, and Mechanisms in Skin Health

Eugenol is a natural phenylpropanoid compound primarily found in clove (Syzygium aromaticum), cinnamon, basil, and other aromatic plants, with clove oil being its richest source (Danthu et al., 2020). Its biosynthesis occurs through the phenylpropanoid pathway, a branch of the shikimate pathway, beginning with the amino acid phenylalanine. Phenylalanine undergoes conversion to cinnamic acid via phenylalanine-ammonia-lyase, which is then hydroxylated to coumaric acid by cinnamate 4-hydroxylase. Subsequent enzymatic reactions transform coumaric acid into coniferyl alcohol through the action of cinnamyl-CoA reductase and cinnamyl alcohol dehydrogenase. Finally, methylation and reduction reactions mediated by coniferyl alcohol acetyl transferase yield eugenol (Ouadi et al., 2022).

While the production of eugenol is energetically costly for plants—diverting resources from growth and reproduction—it confers a strong evolutionary advantage. It enhances plant defenses against herbivores and pathogens, reduces interplant competition through allelopathy, and attracts pollinators via its aromatic profile, supporting reproductive success (Ouadi et al., 2022; Hirose et al., 2024).

Eugenol's diverse bioactivities have led to its wide adoption in traditional and modern medicine. It is best known for its antimicrobial, antioxidant, anti-inflammatory, and analgesic effects. In dentistry, it is used as an anesthetic and antiseptic, particularly in root canal treatments and pain relief (Nisar et al., 2021). It also displays cardioprotective effects—including inhibition of platelet aggregation comparable to aspirin (Devi et al., 2024)—and has shown neuroprotective, antipyretic, antiparasitic, and lipid-lowering activity (Nisar et al., 2021).

In dermatology, eugenol stands out for its therapeutic potential in managing inflammation, oxidative stress, and microbial overgrowth—factors central to skin aging and disease. It has been incorporated into nano-emulsions and topical formulations for treating dermatitis, acne, and fungal infections. Nano-encapsulation enhances its skin penetration and reduces cytotoxicity, increasing its suitability for cosmetic use (Esmaeili et al., 2016; Nisar et al., 2021).

Eugenol exhibits potent antioxidant activity, outperforming classical antioxidants such as α-tocopherol in lipid peroxidation inhibition. It effectively scavenges free radicals including DPPH, ROS, and $H_2O_2$ (Gülçin, 2011). Its anti-inflammatory properties are mediated by suppression of the NF-κB signaling pathway, leading to reduced expression of pro-inflammatory cytokines such as TNF-α and IL-6, as demonstrated in models of acute lung injury (Magalhaes et al., 2019). Eugenol also enhances the Nrf2/ARE signaling pathway, boosting the transcription of endogenous

antioxidant enzymes—an effect particularly relevant for countering oxidative skin aging (Thiruvengadam et al., 2021). In oncology research, eugenol has shown synergistic potential when combined with chemotherapeutics like gemcitabine and cisplatin (Zari et al., 2021).

In skin keratinocytes cells, eugenol activates the TRPV1 receptor, leading to calcium influx that modulates inflammatory signaling (Meotti et al., 2014). It suppresses oxidative and inflammatory stress at the molecular level, while also promoting tissue resilience and matrix preservation—two key features in anti-aging dermatology.

Beyond its antioxidant and anti-inflammatory properties, eugenol also plays a significant role in preventing protein glycation. It reduces the formation of AGEs by inhibiting intestinal α-glucosidase activity and competing with sugars for protein binding sites via its 4'-hydroxyl group. This antiglycation mechanism is crucial for preserving the structure and function of dermal proteins such as collagen and elastin, thereby maintaining skin elasticity. Additionally, eugenol acts as a reactive carbonyl species (RCS) scavenger, further inhibiting AGE formation and preventing collagen cross-linking and degradation. Together, its antiglycation and antioxidant actions help protect fibroblasts from oxidative stress and age-related cellular damage (Singh et al., 2016).

Despite its broad therapeutic potential, high concentrations of eugenol can be toxic, with risks including liver and DNA damage, as well as skin irritation, particularly in sensitive individuals or with prolonged exposure (Nisar et al., 2021). Its reactive structure enables covalent binding to proteins like keratin, which may lead to allergic sensitization or cross-reactivity (Lepoittevin and Lafforgue, 2020).

Compared to other natural oils used in skincare, such as olive oil, eugenol demonstrates superior antioxidant capacity, antimicrobial potency, and anti-aging efficacy. While olive oil provides excellent emollient and barrier-supporting properties, it lacks the targeted bioactivity of eugenol in treating oxidative and inflammatory skin conditions (Surber et al., 2018). Additionally, eugenol's pleasant aroma and high compatibility with emulsions and serums make it particularly well-suited for lightweight, multifunctional formulations—such as under-eye creams or anti-aging serums (Guzman and Lucia, 2021).

All things considered, eugenol is a potent natural agent with antioxidant, anti-inflammatory, antimicrobial, and AGE-inhibitory properties, making it a highly promising candidate for anti-aging skincare. However, its therapeutic use requires careful formulation to balance efficacy with safety, and further studies are essential to fully elucidate its molecular mechanisms and optimize its application.

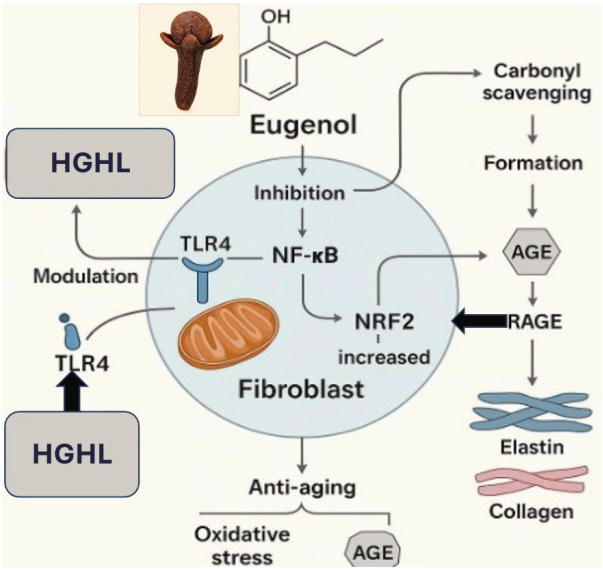


**Figure 7.** Eugenol's protective effects on fibroblasts. Eugenol inhibits TLR4 and NF-κB signaling, activates NRF2 antioxidant pathways, scavenges reactive carbonyls, and prevents AGE–RAGE

interaction, thereby reducing inflammation and collagen degradation. Figure generated using OpenAI's ChatGPT (Pro version with DALL·E image generation), 2025.

### 1.13. Hypotheses

A comprehensive understanding of dermal aging necessitates the investigation of biological and molecular processes that impact skin structure and function. Central to this phenomenon is the reduced synthesis and/or increased degradation of ECM proteins, particularly collagen and elastin, which are fundamental for maintaining skin integrity and resilience. Concurrently, cellular mechanisms such as metabolic dysregulation, oxidative stress, and impaired cell cycle control contribute to aging at the cellular level. These multifaceted processes collectively drive the progressive decline in skin elasticity, hydration, and regenerative capacity, forming the basis for ongoing research into the pathways that underpin visible and structural changes in aging skin (Samizadeh, 2024).

Despite significant progress in elucidating the mechanisms of skin aging, critical gaps remain in understanding the factors that counteract these effects and promote skin rejuvenation. Environmental and intrinsic stressors—particularly UV radiation and oxidative stress—induce persistent damage and trigger stress-induced cell cycle arrest, culminating in cellular senescence. Senescent cells, marked by pro-inflammatory secretory profiles and disrupted homeostasis, contribute substantially to functional deterioration in aged skin (Gu et al., 2020). Oxidative stress arises from diverse sources, including dietary habits, which serve as a primary focus of this study. HGHL diets are known to exacerbate oxidative stress by promoting mitochondrial dysfunction, increasing AGEs formation and elevating inflammatory responses. These changes accelerate the aging process and emphasize the critical need to explore dietary influences on skin health (Meenakshi et al., 2024).

This study has two central objectives.

1. We aim to demonstrate how an HGHL diet induces mitochondrial dysfunction, inflammation and AGE formation, ultimately increasing oxidative stress and accelerating skin aging.
2. We plan to investigate the protective effects of natural compounds—specifically psilocybin and eugenol—which have demonstrated antioxidant and anti-inflammatory properties with anti-aging potential in prior research (Nkadimeng et al., 2021; Ulanowska & Olas, 2021).

Our goal is to evaluate the extent to which psilocybin and eugenol mitigate the detrimental effects of HGHL exposure on skin fibroblasts (BJ-5TA), with particular emphasis on reducing cellular senescence and preserving cellular health. The study will involve both co-treatment (simultaneous exposure) and post-treatment (following HGHL exposure) approaches to capture the full scope of their protective and curative capacities.

**Specifically, we hypothesize that:**

1. Exposure of fibroblasts to a HGHL mixture will induce stress-induced premature senescence (SIPS) via increased oxidative stress and ROS generation.
2. Psilocybin extract will reduce senescence-associated changes, including enhanced apoptotic resistance, dysregulated cell cycle progression, impaired wound closure, and inflammatory gene expression.
3. Eugenol extract will counteract senescence-associated features, including enhanced apoptotic resistance, dysregulated cell cycle progression, impaired wound closure, and inflammatory cytokines gene expression.

4. The magnitude of these effects will be concentration-dependent, with varying responses based on psilocybin and eugenol dosage.
5. The underlying anti-aging mechanisms of both compounds will involve inhibition of inflammatory mediators, suppression of ROS-related pathways, and delayed onset of cellular senescence.

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## 2. Chapter 2: Psilocybin Alleviates High-Glucose and High-Lipid-Induced Skin Aging in BJ-5Ta Fibroblasts

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*This chapter is included with the permission of all co-authors. Author contributions are detailed in the "Contributions of Authors" section in the Preface, in accordance with the University of Lethbridge manuscript-based thesis guidelines.*

### 2.1. Abstract

Cellular aging, driven by oxidative stress, mitochondrial dysfunction, and inflammation, is exacerbated by a high-glucose and high-lipid (HGHL) diet, ultimately leading to collagen degradation and skin aging. Psilocybin, a naturally occurring compound, has shown potential in reducing symptoms of aging. However, its effects on BJ-5ta skin fibroblasts under HGHL conditions remain largely unknown, with only a few studies available on psilocybin. This study explores the protective effects of psilocybin on BJ-5ta fibroblasts exposed to HGHL, focusing on cellular viability, apoptosis, senescence, the inflammatory responses, the extracellular matrix (ECM) preservation, and wound healing. First, fibroblasts were exposed to 25 mM glucose and 400 μM palmitic acid (HGHL conditions) to establish cell aging. Then, psilocybin effects were tested in co- and post-treatment with HGHL. Post-treatment with psilocybin at 15 μM (P15) and co-treatment with psilocybin at 10 μM (P10) preserved cellular viability and decreased beta-galactosidase activity. P10 was most effective in reducing apoptosis and alleviating HGHL-induced S phase arrest, thereby promoting cell cycle progression. P15 also reduced senescence markers and decreased the expression of inflammatory cytokines *IL-1β, IL-6,* and *COX-2*. Additionally, psilocybin promoted non-significant fibroblast migration, and P10 co-treated with HGHL significantly upregulated *elastin* (*ELN*) gene expression. These findings suggest that psilocybin's antioxidative, anti-inflammatory, and regenerative properties make it a promising

natural compound for reducing skin aging, particularly under oxidative stress conditions. Further research is needed to explore its long-term effects, optimal dosages, and clinical applications.

## 2.2. Introduction

Nowadays, people live longer, and countries like South Korea and Japan are at the forefront of this global aging trend (He et al., 2023). While increased life expectancy enables individuals to lead more active and productive lives, it also brings challenges, such as a higher prevalence of age-related diseases (ARDs) like cardiovascular disease, diabetes, cancer, Parkinson's, and Alzheimer's. These conditions often result in prolonged impairment, highlighting the importance of understanding the aging process and developing effective treatments (Gladyshev, 2016; Bonte et al., 2019).

Skin aging, a significant focus in aging research due to its role as the body's primary protective barrier, has gained notable societal and scientific interest (Parrado et al., 2019; Fuchs and Blau, 2020). This process is influenced by both internal and external factors, including genetic changes, UV radiation, diet, pollution, and lifestyle choices. These contribute to a decline in collagen production and degradation, leading to wrinkles, loss of elasticity, and sagging, but also impact skin barrier function, wound healing and overall skin health-making skin aging a significant concern beyond aesthetics(Gruber et al., 2021; Papaccio et al., 2022). Oxidative stress, a key player in both intrinsic and extrinsic aging, damages skin cells and impairs collagen synthesis. In intrinsic aging, oxidative stress is linked to a weakened antioxidant defense system, while in extrinsic aging, environmental factors like UV rays, smoking, and poor diet exacerbate this damage, resulting in wrinkles, hyperpigmentation, and changes in skin texture (Rinnerthaler et al., 2015; Dyer et al., 2018). Maintaining mitochondrial health and enhancing damage repair mechanisms are critical to slowing the process (Walker et al., 2022).

Diet plays a critical role in mitochondrial health and the aging process. Elevated glucose levels have been linked to increased oxidative stress, driven by the overproduction of reactive oxygen species (ROS) during mitochondrial electron transport (Jiang et al., 2021). This oxidative stress can damage mitochondrial components, including proteins, lipids, and mitochondrial DNA (mtDNA), impairing ATP production (ATP) and contributing to tissue aging (Longo et al., 2015; Quan et al., 2015; Gems et al., 2024). Chronic high glucose exposure has also been associated with disrupted mitochondrial dynamics, aggravating cellular dysfunction and senescence. These processes are implicated in aging-related conditions such as cardiovascular disease and type 2 diabetes (T2D) (Jiang et al., 2021).

Additionally, a diet high in glucose and lipids accelerates skin aging through glycation, glycoxidation, and oxidative damage. Elevated HGHL levels—often linked to a Westernized diet, obesity, and type 2 diabetes (T2D)—promote the formation of advanced glycation end-products (AGEs) (Lipsky and King, 2015; Ashraf et al., 2016). These AGEs impair the structure and function of key proteins like collagen and elastin, reducing skin elasticity and contributing to visible signs of aging, such as wrinkles and sagging. Moreover, chronic low-grade inflammation triggered by AGEs degrades skin structures, compromising its integrity. Elevated lipid levels further disrupt the skin's lipid matrix, contributing to dryness, inflammation, and other aging markers (Jiang et al., 2021).

Furthermore, mitochondrial dysfunction caused by an HGHL diet plays a central role in both systemic and skin aging by driving the overproduction of ROS, which exacerbates oxidative stress and damages cellular components such as DNA, lipids, and proteins. This accelerates markers of aging, including DNA mutations, telomere shortening, and cellular senescence (Williamson et al., 2020). In the skin, mitochondrial dysfunction manifests as dryness, wrinkles, and reduced

elasticity. Additionally, it activates pathways such as mitogen-activated protein kinase (MAPK), leading to the production of matrix metalloproteinases (MMPs) that degrade collagen and elastin, weakening the extracellular matrix and worsening visible signs of aging. These interconnected processes demonstrate the essential role of mitochondrial health not only in systemic aging but also in maintaining skin integrity and delaying age-related changes (Zouboulis et al., 2019; Ahuja and Bajpai, 2024).

Various strategies have been developed to combat skin aging by targeting its underlying mechanisms. These approaches aim to mitigate oxidative stress, improve cellular function, and enhance skin integrity. For example, compounds that inhibit glycation, promote collagen production, and regulate lipid metabolism help maintain skin elasticity and hydration. Additionally, treatments that enhance mitochondrial function and reduce inflammation are being explored to counteract oxidative damage (Mohiuddin, 2019; Shin et al., 2023). Emerging natural compounds, like psilocybin, have shown promise for their antioxidant and anti-inflammatory benefits, offering a potential alternative to traditional synthetic treatments. Their biocompatibility makes them a safer alternative, with potential applications in reducing oxidative stress and inflammation associated with aging (Lowe et al., 2021; Park et al., 2021).

Psilocybin, a compound produced by fungi in the Basidiomycota phylum, likely evolved as a defense mechanism and plays a role in ecological interactions, such as mycorrhizal associations that enhance plant growth. Biosynthesized from tryptophan, psilocybin is converted into its active form, psilocin, after ingestion (Dinis-Oliveira, 2017; Meyer and Slot, 2023). Psilocin interacts with serotonin receptors, particularly 5-HT2A, to regulate mood, stress, and inflammation, supporting anti-inflammatory responses and cellular repair (Ling et al., 2022; Njenga et al., 2024; Thomann et al., 2024). This highlights its potential therapeutic value in various biological processes.

Psilocybin's interaction with serotonin receptors (5-HT2 and 5-HT7), along with its strong anti-inflammatory and antioxidant properties, suggests that it can be used in dermatology for managing inflammatory conditions, promoting skin rejuvenation, and mitigating cellular senescence (Park et al., 2021; Hecker et al., 2023). It reduces pro-inflammatory cytokines while enhancing anti-inflammatory responses, modulates chronic inflammation in animal studies, and combats oxidative stress—a key factor in aging and cellular damage—particularly in species like *Psilocybe natalensis* (Flanagan and Nichols, 2018; Nkadimeng et al., 2020).

This study is the first to investigate psilocybin's protective effects on skin fibroblasts under HGHL conditions, which accelerate skin aging through oxidative stress and impaired cellular function (Zhang et al., 2021; Zhang et al., 2024). Psilocybin demonstrated concentration-dependent benefits, reducing oxidative damage and inflammation, inhibiting ROS pathways, and delaying cellular senescence, thereby confirming its potential as a therapeutic agent for skin health. Our findings highlight the importance of lifestyle changes to manage oxidative stress, glucose levels, and metabolic balance in combating skin aging.

## 2.3. Materials and Methods

### 2.3.1. Chemicals and Reagents

For our cell culture experiments, we used ISCOVE's Modified Dulbecco's Medium (IMDM) 1X (MULTICELL, Cat# 319-106-CL; Wisent Inc., Saint-Jean-Baptiste, QC, Canada) as the base medium. The medium was supplemented with heat-inactivated Premium Grade Fetal Bovine Serum (FBS) (Cat# 97068-085, VWR International LLC, Radnor, USA) and 100× Penicillin-Streptomycin (10,000 IU Penicillin and 10 mg/ml Streptomycin; Cat# 450-201-EL, Wisent Inc., Quebec, Canada) to support optimal cell growth and prevent contamination. For washing and

handling procedures, we used 1X phosphate-buffered saline (PBS) (Cat# 311-010-CL, Wisent Inc.). Human foreskin BJ-5ta hTERT-immortalized fibroblast cell lines (CRL-4001™) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained under standard culture conditions.

### 2.3.2. Cell Culture and Treatments

Human foreskin BJ-5ta hTERT-immortalized fibroblast line (CRL-4001™), referred to as "fibroblasts" in this study, was cultured in ISCOVE's Modified Dulbecco's Medium (IMDM), supplemented with 10% heat-inactivated Premium Grade Fetal Bovine Serum ((10082147) acquired from Fisher Scientific Company (Ottawa, ON, Canada) and 1% Penicillin-Streptomycin (10,000 IU Penicillin and 10 mg/ml Streptomycin). Mycoplasma testing was performed using a PCR-based detection kit (e.g., MycoAlert™ Mycoplasma Detection Kit, Lonza) according to the manufacturer's instructions to confirm cultures were contamination-free (Supplementary Figure 1).

All fibroblast culturing and harvesting were conducted in a BSL-2 laboratory. The fibroblasts were incubated at 37°C with 5% $CO_2$ in a Forma Steri-Cycle $CO_2$ Incubator (Thermo Scientific, Montreal, Canada) with the culture medium replaced every 48 hours until the fibroblasts reached 90%-100% confluency for experimental use. Subculturing was performed weekly. The population doubling (PD) number for each subculture was calculated using the formula $\Delta PD = \log_2(nf/ni)$, where ni represents the initial number of seeded fibroblasts and nf the final cell count. Cells between passages 17 and 24 were selected for all experiments. Subsequently, fibroblasts underwent treatment with a specified dosages of freshly prepared psilocybin (CAS No. 520-52-50, received from Applied Pharmaceutical Innovation, Edmonton, AB, Canada). To induce cell senescence, fibroblasts were exposed to the combination of high-glucose (HG, 25 mM glucose)

and high-lipid (HL, 400 μM palmitic acid) (HGHL) conditions for 48 h. This approach aligns with findings from the recent study by Ghasemi Gojani et al. (2024), which demonstrated that such conditions reduce cell viability in beta cells, promote apoptotic behavior, and exacerbate other aging-related effects. A 1.0 M glucose stock solution was prepared by dissolving glucose in the medium, followed by filter sterilization, and stored at 4 °C. The palmitic acid (PA) stock solution was prepared by dissolving PA in 100% ethanol with heating at 70 °C. The dissolved PA was then combined with sterile 10% BSA and underwent two cycles of heating at 55 °C for 15 min with mixing. The PA stock was aliquoted and stored at −20 °C. Prior to use, the PA stock was reheated at 55 °C for 15 min.

Building on Gojani et al.'s (2024) findings, which utilized a 10 μM concentration of psilocybin on beta-cells, we optimized psilocybin application for co-treatment and post-treatment conditions. Informed by pilot studies and repeated tests assessing cell toxicity and viability, we selected 10 μM for co-treatment and 15 μM for post-treatment. To evaluate psilocybin's effects, two experimental approaches were employed: in post-treatment, psilocybin was applied after HGHL-induced senescence to assess its reparative potential, whereas in co-treatment, it was administered simultaneously with HGHL to examine its protective effects. These methods provide insight into psilocybin's dual role in prevention of damage to skin cells and repair of such damage. For all experiments, psilocybin was used at 15 μM (P15) in post-treatment (HGHL/P15) and 10 μM (P10) in co-treatment (HGHL+P10), along with P15 applied to untreated fibroblasts to assess psilocybin's effect in the absence of stress.

For post-treatment, HGHL conditions were applied for 48 h to induce senescence in cells. HGHL was induced in two out of four wells, while the remaining two wells were controls. Of the two control wells, one served as a control containing untreated cell with media (CT), and the other

contained untreated fibroblasts supplemented with 15 μM psilocybin (P15). Following the 48-hour HG and HL treatments, fibroblasts in the two HGHL-induced wells were washed twice with 1X PBS. One well was treated with 15 μM psilocybin, while the other was treated with media alone for an additional 48 h. The media-only treatment in HGHL-induced fibroblasts served as a control to assess psilocybin's effects (P15) under these conditions.

In the co-treatment approach, fibroblasts were simultaneously treated with psilocybin at the designated concentration (P10) and HGHL for 48 h. The experimental setup included four wells: one with HGHL treatment alone serving as a control, one with untreated fibroblasts (CT) control, one with psilocybin at 15 μM added to untreated fibroblasts, and one with the combined psilocybin (P10) and HGHL treatment. These concentrations were selected for all assays, including wound healing, beta-galactosidase, apoptosis, and cell cycle analyses, along with RNA isolation for quantitative reverse transcription PCR (qRT-PCR). RNA was extracted from treated fibroblasts for qRT-PCR to assess the effects of treatment.

### 2.3.3. MTT Assay

Cell viability and cytotoxicity of BJ-5ta human skin fibroblasts were evaluated using the MTT assay, which relies on the ability of metabolically active cells to reduce the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) into insoluble purple formazan crystals. This redox reaction predominantly occurs in the mitochondria through the action of succinate dehydrogenase, though other oxidoreductases located in the cytosol and endoplasmic reticulum also contribute, utilizing NADH and NADPH as electron donors (van Meerloo et al., 2011). As a result, formazan production is directly proportional to the number of viable cells, since only cells with intact metabolic activity can drive this reduction. In contrast,

dead or metabolically inactive cells do not generate formazan, making the MTT assay a reliable indicator of overall cellular metabolic activity rather than purely mitochondrial function (Ghasemi et al., 2022).

BJ-5ta fibroblasts were seeded at a density of $5.0 \times 10^3$ cells per well in 100 µl of complete culture medium in 96-well plates and incubated for 24 to 48 hours to reach 70–80% confluency prior to treatment. Treatments—including high glucose and high lipid (HGHL) conditions, psilocybin, and various concentrations of eugenol—were applied to determine cytotoxic and effective doses. Cell viability was assessed at three time points: days 0, 2, and 4 post-treatment. Each condition was tested in triplicate, and the experiments were independently repeated three times to ensure reproducibility.

Following treatment, 10 µl of MTT reagent from the Cell Proliferation Kit I (#11465007001, Roche, Ontario, Canada) was added directly to each well. Plates were incubated for 4 hours at 37 °C in a humidified atmosphere containing 5–6.5% $CO_2$. After incubation, 100 µl of solubilization solution (10% SDS in 0.01 M HCl) was added to dissolve the formazan crystals, followed by overnight incubation under the same conditions. Absorbance was measured at 595 nm using a FLUOstar Omega microplate reader (BMG LABTECH, Offenburg, Germany). Cell viability was calculated relative to untreated control cells.

### 2.3.4. β-Galactosidase Assay

To assess cellular senescence, fibroblasts were treated under various conditions, including HGHL to induce premature aging, psilocybin co-treatment and post-treatment, as well as psilocybin treatment on untreated fibroblasts. The cells were then washed twice with 1X PBS. To quantify β-galactosidase activity, fibroblast lysates from both normal and HGHL-induced prematurely aged

fibroblasts were prepared using the protein lysis buffer from the Beta-Galactosidase Detection Kit (Fluorometric) (ab176721, Abcam, Toronto, ON, Canada). Protein concentration in each sample was measured with the Bradford assay, and the samples were diluted to a final protein concentration of 1 µg/ml. Following this, 50 µl of each standard and sample (diluted in 1× lysis buffer) were transferred to a black 96-well plate. To each well, 50 µl of fluorogenic fluorescein digalactoside (FDG) working solution was added, and the plate was incubated at 37 °C for 4 h. Subsequently, 50 µl of stop buffer was added, and fluorescence was measured for each sample using a FLUOstar Omega (BMG LABTECH, Offenburg, Germany) plate reader with excitation and emission wavelengths of 490 nm and 525 nm, respectively. β-Galactosidase levels, indicating cellular senescence, were determined by comparing fluorescence values to a β-galactosidase standard curve prepared for each experiment. Each experiment was conducted in triplicate for reliability.

### 2.3.5. Apoptosis Assay

To evaluate apoptosis, the BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit II (Cat No. BDB556570; BD Biosciences, Mississauga, ON, Canada) was employed to detect the externalization of phosphatidylserine (PS) on the cell membrane. FITC Annexin V specifically binds to PS exposed on apoptotic cells, while propidium iodide (PI) helps differentiate live cells from those that are necrotic or late-apoptotic. Cells labeled only with FITC Annexin V were categorized as apoptotic, those positive for PI were identified as necrotic or in late stages of apoptosis, and cells negative for both dyes were considered viable. To begin, cells were rinsed twice with cold D-PBS and resuspended in 1X Annexin V Binding Buffer at a concentration of $1 \times 10^6$ cells/mL. A 100 µL sample (containing $1 \times 10^5$ cells) was transferred to a 5 mL tube, and 5 µL of FITC Annexin V along with 5 µL of PI were added. The mixture was gently mixed and

incubated in darkness at room temperature (approximately 25°C) for 15 minutes. Following incubation, 400 µL of 1X Binding Buffer was added to the tube, and the samples were analyzed within one hour using a BD FACSAria™ Fusion Flow Cytometer. Fibroblasts that were Annexin V-positive and PI-negative were classified as early apoptotic, while fibroblasts that were both Annexin V- and PI-positive were considered late apoptotic or necrotic, as PI can only stain cells with compromised membrane integrity. This assay allowed quantification of the apoptotic and necrotic cell populations, providing insights into the treatment's effects on cell viability and apoptosis. Apoptosis analysis was performed in triplicate.

**2.3.6. Cell Cycle Assay**

After treatment with HGHL, the fibroblasts were washed twice with cold 1X PBS to remove residual media and non-adherent fibroblasts. Next, they were trypsinized to detach them from the culture surface, and then centrifuged to collect the cell pellet. The pellet was resuspended in cold PBS and fixed by adding cold 70% ethanol dropwise while gently vortexing. The fibroblasts were then incubated at 4°C for at least 2 h (or overnight) to ensure proper fixation.

Following fixation, the fibroblasts were washed again with cold PBS to remove ethanol. To eliminate RNA, 50 µl of RNase solution (100 µg/ml) was added to each sample, followed by the addition of 200 µl of a propidium iodide (PI) solution prepared at 50 µg/ml to stain the DNA. The stained cells were incubated in the dark at room temperature for 30-60 min to ensure consistent dye binding.

Finally, the cell cycle distribution was analyzed by BD FACSAria™ Fusion Flow Cytometer (BD Biosciences, San Diego, CA, USA). Fibroblasts were categorized based on DNA content into different phases: G0/G1 (resting or initial growth phase), S (DNA synthesis phase), and G2/M

(second growth and mitosis phase), providing insights into the cell population's distribution across the cell cycle stage. Cell cycle distribution was analyzed in triplicate.

### 2.3.7. Wound Healing Assay

Fibroblasts were grown in 6-well plates until they reached over 90% confluence. A 100 µl pipette tip was employed to create a scratch down the center of each well, mimicking a wound. Following this, the fibroblasts were washed twice with 1X PBS before adding the appropriate cell culture growth medium or specific treatments. Images of the wound healing process were captured at various time points: day 0 (2 h after wound creation, treatments began at this time), day 1 (24 h after treatment), day 2 (48 h after treatment), day 3 (72 h after treatment) and day 4 (96 h after treatment) throughout the experiment. The Infinity3 camera with OLYMPUS CKX41 microscope was utilized to obtain images within its linear dynamic range, which ensures a consistent relationship between signal intensity and the amount of material present. The migration distance of fibroblasts (µm), indicating their wound-closing ability (Beegum et al., 2022), was measured throughout the experiment. Image analysis was conducted using ImageJ (IJ 1.46r) software, and all samples were analyzed in triplicate.

### 2.3.8. qRT-PCR

RNA was extracted from fibroblast monolayer cultures using TRIzol® Reagent (Invitrogen, Carlsbad, CA), followed by purification with the RNeasy kit (Qiagen, Toronto, ON, Canada) as per the manufacturer's protocol. RNA concentration was measured using a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, Wilmington, DE). A fraction of the total RNA was employed to synthesize complementary DNA (cDNA), utilizing 1 µg of total RNA and a specialized cDNA synthesis kit known as iScript™ Reverse Transcription Supermix (Cat:

1708841; BioRad Laboratories, Saint-Laurent, QC, Canada). The resulting cDNA product was then employed as a template for q-PCR, with 1 µl of cDNA being utilized in each reaction. The qRT-PCR reactions were carried out using a SsAdvancedTM Universal Inhibitor-Tolerant SYBR Green Supermix (Cat: 1725017; BioRad Laboratories, Saint-Laurent, QC, Canada). The primers required for the q-RT-PCR analysis were designed employing an online IDT software (Table 1). Beta-actin was used as the reference gene for normalization in the experiment. Each group included three biological replicates, with three technical replicates per sample.

**Table 1. Primers Used in the Experiments.**

| **Gene** | **Primer Sequence** |
|---|---|
| *Col3a1* | |
| H-F-Col3a1 | F: 5` AAGTCAAGGAGAAAGTGGTCG 3` |
| H-R-Col3a1 | R: 5` CTCGTTCTCCATTCTTACCAGG 3` |
| *Col1a1* | |
| H-F-Col1a1 | F: 5` CCCCTGGAAAGAATGGAGATG 3` |
| H-R-Col1a1 | R: 5` TCCAAACCACTGAAACCTCTG 3` |
| *Elastin* | |
| H-F-Eln | F: 5` GGCTTTGGTGTCGGAGTC 3` |
| H-R-Eln | R: 5` AACTAACCCGTACTTGGCAG 3` |
| *b-Actin* | |
| Forward | F: 5` GGCATCCTCACCCTGAAGTA 3` |
| Reverse | R: 5` CACACGCAGCTCATTGTAGAAG 3` |
| *IL-6* | |

| | |
|---|---|
| Forward | F: 5` GGAGACTTGCCTGGTGAAA 3` |
| Reverse | R: 5` CTGGCTTGTTCCTCACTACTC 3` |
| *COX-2* | |
| Forward | F: 5` TACTGGAAGCCAAGCACTTT 3` |
| Revers | R: 5` GGACAGCCCTTCACGTTATT 3` |
| *TNF-alpha* | |
| Forward | F: 5` CCAGGGACCTCTCTCTAATCA 3` |
| Reverse | R: 5` TCAGCTTGAGGGTTTGCTAC 3` |
| *IL-1b* | |
| Forward | F: 5` CCTTAGGGTAGTGCTAAGAGGA 3` |
| Reverse | R: 5` AAGTGAGTAGGAGAGGTGAGAG 3` |

### 2.3.9. Statistics

The data were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's test for comparing mean values. GraphPad Prism 6 software version 9.5.1 (GraphPad Software, San Diego, CA, USA) was used to perform the analysis and prepare the graphics.

## 2.4. Results

### 2.4.1. Psilocybin Increases Cellular Viability in Untreated and HGHL-Treated Samples

A pilot study was conducted to assess cell viability across psilocybin concentrations ranging from 5 to 20 μM. The findings revealed that concentrations between 5 and 15 μM enhanced cell viability, whereas 20 μM decreased it (Supplementary Figure 1). The effects became clear already on the 2$^{nd}$ day of culturing.

In the co-treatment setup, HGHL was induced simultaneously with varying concentrations of psilocybin, and the exposure time for HGHL was optimized to two days. Therefore, co-treatment was also applied for two days. In the post-treatment setup, HGHL was first induced for the optimized duration of two days. After that, psilocybin was applied for an additional two days to evaluate its curative effects following HGHL exposure.

In the main experiment, we tested psilocybin's effect on cell viability in concentrations ranging from 1 to 15 µM. Analysis showed that exposure of untreated fibroblasts to P15 resulted in a significant increase in cell viability in both post-treatment and co-treatment experiments (Figure 1A, B) compared to CT. Thus, P15 was used as a control (CT+P15) in all subsequent experiments.

In the post-treatment experiment, P15 appeared to be the most effective concentration among other concentrations increasing the cell viability altered by HGHL (Figure 1A) compared to HGHL/M, while in the co-treatment experiment, P10 was the best (Figure 1B). Thus, for all further experiments, the post-treatment experiments were done with P15, while co-treatment was with P10.

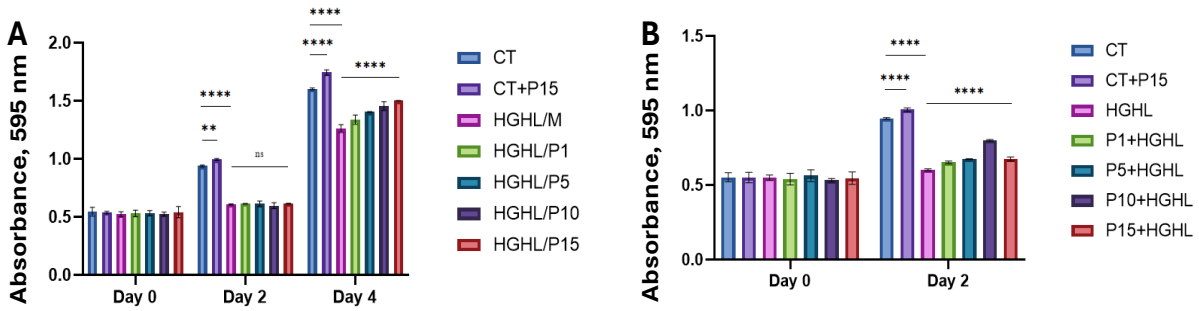



**Figure 1. Effects of different concentrations of psilocybin on the cell viability in post-treatment (A) and co-treatment (B) of HGHL-induced fibroblasts.** Data shows the cell viability (MTT assay); Y axis shows absorbance at 595 nm of fibroblasts measured at days 0, 2 and 4 in post HGHL treatment (A), and days 0 and day 2 in co-treatment of psilocybin and HGHL (B). CT - Control (untreated fibroblasts); CT+P15 - psilocybin 15 µM added to untreated

fibroblasts; P1, P5, P10, P15 - psilocybin at 1, 5, 10, 15 µM; HGHL - high glucose (25 mM) and high lipid (400 µM) treatment; HGHL/M – media added to HGHL treatment sample; HGHL/P1, P5, P10, P15 - psilocybin at various doses added post HGHL induction; P1, P5, P10, P15+HGHL- psilocybin at different doses added together with HGHL as a co-treatment. Data are presented as mean ± SD. N=3. The collected data were subjected to statistical analysis using one-way analysis of variance (ANOVA), followed by Dunnett's test for comparing mean values. GraphPad Prism 6 software was used to perform this analysis. Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns - not significant.

### 2.4.2. Psilocybin Reduces β-Galactosidase Activity

β-galactosidase levels increase with aging, reflecting cellular senescence. Analysis showed the highest β-galactosidase activity in an HGHL-treated sample in co-treatment (Figure 2B), and in an HGHL-treated sample followed by the addition of culture media in post-treatment (Figure 2A). β-galactosidase activity of other samples was significantly lower than HGHL-treated samples in both co- and post-treatments. This experiment showed that psilocybin is able to prevent the increase in cellular senescence in response to HGHL in the co-treatment experiment (Figure 2B) as well as decreases the cellular senescence caused by HGHL in the post-treatment experiment (Figure 2A).

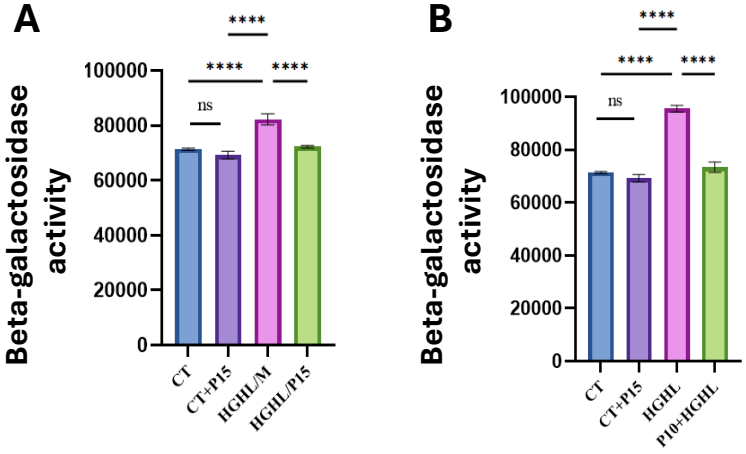



**Figure 2. Effects of psilocybin on β-galactosidase activity in fibroblasts treated with HGHL.** Data shows the β-galactosidase activity (measured in fluorescence intensity, arbitrary units, Y axis) after HGHL treatment in post- (A) and co-treatment experiments (B) with psilocybin. CT - Control (untreated fibroblasts); CT+P15 - psilocybin 15 µM added to untreated fibroblasts; HGHL - high glucose (25 mM) and high lipid (400 µM) treatment; HGHL/M – media added to HGHL treatment sample; HGHL/P15 - 15 µM psilocybin added post HGHL induction;

P10+HGHL - 10 µM psilocybin added together with HGHL as a co-treatment. Data are presented as mean ± SD. N=3. Statistical analysis was performed using one-way ANOVA, followed by Dunnett's test for mean comparisons, with GraphPad Prism 6 software. Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns - not significant.

### 2.4.3. Psilocybin Reduces Apoptosis in HGHL-Treated Fibroblasts

The apoptosis test is designed to detect and measure the occurrence of apoptosis in fibroblasts, providing important insights into cellular health, treatment effects, or disease progression. The primary goal of this experiment was to assess the extent and rate at which fibroblasts undergo apoptosis. For both co- and post-treatment with psilocybin, the results showed that HGHL and HGHL/Media significantly incresed the number of apoptotic cells compared to the CT (Figure 3). Post-treatment with P15 did not decrease the apoptosis compared to HGHL/M (Figure 3A), while co-treatment with P10 decreased it slightly but significantly compared to HGHL(Figure 3B).

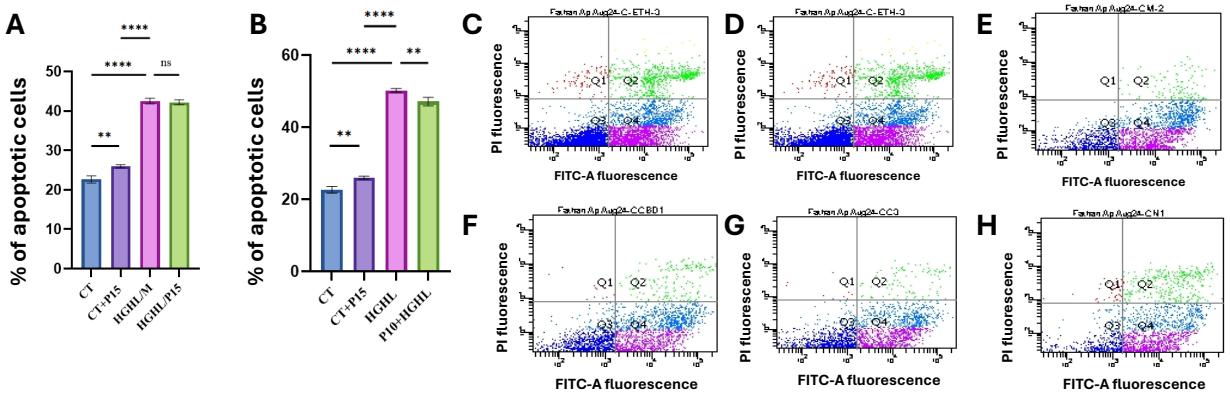



**Figure 3. Effects of psilocybin on apoptosis in untreated and HGHL-treated fibroblasts.** Data shows the percentage of apoptotic cells after HGHL treatment in post- (A) and co-treatment (B) experiments with psilocybin. Representative images of apoptosis in CT (C), CT+P15 (D), HGHL (E), HGHL/M (F), HGHL/P15 (G), P10+HGHL (H). CT - control (untreated fibroblasts); CT+P15 - psilocybin 15 µM added to untreated fibroblasts; HGHL - high glucose (25 mM) and high lipid (400 µM) treatment; HGHL/M – media added to HGHL treatment sample; HGHL/P15 - 15 µM psilocybin added post HGHL induction; P10+HGHL - 10 µM psilocybin added together with HGHL as a co-treatment. Data are presented as mean ± SD. N=3. Statistical analysis was performed using one-way ANOVA, followed by Dunnett's test for mean comparisons, with GraphPad Prism 6 software. In panels C to H, the X-axis represents FITC-A (Annexin V–FITC fluorescence, indicating phosphatidylserine exposure), and the Y-axis represents Propidium Iodide (PI) fluorescence, which measures membrane permeability associated with late apoptosis or necrosis. Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns - not significant.

### 2.4.4. Psilocybin Reduces Stress-Induced Cell Cycle Disruption in HGHL-Treated Fibroblasts

In the cell cycle assay, fibroblasts were categorized based on DNA content into different phases: G0/G1 (resting or initial growth phase), S (DNA synthesis phase), and G2/M (second growth and mitosis phase), allowing for an assessment of the fibroblast population's distribution across the various stages of the cell cycle. Both during co-treatment and post-treatment, HGHL and HGHL/M induced significant cellular stress, shifting many fibroblasts into the S phase and disrupting normal cell cycle progression (Figure 4).

When treated with P15 alone, there were no significant changes in the cell cycle compared to CT conditions, with the distribution of fibroblasts being similar to that of the untreated control (Figure 4A, B). In contrast, post-treatment with psilocybin significantly alleviated the stress induced by the HGHL environment, compared to media added after HGHL induction, maintaining a higher proportion of fibroblasts in the G1 phase and reducing the number of fibroblasts in the S phase (Figure 4A).

Co-treatment with psilocybin appeared to reverse the HGHL stress effects entirely, restoring the cell cycle distribution to a near-normal state (Figure 4B). Psilocybin exhibited protective and restorative effects on the cell cycle under HGHL conditions, showing its potential to mitigate stress-induced disruptions in cellular function, with the most significant impact observed during co-treatment.

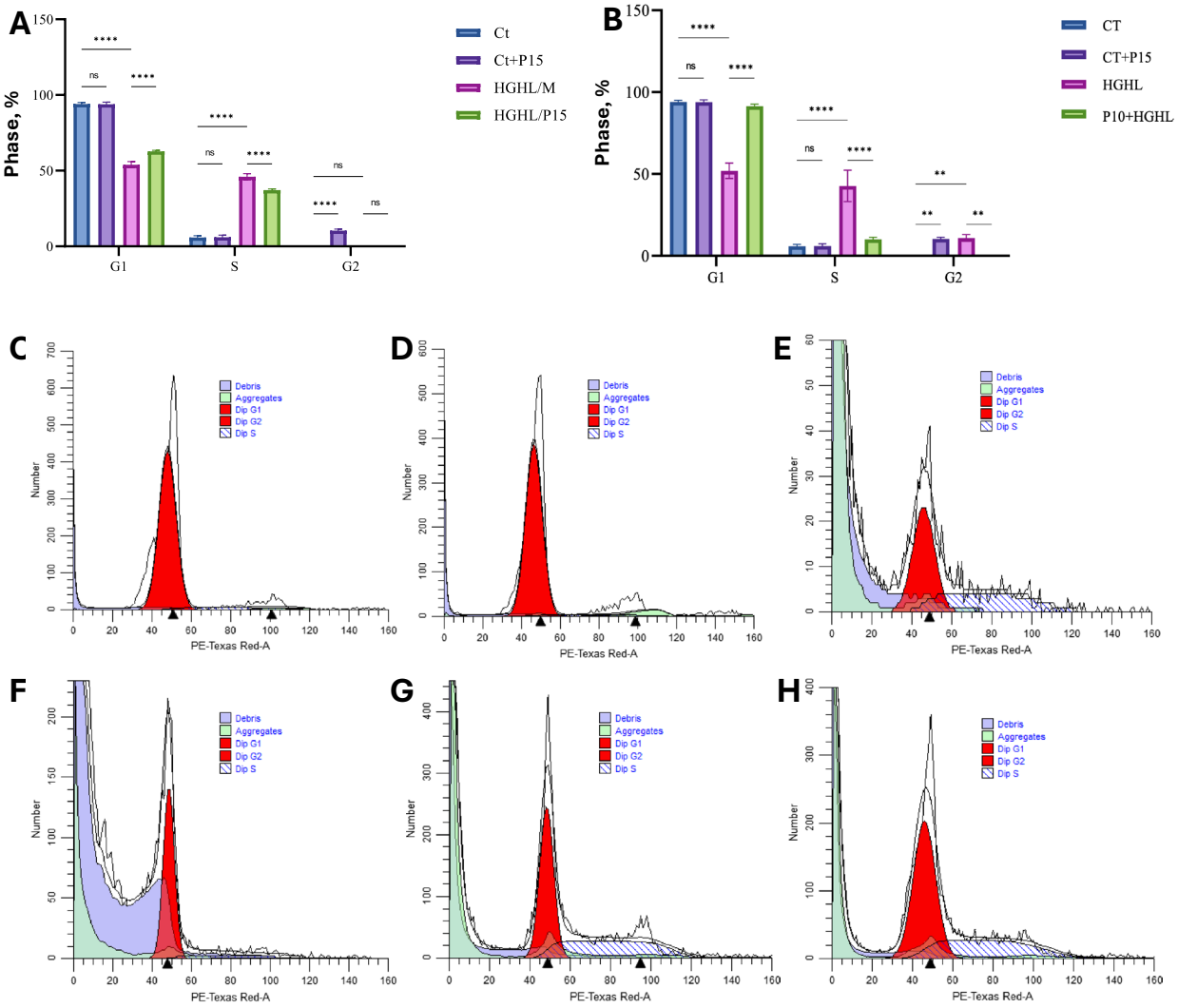


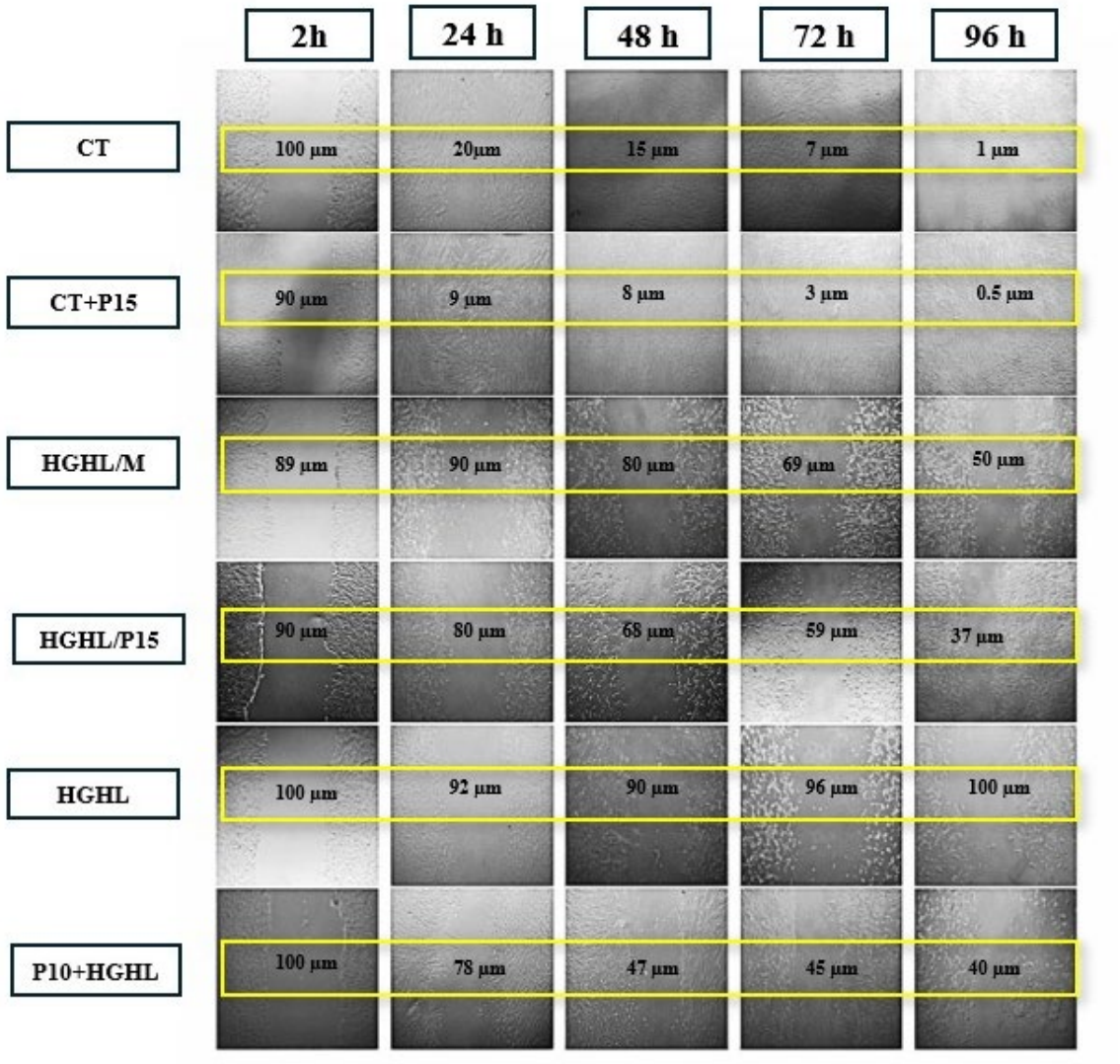

**Figure 4. Impact of psilocybin on the cell cycle in untreated and HGHL-treated fibroblasts.** Data shows the percentage of cells in specific cell cycle phase after HGHL treatment in post- (A) and co-treatment (B) experiments with psilocybin. Representative images of cell cycle in CT (C), CT+P15 (D), HGHL (E), HGHL/M (F), HGHL/P15 (G), P10+HGHL (H). CT - control (untreated fibroblasts); CT+P15 – psilocybin 15 µM added to untreated fibroblasts; HGHL - high glucose (25 mM) and high lipid (400 µM) treatment; HGHL/M – media added to HGHL treatment sample; HGHL/P15 - 15 µM psilocybin added post HGHL induction; P10+HGHL - 10 µM psilocybin added together with HGHL as a co-treatment. Data are presented as mean ± SD. N=3. Statistical analysis was conducted using one-way ANOVA, followed by Dunnett's test for mean comparisons, using GraphPad Prism 6 software. Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns - not significant.

### 2.4.5. Psilocybin Does Not Significantly Improve the Wound Healing

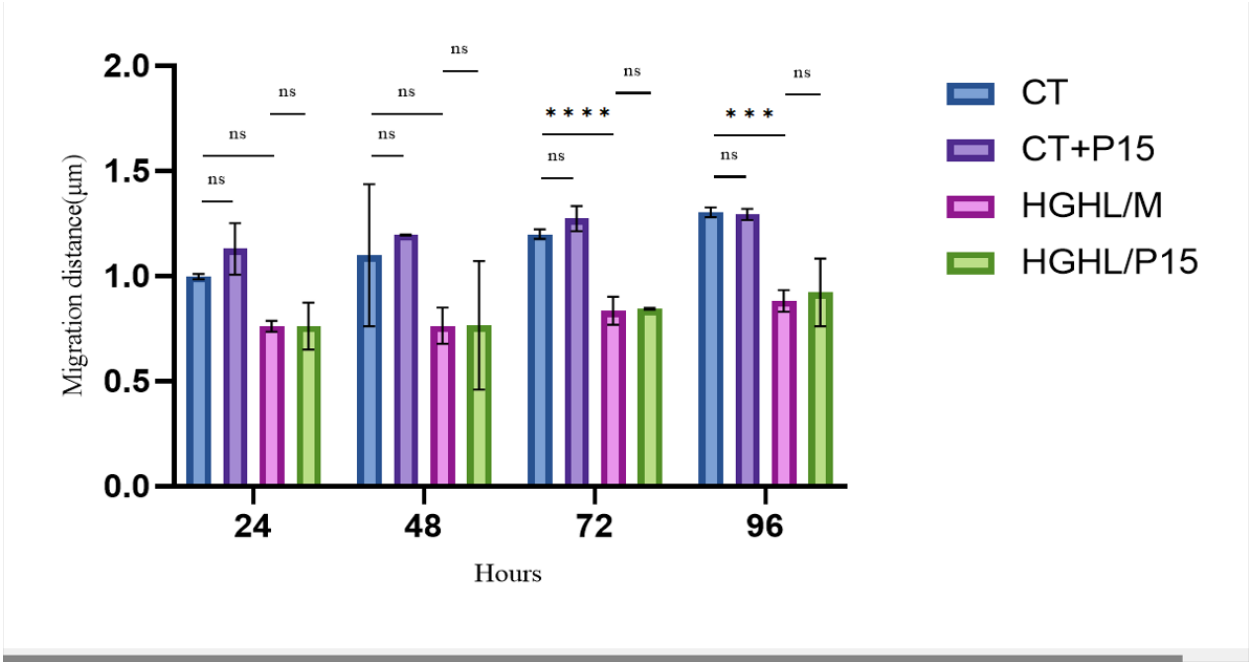
The wound healing assay is a widely used method to evaluate the anti-aging properties of various compounds by assessing cell proliferation and migration (Beegum et al., 2022). We found that P15

applied to untreated fibroblasts showed the tendency to improve wound healing as compared to the CT, but the differences were not significant (Figure 5). Exposure to HGHL significantly decreased wound healing as compared to the CT group, and the addition of media (HGHL/M group) did not significantly improve it (Figure 5). Co-treatment with P10 and HGHL showed improvement of wound healing comparing to HGHL, although the differences were not significant. Post-treatment with P15 result in minimal and statistically non-significant improvement of wound healing compared to HGHL/M on day 4.

*A)*



*B)*




*C)*

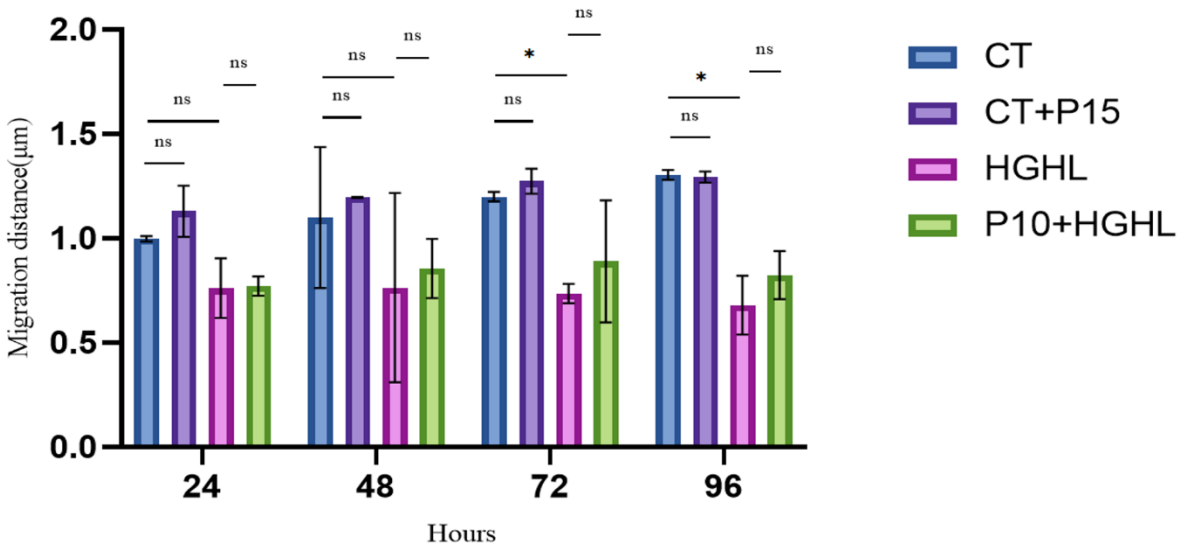



**Figure 5. Effects of psilocybin on wound healing progress in untreated and HGHL-treated fibroblasts.** A. Microscope images depict the wound healing process in untreated and HGHL-treated fibroblasts under various treatment conditions. For post-treatments, HGHL was applied 2 hours after wound creation and continued for 48 hours. After 48 hours, HGHL was removed, fibroblasts were washed with 1X PBS, and treatments with P15 and media were applied for an additional 48 hours (HGHL/P15). Additional conditions included: untreated fibroblasts treated

with P15 (CT+P15), where P15 was added 2 hours after wound creation; fibroblasts co-treated with HGHL and P10 simultaneously 2 hours post-wound creation (P10+HGHL); fibroblasts treated with HGHL alone 2 hours after wound creation (HGHL), and CT. Images were taken at specific intervals: 2 hours post-wound creation, and at 24-, 48-, 72- and 96-hours following treatment. Wound closure was quantified using ImageJ software. The 2-hour images for each well were intended to be consistent; however, manual processing with 100-µL pipette tips introduced variability. Thus, images at subsequent time points were normalized using their respective 2-hour images as references. B. Quantitative analysis of wound healing progression in HGHL-induced fibroblasts post-treated with P15. C. Quantitative analysis of wound healing progression in fibroblasts co-treated with HGHL and P10. Data are shown as mean ± SD. N=3. Statistical significance was determined using one-way ANOVA, followed by Dunnett's post hoc test in GraphPad Prism 6 software. Significance levels: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; ns - not significant.

### 2.4.6. Protective Effects of Psilocybin on the Expression of Genes Involved in ECM Maintenance and Inflammatory Response

Fibroblasts are key producers of essential extracellular matrix components—such as collagen, elastin, and hyaluronan—that are critical for maintaining the structure and function of the skin as well as other tissues and organs throughout the body. Additionally, inflammatory cytokines play a crucial role in skin health, as they regulate collagen synthesis and influence the skin's ability to repair and renew, highlighting the importance of assessing these factors in studies of cellular aging and tissue resilience.

Here, we first tested the expression of two collagen encoding genes, *Collagen type I alpha 1* (*COL1A1*), *Collagen type III alpha 1* (*COL3A1*) and *elastin* (*ELN*) genes. Exposure to P15 of untreated fibroblasts (CT+P15) significantly decreased the expression of *COL1A1* and *ELN* genes (Figure 6). It did not significantly change *COL3A1* expression, although there was a trend toward an increase in expression. HGHL treatment decreased the expression of all three genes in both co- and post-treatments. Addition of P15 in post-treatment did not change the expression of *COL1A1* and *COL3A1* genes but further downregulated the *ELN* gene (Figure 6A, C, E). Notably, P10 co-

treatment with HGHL did not significantly increase *COL1A1* or *COL3A1* expression compared to HGHL alone but did result in a significant increase in *ELN* expression (Figure 6B, D, F).

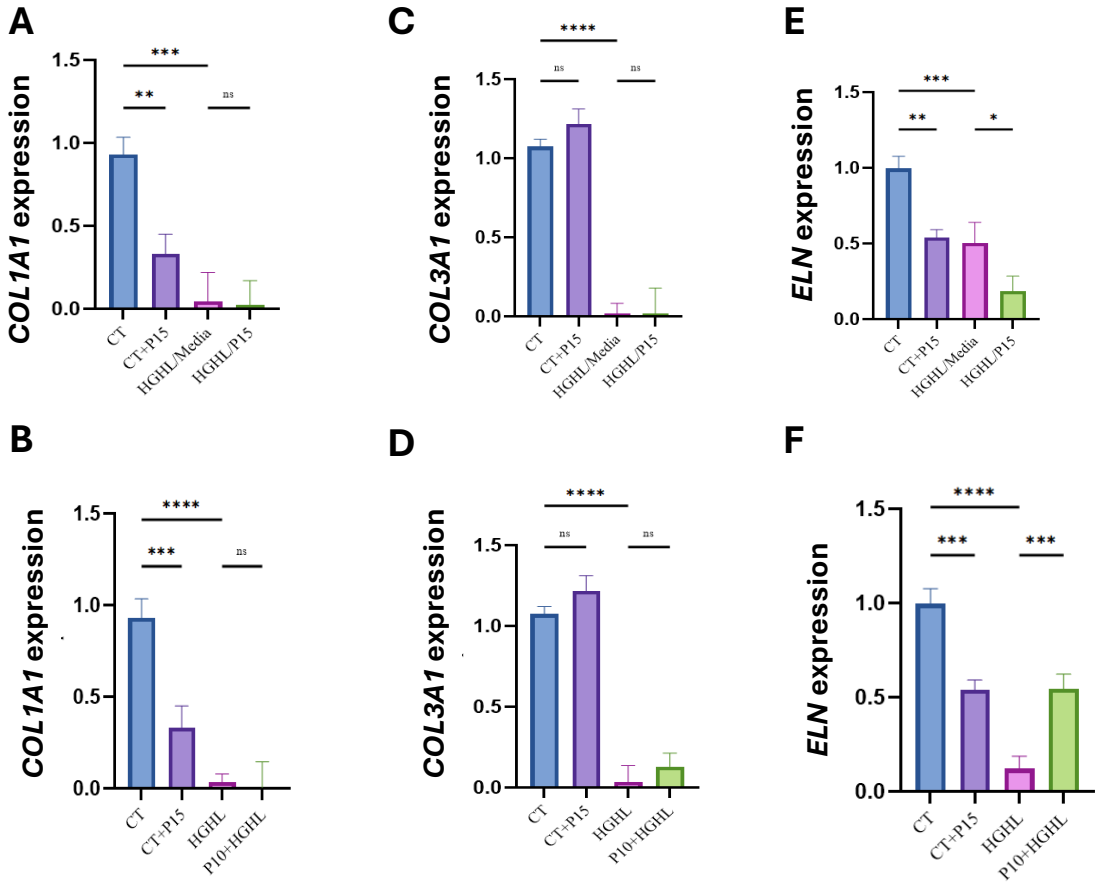



**Figure 6. Effect of psilocybin on the expression of *COL1A1*, *COL3A1* and *ELN* genes in untreated and HGHL-treated fibroblasts.** *COL1A1* expression in post-treatment (A) and co-treatment (B). *COL3A1* expression in post-treatment (C) and co-treatment (D). *ELN* expression in post-treatment (E) and co-treatment (F). CT - control (untreated fibroblasts); CT+P15 – psilocybin 15 µM added to untreated fibroblasts; HGHL - high glucose (25 mM) and high lipid (400 µM) treatment; HGHL/M – media added to HGHL treatment sample; HGHL/P15 - 15 µM psilocybin added post HGHL induction; P10+HGHL - 10 µM psilocybin added together with HGHL as a co-treatment. Data are presented as mean ± SD. N=3. Statistical analysis was performed using one-way ANOVA, followed by Dunnett's test for mean comparisons, using GraphPad Prism 6 software. Significance indicators: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns - not significant.

In the analysis of inflammatory cytokine gene expression, the addition of P15 to untreated fibroblasts significantly decreased the expression of *IL-1β*, *IL-6*, and *COX-2* genes while increased the expression of *TNF-α* gene (Figure 7). Treatment with HGHL increased the expression of all

tested genes in both, co- and post-treatment experiments. In the post-treatment experiment, the addition of P15 after HGHL treatment significantly reduced the expression of all genes but *TNF-α* (Figure 7A, C, E, G). In the co-treatment experiment, P10 reduced the expression of all genes except *IL-6*, as compared to HGHL alone treatment (Figure 7B, D, F, H).

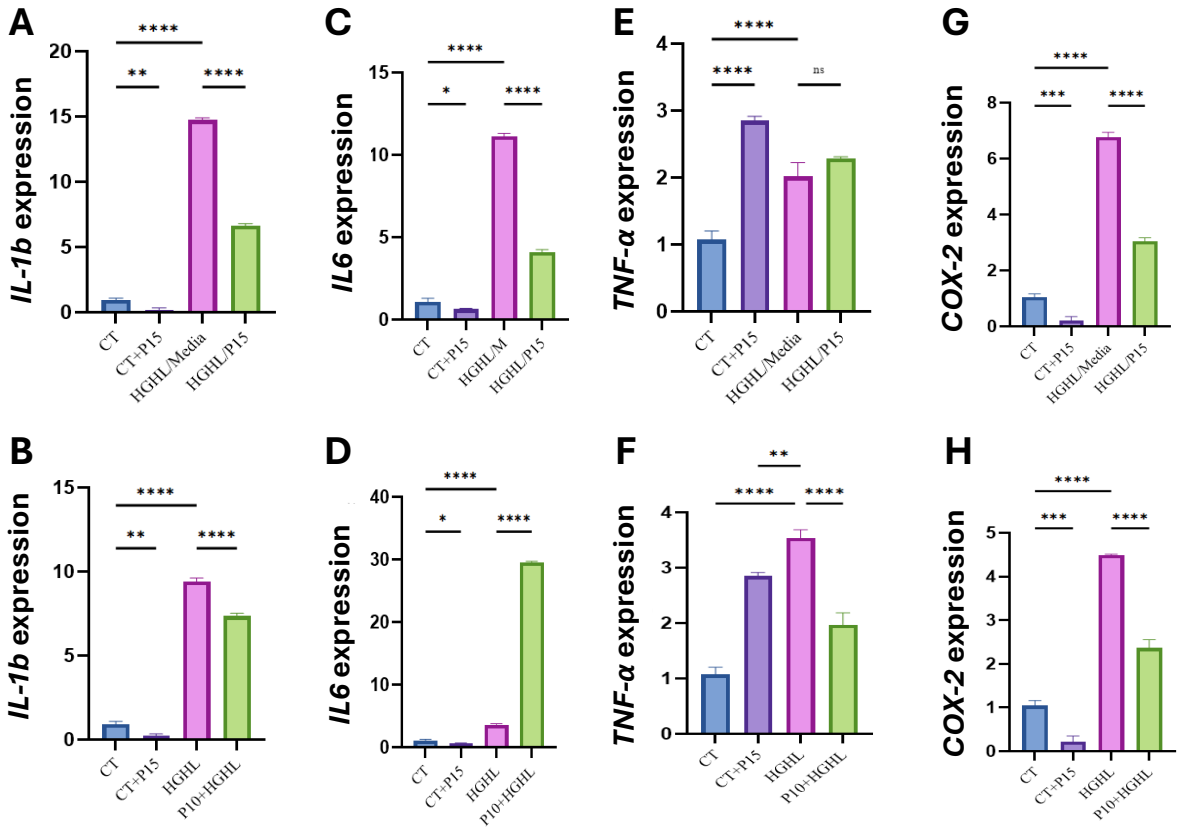



**Figure 7. Effect of psilocybin on the expression of inflammatory cytokines in untreated and HGHL-treated fibroblasts.** *IL-1β* expression in the post-treatment (A) and co-treatment (B). *IL-6* expression in the post-treatment (C) and co-treatment (D). *TNF-α* expression in the post-treatment (E) and co-treatment (F). *COX-2* expression in post-treatment (G) and co-treatment (H). CT - control (untreated fibroblasts); CT+P15 – psilocybin 15 µM added to untreated fibroblasts; HGHL - high glucose (25 mM) and high lipid (400 µM) treatment; HGHL/M – media added to HGHL treatment sample; HGHL/P15 - 15 µM psilocybin added post HGHL induction; P10+HGHL - 10 µM psilocybin added together with HGHL as a co-treatment. Data are presented as mean ± SD. N=3. Statistical analysis was performed using one-way ANOVA, followed by Dunnett's test for mean comparisons, using GraphPad Prism 6 software. Significance indicators: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns - not significant.

## 2.5. Discussion

In this study, we developed an HGHL-based model of skin aging using fibroblasts to investigate the anti-aging properties of psilocybin. By simulating HGHL conditions, we aimed to replicate the metabolic stress associated with aging, uncovering its impact on fibroblast viability, ECM integrity, inflammation, and cell cycle dynamics. Our results revealed significant oxidative stress, elevated ROS levels, and mitochondrial dysfunction, all of which contributed to apoptosis (Figure 3), cellular senescence (Figures 2 and 4), cell viability (Figures 1 and Supplementary 1), inflammation (Figure 7), impaired ECM integrity (Figure 6), and reduced wound healing development (Figure 5). These findings align with earlier studies demonstrating that prolonged exposure to high glucose accelerates fibroblast aging through glycation, glycoxidation, mitochondrial dysfunction, and chronic inflammation (Wang and Dreesen, 2018; Jiang et al., 2022).

While various *in vitro* models have been developed to study skin aging, most have focused on oxidative stress, UV exposure, $H_2O_2$-induced damage, and glycation-induced damage. However, the combined effects of HGHL conditions on fibroblast aging remain relatively underexplored. Previous research primarily examined high glucose-induced cellular damage in vascular endothelial and mesothelial cells (Książek, 2013; Lipsky and King, 2015), but studies specifically addressing dermal fibroblasts under HGHL conditions are limited.

### 2.5.1. Effect of HGHL and Psilocybin on Cell Viability and Senescence

We tested the ability of psilocybin to alleviate aging-related changes by inducing HGHL conditions in fibroblasts through both co-treatment and post-treatment experiments. Building on the findings of Ghasemi Gojani et al. (2024), which demonstrated that induction with high glucose

(25 mM) and high lipid (palmitic acid, 400 μM) reduces beta-cell viability, promotes apoptotic behavior, and exacerbates aging-related effects (Gojani et al., 2024; Ghasemi Gojani et al., 2024), our study focused on optimizing these concentrations for skin fibroblasts. This finding is consistent with prior research showing that chronic metabolic stress reduces fibroblast survival by increasing mitochondrial ROS production and impairing ATP synthesis (Cialdai et al., 2022).

In their experiment with beta cells, Ghasemi Gojani et al. (2024) used a 10 μM concentration of psilocybin as a treatment. We wanted to test a broader range of concentrations, with the goal of determining the safest and most effective psilocybin concentrations for skin fibroblasts. The pilot study tested concentrations ranging from 5 to 20 μM. The results showed that 5 to 15 μM improved cell viability without inducing toxicity, while 20 μM caused significant toxic effects, reducing cell viability compared to the controls. These safe concentrations were further refined through MTT assays, which helped identify the optimal doses for co-treatment (10 μM) and post-treatment (15 μM) applications (Figures 1 and Supplementary 1).

Previous studies have demonstrated that psilocybin exhibits minimal toxicity in normal cells while promoting neuroplasticity and cellular resilience (Johnson et al., 2017; Smedfors et al., 2022). In our study, we confirmed that psilocybin did not adversely affect untreated fibroblasts, supporting its safety profile in non-stressed conditions. This contrasts with other psychoactive compounds, such as ketamine and MDMA, which have been reported to induce oxidative stress and mitochondrial damage (Weinstein, 2023).

Recent studies have demonstrated that psilocybin exerts protective effects against cellular stressors by reducing ROS accumulation, enhancing mitochondrial function, and mitigating cellular senescence. For instance, research has shown that psilocybin treatment leads to a dose-dependent decrease in oxidative stress levels and cell-cycle arrest markers, while increasing markers of DNA

replication and proliferation, thereby decelerating cellular senescence (Hecker et al., 2023). Additionally, psilocybin has been observed to induce rapid and sustained growth of dendritic spines in neuronal cells, suggesting enhanced mitochondrial efficiency and stress-related gene expression modulation (Shao et al., 2021). Furthermore, studies propose that serotonergic psychedelics like psilocybin may induce transdiagnostic effects by modulating mitochondrial function, indicating a potential subcellular mechanism underlying their therapeutic benefits (Fissler et al., 2023). Notably, psilocybin's ability to regulate key molecular pathways involved in cellular longevity, such as FOXO transcription factors, suggests its potential role in enhancing fibroblast resilience against metabolic stress (Gallezot et al., 2011).

Cellular senescence, a key feature of aging marked by irreversible cell cycle arrest (Kumari and Jat, 2021), was assessed through beta-galactosidase activity. HGHL conditions significantly elevated beta-galactosidase levels, indicating increased senescence. We observed a marked increase in s β-galactosidase (SA-β-gal) activity in HGHL-treated fibroblasts (Figure 2), reinforcing the strong link between metabolic stress and premature cellular death. Our findings align with a study on human periodontal ligament fibroblasts (PDLFs), where culturing cells in high-glucose medium (50 mM) led to decreased viability and impaired wound healing (Aldoss et al., 2023). Psilocybin treatment, with P10 in co-treatment and P15 in post-treatment, notably reduced beta-galactosidase activity compared to HGHL and HGHL/M. Additionally, P15 on untreated fibroblasts non-significantly alleviated enzyme levels comparing to CT (Figure 2).

### 2.5.2. Effect of HGHL and Psilocybin on the Expression of ECM Components

In our study, psilocybin displayed differential effects on key ECM components, such as *COL1A*, *COL3A*, and elastin (Figure 6), which are vital for maintaining skin structural integrity. Fibroblasts, responsible for collagen and elastin production, support skin elasticity and resilience. Under

HGHL conditions, the expression of *COL1A* and *elastin* was significantly reduced compared to untreated fibroblasts, indicating that HGHL treatment impairs ECM synthesis. However, psilocybin treatment, particularly with P10, led to an increase in the expression of *elastin*, suggesting that psilocybin may help preserve skin structure and function under stress. P10 co-treatment significantly increased elastin expression, compared to HGHL (Figure 6).

Our data highlight the central role of mitochondrial dysfunction and oxidative stress in ECM degradation under HGHL conditions. Elevated ROS levels activate the MAPK pathway, leading to the upregulation of MMP-2 and MMP-9, which degrade collagen and elastin, contributing to skin sagging and wrinkles (Ahuja and Bajpai, 2024). Additionally, disruption of the TGF-β/Smad pathway impairs collagen synthesis, further accelerating dermal deterioration. These effects are compounded by the senescence-associated secretory phenotype (SASP), in which fibroblasts release pro-inflammatory cytokines and proteolytic enzymes, creating a pro-degradative microenvironment (Nikolakis et al., 2016; Wang and Dreesen, 2018). Furthermore, high glucose promotes excessive ECM protein synthesis, leading to tissue fibrosis and rigidity due to AGE cross-linking (Gui et al., 2024), while high lipid levels disrupt MMP activity and impair ECM remodeling (Lee et al., 2021).

While direct studies on psilocybin's effects on collagen synthesis in fibroblasts are limited, existing research suggests that serotonin receptor activation plays a crucial role in modulating fibroblast behavior and ECM remodeling. Serotonin has been shown to stimulate DNA synthesis in fibroblasts through 5-HT1B receptors linked to Gi-protein signaling, promoting cellular proliferation and repair (Tarbit, 2019). Additionally, serotonin enhances type IV collagen production in human mesangial cells via 5-HT2A receptor activation, highlighting its potential influence on ECM integrity (Barrett, 2017). These findings of non-significant improvment of

*COL1A* and *COL3A* gene exprsion under the stress and significant gene expresion of *ELN in co-tretment* comparing to HGHL (Figure 6), support the hypothesis that psilocybin, a serotonin receptor agonist, may contribute to ECM repair by upregulating collagen synthesis, aligning with its broader effects on cellular resilience and stress adaptation.

The metabolic consequences of an HGHL diet extend beyond glycation and oxidative stress. Elevated lipid levels contribute to dysregulated sebaceous gland activity, leading to imbalances in skin hydration and increased susceptibility to inflammation (Passeron et al., 2020; Choi, 2021). The interplay between lipid metabolism and skin aging is particularly evident when excessive lipid accumulation disrupts the epidermal barrier function, promoting trans-epidermal water loss and resulting in a dry, fragile skin phenotype. Additionally, lipid peroxidation products generated during oxidative stress impair keratinocyte and dermal fibroblast function and contribute to premature aging by reducing the gene expresion and synthesis of collagen and elastin fibers (Figure 6).

### 2.5.3. Effect of HGHL and Psilocybin on the Expression of Proinflammatory Cytokines

Psilocybin's influence on inflammatory pathways supports its potential as an anti-aging agent. Building on prior research, such as the study by Nkadimeng et al. (2021), which investigated the anti-inflammatory properties of four psilocybin-containing mushrooms on LPS-induced inflammation in human U937 macrophage fibroblasts, our study further examined inflammatory cytokine gene expression. Nkadimeng et al. (2021) explored the effects of psilocybin-containing mushrooms on inflammatory mediators, including TNF-α, IL-6, COX-2, and 15-LOX, as well as the regulation of anti-inflammatory cytokines like IL-10, which are linked to chronic diseases. Extracts effectively decreased pro-inflammatory cytokines while enhancing anti-inflammatory cytokines such as IL-10. Although its anti-inflammatory effects are minimal in healthy individuals,

they are significantly amplified in clinical populations, emphasizing its therapeutic importance (Nichols, 2020; Burmester et al., 2023).

Nkadimeng et al. (2021) findings also demonstrated a significant, dose-dependent reduction in nitric oxide, prostaglandin E2, and interleukin-1β production, comparable to positive controls. Phytochemical analysis confirmed the presence of antioxidant and anti-inflammatory compounds, indicating the safety and effectiveness of the mushroom extracts in mitigating inflammation (Nkadimeng et al., 2020; Nkadimeng et al., 2021). Furthermore, psilocybin facilitates cellular repair by modulating key transcription factors such as NF-κB, STAT3, and STAT1 (Ghasemi Gojani, 2023). In alignment with these results, our study investigated inflammatory cytokine gene expression using qPCR, providing additional support for these earlier findings.

Inflammatory cytokines such as TNF-α, IL-6, COX-2, and IL-1β play a critical role in aging by promoting ECM degradation and cellular senescence (Li et al., 2023). Our results demonstrated that psilocybin reduced the expression of some pro-inflammatory cytokines, particularly *IL-1β* and *IL-6*, in untreated fibroblasts and mitigated their upregulation in HGHL-induced conditions. Specifically, P15 reduced *IL-1β* and *IL-6* expression in untreated fibroblasts and post-treatment conditions (Figure 7A, C). Additionally, co-treatment with P10 significantly alleviated *IL-1β* levels, whereas P10 significantly increased *IL-6* gene expression comparing to HGHL in co-treatment condition (Figure 7B, 7D). This anti-inflammatory effect is vital for maintaining ECM integrity, as chronic inflammation is a hallmark of aging and a key driver of collagen degradation and ECM remodeling. Psilocybin's ability to modulate these inflammatory cytokines suggests its potential in counteracting the inflammatory response associated with skin aging and cellular senescence (Figure 7).

Interestingly, psilocybin's effects on *TNF-α* expression were somewhat unexpected. While P15 treatment in untreated fibroblasts led to a significant increase in *TNF-α* expression, it did not reduce *TNF-α* levels in HGHL-induced fibroblasts under post-treatment conditions. However, P10 in co-treatment alleviated *TNF-α* expression compared to HGHL-induced fibroblasts, suggesting that psilocybin's impact on cytokine expression may vary depending on the cellular context (Figure 7E, 7F).

Psilocybin exhibited a mitigating effect on the inflammatory marker *COX-2*. P15 treatment significantly reduced *COX-2* expression in untreated fibroblasts and in post-treated HGHL-induced fibroblasts, while P10 in co-treatment with HGHL also reduced *COX-2* expression compared to HGHL-induced fibroblasts. This suggests that psilocybin's anti-inflammatory action is robust, though its effects may vary depending on the context. The dual regulation of pro-inflammatory cytokines and ECM components by psilocybin highlights its potential as a multifaceted therapeutic for skin aging (Figure 7G, 7H).

### 2.5.4. The Effects of HGHL and Psilocybin on Apoptosis

The apoptosis assay showed that psilocybin alone resulted in a small but significant increase in apoptosis. It was not effective in reducing apoptosis triggered by HGHL in post-treatment but was in co-treatment (Figure 3).

Prolonged metabolic stress pushes fibroblasts beyond cell cycle arrest into programmed cell death, further impairing tissue homeostasis. This aligns with studies on human foreskin fibroblasts under high glucose conditions, where elevated glucose levels increased ROS production, activating the MAPK signaling pathway and leading to the upregulation of MMPs, which contribute to ECM degradation and apoptosis (Xuan et al., 2014). Excessive glycation profoundly affects structural

skin proteins, including collagen and elastin, leading to increased dermal stiffness and fragility, as confirmed by our q-PCR analysis (Figure 6). Studies have demonstrated that AGE accumulation impairs wound healing, heightens skin vulnerability to injuries, and contributes to skin laxity—findings that are clearly reflected in our results (Figure 5) (Van Putte et al., 2016).

Ghasemi Gojani et al. (2024) demonstrated that psilocybin administration mitigates HGHL-stimulated β-cell loss, potentially through the modulation of apoptotic biomarkers and the mitigation of TXNIP, STAT-1, and STAT-3 phosphorylation. Our experiments demonstrate that caution needs to be exercised, as psilocybin alone may increase apoptosis. This could be a cell-specific response.

### 2.5.5. The Effects of HGHL and Psilocybin on the Cell Cycle

Beyond ECM degradation, our study reveals significant alterations in fibroblast cell cycle dynamics under HGHL conditions, driven by oxidative stress and metabolic disruption. High glucose and lipid accumulation impair mitochondrial function, reduce ATP production, and induce ER stress, activating the unfolded protein response (UPR) and triggering key cell cycle arrest pathways such as p53/p21 and p16/Rb, leading to senescent cell accumulation (Casas-Martinez et al., 2024). As a result, oxidative stress disrupts cell cycle checkpoints, increasing the proportion of fibroblasts in the S phase and decreasing the proportion in the G1 phase (Figure 4), suggesting heightened replication stress, a hallmark of premature senescence and genomic instability (Avelar, 2023).

Our findings demonstrate that psilocybin treatment helps restore cell cycle balance under HGHL-induced stress. Specifically, P10 co-treatment and P15 post-treatment improved cell cycle progression, with post-treatment significantly reducing HGHL-induced stress by maintaining more

fibroblasts in the G1 phase and decreasing S phase entry. Co-treatment fully reversed these stress effects, restoring the cell cycle to a near-normal state. A reduction in the proportion of cells in the S phase compared to other treatments in a cell cycle assay suggests that the treatment does not disrupt DNA synthesis and promotes cell proliferation. These results suggest that psilocybin plays a protective role in stabilizing cell cycle dynamics under metabolic stress and promotes proliferation. By maintaining DNA integrity and supporting controlled cellular proliferation, psilocybin may contribute to skin repair and regeneration, highlighting its potential as a protective agent against stress-induced premature aging (Figure 4).

Similar findings were observed by Hecker et al. (2023), who investigated psilocybin's impact on replicative senescence using a validated human cell model; their findings showed a dose-dependent reduction in cell-cycle arrest markers, an increase in DNA replication and proliferation markers, and a decrease in oxidative stress and SASP factors. Notably, their study identified psilocybin as a geroprotective (senescence-inhibiting) agent that slowed cellular senescence without exhibiting senolytic (senescence-targeting) activity. Given the central role of senescence and inflammation in age-related diseases, these findings collectively support the potential of psilocybin as a therapeutic strategy for mitigating cellular aging.

### 2.5.6. Other Effects of Psilocybin and Potential Mechanisms

Psilocybin's effects extend beyond its influence on inflammatory markers, as it primarily interacts with serotonin 5-$HT_2A$ receptors, exerting potent anti-inflammatory and antioxidant effects. Studies indicate that it reduces inflammation-induced oxidative stress and modulates immune responses at sub-hallucinogenic doses (Flanagan and Nichols, 2018; Ly et al., 2018).

Psilocybin, known for its neuroregenerative and anti-inflammatory properties, may also benefit skin health by modulating oxidative stress pathways and enhancing cellular resilience via serotonin receptors, particularly 5-HT2A (Dewhirst, 2023; Rahman et al., 2024). While its effects on fibroblasts and ECM integrity remain unclear, its role in neuroplasticity and systemic stress resilience may indirectly support skin homeostasis and combat aging (Smedfors et al., 2022).

Compared to other anti-aging compounds such as metformin and vitamin D, psilocybin presents a distinct therapeutic profile. While metformin primarily targets AMPK pathways to enhance mitochondrial function, psilocybin appears to exert broader regulatory effects through serotonin signaling, influencing both metabolic and inflammatory pathways (He et al., 2023; Shin et al., 2023). Additionally, psilocybin has been shown to modulate epigenetic markers associated with longevity and stress resistance, distinguishing it from other interventions (Meloni, 2024). Unlike synthetic antioxidants, psilocybin's ability to modulate mitochondrial biogenesis via serotonin receptor activation represents a novel mechanism in skin aging research (Larrea et al., 2024).

### 2.6. Conclusions

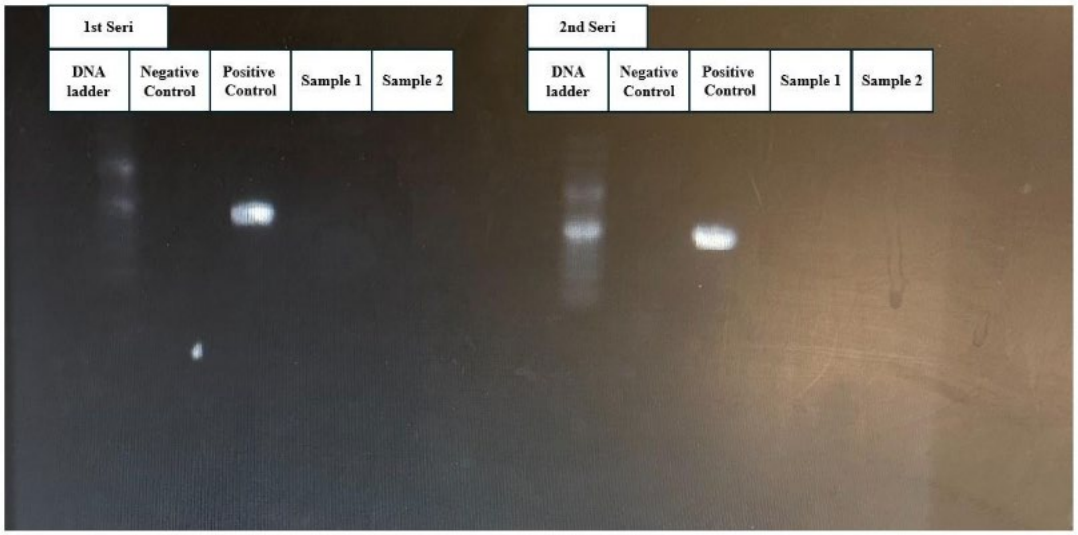
This study highlights psilocybin's multifaceted potential as a natural compound mitigating skin aging under HGHL conditions. A key finding was psilocybin's ability to restore normal cell cycle dynamics, alleviating HGHL-induced S phase arrest and promoting proper cell cycle progression essential for tissue integrity. Additionally, reduced senescence, as evidenced by decreased beta-galactosidase activity, reinforced psilocybin's rejuvenating effects. Psilocybin also showed a tendency to enhance fibroblast movement and accelerated wound closure in both post-treatment and co-treatment conditions. Also, psilocybin increased elastin gene expression, comparing to HGHL a critical ECM component responsible for maintaining skin structure and elasticity.

Furthermore, psilocybin significantly reduced pro-inflammatory markers such as *IL-1β*, *IL-6*, and *COX-2*, which are involved in ECM degradation and collagen breakdown. Its combined anti-inflammatory and antioxidative properties reinforce its ability to counteract age-related skin deterioration. By modulating apoptosis, improving cell cycle distribution, and preserving ECM integrity, psilocybin supports cellular resilience and skin rejuvenation.

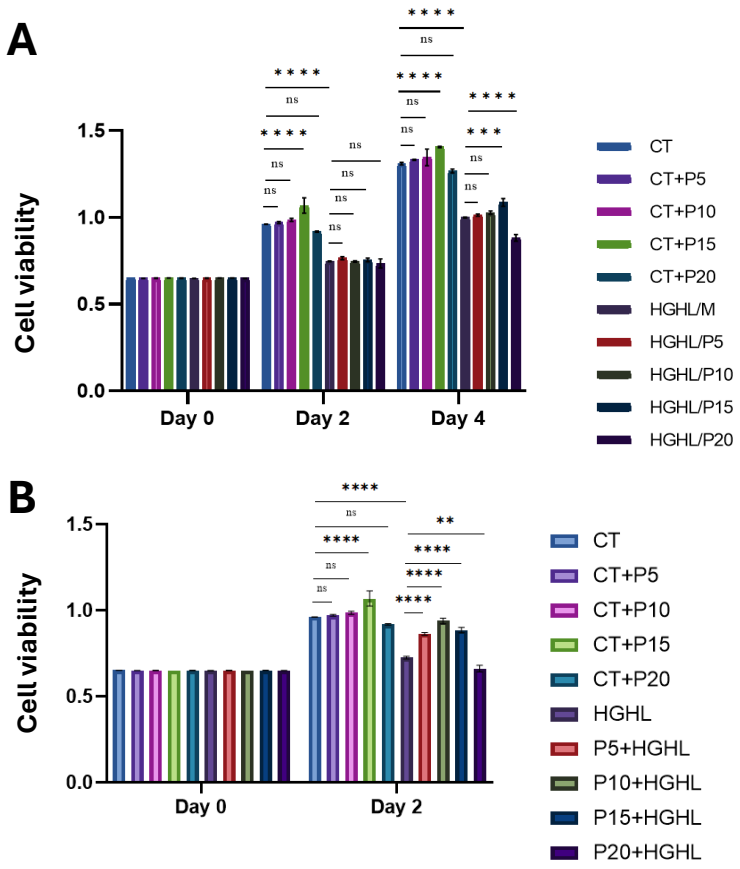
### 2.7. Future Directions

While the *in vitro* nature of this study limits its direct translation to *in vivo* settings, these findings highlight the potential of psilocybin in combating age-related skin changes and promoting overall skin health. Further research is needed to investigate its long-term effects, optimal dosage, and mechanisms of action *in vivo* to validate its therapeutic potential. Additionally, exploring interactions with other anti-aging compounds and its clinical applications will be essential.

## 2.8. Supplementary Materials




**Supplementary Figure 1.** PCR-based mycoplasma detection in BJ-5TA fibroblast cultures. *Left gel:* DNA ladder (lane 1), negative control (lane 2), positive control (lane 3), BJ-5TA samples (lanes 4–5) — all samples tested negative. *Right gel:* Replicate assay showing identical lane arrangement and confirming negative results for both BJ-5TA samples.




**Supplementary Figure 2. Effects of different concentrations of psilocybin on the cell viability in post-treatment (A) and co-treatment (B) of untreated and HGHL-induced fibroblasts.** Data shows the cell viability (MTT assay) of fibroblasts measured at days 0, 2 and 4 after HGHL treatment (A) and day 0 and day 2 co-treatment of psilocybin and HGHL (B). CT - Control (untreated fibroblasts); CT+P5, P10, P15 and P20 - psilocybin 5, 10, 15 and 20 µM added to untreated fibroblasts; P5, P10, P15, P20 - psilocybin at 5, 10, 15, 20 µM; HGHL - high glucose (25 mM) and high lipid (400 µM) treatment; HGHL/M – media added to HGHL treatment sample; HGHL/P5, P10, P15, P20 - psilocybin at various doses added post HGHL induction; P5, P10, P15, P20+HGHL- psilocybin at different doses added together with HGHL as a co-treatment. Data are presented as mean ± SD. N=3. The collected data was subjected to statistical analysis using one-way analysis of variance (ANOVA), followed by Dunnett's test for comparing mean values. GraphPad Prism 6 software was used to perform this analysis. Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns - not significant.

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# 3. Chapter 3: Eugenol Mitigates Skin Aging Induced by High-Glucose and High-Lipid Conditions in BJ-5Ta Fibroblasts

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*This chapter is included with the permission of all co-authors. Author contributions are detailed in the "Contributions of Authors" section in the Preface, in accordance with the University of Lethbridge manuscript-based thesis guidelines.*

## 3.1. Abstract

Eugenol, a phenolic compound derived from clove oil, has garnered considerable attention for its anti-aging properties, particularly in relation to skin health. This study investigated the effects of eugenol on human dermal fibroblasts exposed to high-glucose and high-lipid (HGHL) conditions—25 mM glucose and 400 µM palmitic acid—to mimic premature skin aging. After establishing metabolic stress, fibroblasts were treated with 15 µM eugenol either as a co-treatment or post-treatment. A comprehensive set of assays was conducted, including 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) for cell viability, β-galactosidase staining for senescence, qPCR for inflammatory cytokines and extra-cellular matrix (ECM)-related gene expression, apoptosis and cell cycle analyses, and wound healing assays. Eugenol significantly reduced oxidative stress, inflammation, and cellular senescence in fibroblasts under HGHL conditions. *IL-1β* and *COX-2* expression levels were significantly decreased in both treatment strategies. *IL-6* expression was significantly reduced in post-treatment, while *TNF-α* decreased in co-treatment. Regarding ECM-related genes, *COL3A1* expression significantly increased in untreated fibroblasts treated with eugenol, and elastin expression was significantly elevated in co-treatment with HGHL. Additionally, under HGHL conditions, eugenol inhibited

apoptosis and positively influenced cell cycle progression, contributing to improved cell survival. Overall, eugenol can be considered as natural compound mitigating signs of metabolic stress-induced skin aging.

### 3.2. Introduction

Eugenol is a phenolic compound with a chemical formula of $C_{10}H_{12}O_2$, primarily found in clove, cinnamon, and basil. It is biosynthesized through the phenylpropanoid pathway, and is contributing to plant defense mechanisms against herbivores and pathogens (Ouadi et al., 2022; Hirose et al., 2024). Beyond its biological role in plants, eugenol has garnered attention for its therapeutic potential in various fields, including medicine, dentistry, and dermatology, owing to its antimicrobial, anti-inflammatory, antioxidant, and analgesic properties (Krasniqi & Daci, 2017; Ulanowska & Olas, 2021; Ghasemi Gojani et al., 2023). In recent years, it was shown to be relevant to dermatology, addressing skin aging—a complex process driven by both intrinsic factors such as genetic programming and extrinsic factors like UV radiation, pollution, and diet (Hussein et al., 2024; Tong et al., 2024).

Skin aging primarily affects the dermis, where extracellular matrix (ECM) degradation, collagen loss, and reduced elasticity contribute to visible signs of aging, including wrinkles, sagging, and skin thinning (Shin et al., 2019). These changes are driven by interconnected mechanisms, including oxidative stress, chronic inflammation ('inflammaging'), mitochondrial dysfunction, and DNA damage, all of which exacerbate tissue breakdown and impair repair processes (Rea et al., 2018).

Oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) and the skin's antioxidant defenses, is a central contributor to aging. ROS cause damage to proteins, lipids, and DNA, disrupting vital processes such as collagen synthesis and ECM remodeling (Gkogkolou & Böhm, 2012; Papaccio et al., 2022). Additionally, inflammation, particularly inflammaging, plays a significant role in aging. This process is driven by the upregulation of pro-inflammatory cytokines like TNF-α and IL-6, which activate pathways such as NF-κB and toll-like receptors (TLRs), contributing to fibroblast senescence and ECM degradation (Chen et al., 2019; Wang et al., 2022).

Mitochondrial dysfunction, another hallmark of aging, leads to decreased ATP production and increased ROS, impairing collagen synthesis and enhancing the vicious cycle of oxidative damage and cellular stress. Dysfunctional mitochondria also activate pro-inflammatory pathways such as AP-1 and NF-κB, further promoting fibroblast senescence and inflammation. The accumulation of senescent fibroblasts in the dermis releases matrix-degrading enzymes, accelerating ECM breakdown and contributing to skin fragility. Additionally, their senescence-associated secretory phenotype amplifies inflammation and ECM degradation, creating a self-reinforcing cycle of aging-related damage (Dyer & Miller, 2018; Sreedhar et al., 2020; Cong et al., 2024).

These pathological processes are significantly intensified under high glucose and high lipid (HGHL) conditions, which elevate oxidative stress and drive the formation of advanced glycation end products (AGEs). AGEs are generated through the non-enzymatic glycation of proteins such as collagen and elastin, leading to stiffening of the ECM and reduced skin elasticity. Additionally, AGEs activate inflammatory responses via receptors like RAGE, further amplifying oxidative damage and accelerating skin aging (Almanza et al., 2019; Bansode & Gacche, 2019; Berlanga-Acosta et al., 2020). Chronic hyperglycemia has been shown to expedite aging in various cell

types, particularly fibroblasts, by enhancing oxidative stress, promoting AGE accumulation, and triggering senescence pathways. For instance, Bian et al. (2020) and Chang et al. (2010) demonstrated that prolonged exposure to elevated glucose levels induced premature senescence in human dermal fibroblasts and endothelial cells, largely through AGE buildup and the subsequent activation of pro-inflammatory signaling (Bian et al., 2020; Chang et al., 2010; Lu et al., 2022).

Similarly, high lipid conditions—often associated with metabolic disorders—have been linked to increased cellular stress and accelerated aging. Lipid overload induces lipotoxicity, mitochondrial dysfunction, and inflammation, all of which contribute to premature cellular senescence. Studies by Romer et al. (2021) and Oberhauser et al. (2021) highlighted these effects in adipocytes, hepatocytes, and pancreatic beta cells under high-lipid conditions (Oberhauser et al., 2021; Romer et al., 2021).

Eugenol, with its antioxidant, anti-inflammatory, and AGE-reducing properties, presents a promising solution for mitigating skin aging (Al-Trad et al., 2019; Damasceno et al., 2024). It has demonstrated potential in treating inflammatory skin conditions like dermatitis and acne while also promoting skin rejuvenation. Advances in nano-encapsulation techniques have further improved eugenol's effectiveness by enhancing its skin penetration and reducing cytotoxicity (Nisar et al., 2021; Sahlan et al., 2021). Despite its potential, the mechanisms underlying eugenol’s role in skin aging remain incompletely understood (Makuch et al., 2021). Eugenol’s anti-inflammatory and antioxidant properties extend far beyond skin health, offering therapeutic benefits in a range of medical fields, including oncology.

Its ability to modulate inflammation and oxidative stress has been shown to enhance the effectiveness of chemotherapy agents such as gemcitabine and cisplatin, demonstrating

synergistic potential in cancer treatment (Barboza et al., 2018; Pezzani et al., 2019; Padhy et al., 2022).

While eugenol has numerous benefits for skin aging and therapeutic applications, high concentrations may lead to adverse effects, including liver damage, skin irritation, and DNA damage, underscoring the importance of precise dosage regulation (Bendre et al., 2016; de Araújo Lopes et al., 2018). Its combination of antioxidant, anti-inflammatory, and ECM-preserving properties, along with its light texture and pleasant aroma, makes it a promising ingredient in skincare formulations (Nagaraju et al., 2021; Wanakhachornkrai et al., 2020).

This study aims to investigate eugenol's antioxidant, anti-inflammatory, and AGE-reducing properties in the context of skin aging, particularly in HGHL-induced conditions.

### 3.3. Methods and Materials

#### 3.3.1. Chemicals and Reagents

For our cell culture experiments, we used ISCOVE's Modified Dulbecco's Medium (IMDM) 1X (MULTICELL, Cat# 319-106-CL; Wisent Inc., Saint-Jean-Baptiste, QC, Canada) as the base medium. The medium was supplemented with heat-inactivated Premium Grade Fetal Bovine Serum (FBS) (Cat# 97068-085, VWR International LLC, Radnor, USA) and 100× Penicillin-Streptomycin (10,000 IU Penicillin and 10 mg/ml Streptomycin; Cat# 450-201-EL, Wisent Inc., Quebec, Canada) to support optimal cell growth and prevent contamination. For washing and handling procedures, we used 1X phosphate-buffered saline (PBS) (Cat# 311-010-CL, Wisent Inc.). Human foreskin BJ-5ta hTERT-immortalized fibroblast cell lines (CRL-4001™) were

obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained under standard culture conditions.

### 3.3.2. Cell Culture and Treatments

Human foreskin BJ-5ta hTERT-immortalized cell lines (CRL-4001™), referred to as "fibroblasts" in this study were cultured in ISCOVE's Modified Dulbecco's Medium (IMDM), supplemented with 10% heat-inactivated Premium Grade Fetal Bovine Serum and 1% Penicillin-Streptomycin (10,000 IU Penicillin and 10 mg/ml Streptomycin). Mycoplasma testing was performed using a PCR-based detection kit (e.g., MycoAlert™ Mycoplasma Detection Kit, Lonza) according to the manufacturer's instructions to confirm cultures were contamination-free (Supplementary Figure 1).

All cell culturing and harvesting were conducted in a BSL-2 laboratory. The cells were incubated at 37°C with 5% $CO_2$ in a Forma Steri-Cycle $CO_2$ Incubator (Thermo Scientific, Montreal, Canada) with the culture medium replaced every two days until the cells reached 90%-100% confluency for experimental use. Subculturing was performed weekly. The population doubling (PD) number for each subculture was calculated using the formula $\Delta PD = \log_2(nf/ni)$, where ni represents the initial number of seeded cells and nf the final cell count. Cells between passages 17 and 24 were selected for all experiments.

To induce cell senescence, fibroblasts were exposed to the combination of high-glucose (HG, 25 mM glucose) and high-lipid (HL, 400 µM palmitic acid) (HGHL) conditions for 48 hours. This approach aligns with findings from the recent study by Gojani et al. (2023), which demonstrated that such conditions reduce cell viability in β-cells, promote apoptotic behavior, and exacerbate other aging-related effects (Gojani et al., 2023). A 1.0 M glucose stock solution was prepared by dissolving glucose in the medium, followed by filter sterilization, and stored at 4°C. The palmitic

acid (PA) stock solution was prepared by dissolving PA in 100% ethanol with heating at 70°C. The dissolved PA was then combined with sterile 10% BSA and underwent two cycles of heating at 55°C for 15 minutes with mixing. The PA stock was aliquoted and stored at −20°C. Prior to use, the PA stock was reheated at 55°C for 15 minutes.

Based on various studies, pilot experiments, and repeated tests assessing cell toxicity and viability, we optimized the application of eugenol (CAS No. 97-53-0, Sigma-Aldrich, Saint Louis, MO, USA) for co-treatment and post-treatment conditions, selecting concentrations of 15 μM for both experimental settings.

To evaluate eugenol's potential under HGHL conditions, post-treatment and co-treatment strategies were employed. These approaches provided a comprehensive understanding of eugenol's reparative and preventive capabilities.

The post-treatment approach assessed eugenol's reparative effects on fibroblasts after HGHL-induced senescence. Fibroblasts were exposed to HGHL conditions for 48 hours to induce senescence. Following this, the HGHL-treated wells were washed twice with 1x PBS and treated either with 15 μM eugenol or media alone for an additional 48 hours. The experimental setup included two additional wells that were not exposed to HGHL. One served as the untreated control containing only media (CT), while the other was treated with 15 μM eugenol (E15) to evaluate its effect on untreated fibroblasts. These conditions allowed comparisons between media-only and eugenol treatments on HGHL-induced fibroblasts and their respective non-HGHL counterparts.

The co-treatment approach investigated eugenol's protective effects during simultaneous exposure to HGHL. Fibroblasts were treated with 15 μM eugenol alongside HGHL for 48 hours. The

experimental design consisted of four wells: one containing untreated fibroblasts with media only (CT), one treated with HGHL alone (control for HGHL-induced damage), one treated with 15 μM eugenol alone (E15) on untreated fibroblasts, and one with the combined treatment of HGHL and 15 μM eugenol. This setup enabled the evaluation of eugenol's protective capabilities under HGHL conditions.

These concentrations were selected for all assays, including wound healing, beta-galactosidase, apoptosis, and cell cycle analyses, along with RNA isolation for qPCR.

### 3.3.3. MTT Assay

Cell viability and cytotoxicity of BJ-5ta human skin fibroblasts were evaluated using the MTT assay, which relies on the ability of metabolically active cells to reduce the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) into insoluble purple formazan crystals. This redox reaction predominantly occurs in the mitochondria through the action of succinate dehydrogenase, though other oxidoreductases located in the cytosol and endoplasmic reticulum also contribute, utilizing NADH and NADPH as electron donors (van Meerloo et al., 2011). As a result, formazan production is directly proportional to the number of viable cells, since only cells with intact metabolic activity can drive this reduction. In contrast, dead or metabolically inactive cells do not generate formazan, making the MTT assay a reliable indicator of overall cellular metabolic activity rather than purely mitochondrial function (Ghasemi et al., 2022).

BJ-5ta fibroblasts were seeded at a density of $5.0 \times 10^3$ cells per well in 100 µl of complete culture medium in 96-well plates and incubated for 24 to 48 hours to reach 70–80% confluency prior to treatment. Treatments—including high glucose and high lipid (HGHL) conditions, psilocybin, and

various concentrations of eugenol—were applied to determine cytotoxic and effective doses. Cell viability was assessed at three time points: days 0, 2, and 4 post-treatment. Each condition was tested in triplicate, and the experiments were independently repeated three times to ensure reproducibility.

Following treatment, 10 µl of MTT reagent from the Cell Proliferation Kit I (#11465007001, Roche, Ontario, Canada) was added directly to each well. Plates were incubated for 4 hours at 37 °C in a humidified atmosphere containing 5–6.5% $CO_2$. After incubation, 100 µl of solubilization solution (10% SDS in 0.01 M HCl) was added to dissolve the formazan crystals, followed by overnight incubation under the same conditions. Absorbance was measured at 595 nm using a FLUOstar Omega microplate reader (BMG LABTECH, Offenburg, Germany). Cell viability was calculated relative to untreated control cells.

### 3.3.4. β-Galactosidase Assay

To assess cellular senescence, fibroblasts were treated under various conditions, including HGHL to induce premature aging, eugenol co-treatment and post-treatment, as well as eugenol treatment on untreated fibroblasts. The cells were then washed twice with 1x PBS. To quantify β-galactosidase activity, fibroblast lysates from both normal and HGHL-induced prematurely aged fibroblasts were prepared using the protein lysis buffer from the Beta-Galactosidase Detection Kit (Fluorometric) (ab176721, Abcam, Toronto, ON, Canada). Protein concentration in each sample was measured with the Bradford assay, and the samples were diluted to a final protein concentration of 1 µg/ml. Following this, 50 µl of each standard and sample (diluted in 1× lysis buffer) were transferred to a black 96-well plate. To each well, 50 µl of fluorogenic fluorescein digalactoside (FDG) working solution was added, and the plate was incubated at 37°C for 4 hours.

Subsequently, 50 µl of stop buffer was added, and fluorescence was measured for each sample using a FLUOstar Omega (BMG LABTECH, Offenburg, Germany) plate reader with excitation and emission wavelengths of 490 nm and 525 nm, respectively. β-Galactosidase levels, indicating cellular senescence, were determined by comparing fluorescence values to a β-galactosidase standard curve prepared for each experiment. Experiments were repeated three times with triplicates in each test.

### 3.3.5. Apoptosis Assay

After treating fibroblasts and washing them twice with 1x PBS to remove any residual media and non-adherent fibroblasts, the fibroblasts were trypsinized to detach them from the culture plate. They were then centrifuged to collect the cell pellet, which was resuspended in cold PBS. To detect apoptosis, the fibroblasts were stained with Annexin V-FITC and PI, which allows to distinguish between live, early apoptotic, late apoptotic, and necrotic cells.

Annexin V binds to phosphatidylserine, which translocates to the outer layer of the cell membrane early in apoptosis. PI, a DNA-binding dye, only enters fibroblasts with compromised membranes, marking late apoptotic and necrotic cells. The staining solution containing Annexin V-FITC and PI was gently mixed with the cell suspension and incubated in the dark at room temperature for 15-20 minutes.

The stained fibroblasts were then analyzed using flow cytometry (BD FACSAria™ Fusion Flow Cytometer, BD Biosciences, San Diego, CA, USA). Fibroblasts that were Annexin V-positive and PI-negative were classified as early apoptotic, while fibroblasts that were both Annexin V- and PI-positive were considered late apoptotic or necrotic. This assay allowed quantification of the

apoptotic and necrotic cell populations, providing insights into the treatment's effects on cell viability and apoptosis.

### 3.3.6. Cell Cycle Assay

After inducing fibroblast treatments, the fibroblasts were washed twice with cold 1x PBS to remove residual media and non-adherent fibroblasts. Next, they were trypsinized to detach them from the culture surface, and then centrifuged to collect the cell pellet. The pellet was resuspended in cold 1x PBS and fixed by adding cold 70% ethanol dropwise while gently vortexing. The fibroblasts were then incubated at 4℃ for at least 2 hours (or overnight) to ensure proper fixation.

Following fixation, the fibroblasts were washed again with cold PBS to remove ethanol. To eliminate RNA, 50 µL of RNase solution (100 µg/mL) was added to each sample, followed by the addition of 200 µL of a propidium iodide (PI) solution prepared at 50 µg/mL to stain the DNA. The stained cells were incubated in the dark at room temperature for 30-60 minutes to ensure consistent dye binding.

Finally, the cell cycle distribution was analyzed by BD FACSAria™ Fusion Flow Cytometer (BD Biosciences, San Diego, CA, USA). Fibroblasts were categorized based on DNA content into different phases: G0/G1 (resting or initial growth phase), S (DNA synthesis phase), and G2/M (second growth and mitosis phase), providing insights into the cell population's distribution across the cell cycle stages.

### 3.3.7. Wound Healing Assay

Fibroblasts were grown in 6-well plates until they reached over 90% confluence. A 100 µL pipette tip was employed to create a scratch or wound line down the center of each well, mimicking a wound scenario. Following this, the fibroblasts were washed twice with 1x PBS before adding the appropriate cell culture growth medium or specific treatments. Images of the wound healing process were captured at various time points: day 0 (2 hours after wound creation, treatments began at this time), day 1 (24 hours after treatment), day 2 (48 hours after treatment), day 3 (72 hours after treatment) and day 4 (96 hours after treatment). The Infinity3 camera with OLYMPUS CKX41 microscope was utilized to obtain images within its linear dynamic range, which ensures a consistent relationship between signal intensity and the amount of material present. The migration distance of fibroblasts (mm), indicating their wound-closing ability, was measured throughout the experiment. Image analysis was conducted using ImageJ (IJ 1.46r) software, and all samples were analyzed in triplicate.

### 3.3.8. qRT-PCR

RNA was extracted from fibroblast monolayer cultures using TRIzol® Reagent (Invitrogen, Carlsbad, CA), followed by purification with the RNeasy kit (Qiagen, Toronto, ON, Canada) as per the manufacturer's protocol. RNA concentration was measured using a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, Wilmington, DE). A fraction of the total RNA was employed to synthesize complementary DNA (cDNA), utilizing 1 µg of total RNA and a specialized cDNA synthesis kit known as iScript™ Reverse Transcription Supermix (Cat: 1708841; BioRad Laboratories, Saint-Laurent, QC, Canada). The resulting cDNA product was then employed as a template for q-PCR, with 1 µl of cDNA being utilized in each reaction. The

qRT-PCR reactions were carried out using a SsAdvancedTM Universal Inhibitor-Tolerant SYBR Green Supermix (Cat: 1725017; BioRad Laboratories, Saint-Laurent, QC, Canada). The primers required for the q-RT-PCR analysis were designed employing an online IDT software (Table 2). Beta-actin was used as the reference gene for normalization in the experiment. Each group included three biological replicates, with three technical replicates per sample.

**Table 1. Primers used in the Experiments**

| **Gene** | **Primer Sequence** |
|---|---|
| *Col3a1* | |
| H-F-Col3a1 | F: 5` AAGTCAAGGAGAAAGTGGTCG 3` |
| H-R-Col3a1 | R: 5` CTCGTTCTCCATTCTTACCAGG 3` |
| *Col1a1* | |
| H-F-Col1a1 | F: 5` CCCCTGGAAAGAATGGAGATG 3` |
| H-R-Col1a1 | R: 5` TCCAAACCACTGAAACCTCTG 3` |
| *Elastin* | |
| H-F-Eln | F: 5` GGCTTTGGTGTCGGAGTC 3` |
| H-R-Eln | R: 5` AACTAACCCGTACTTGGCAG 3` |
| *b-Actin* | |
| Forward | F: 5` GGCATCCTCACCCTGAAGTA 3` |
| Reverse | R:5`CACACGCAGCTCATTGTAGAAG 3` |
| *IL-6* | |
| Forward | F: 5` GGAGACTTGCCTGGTGAAA 3` |
| Reverse | R: 5` CTGGCTTGTTCCTCACTACTC 3` |

| | |
|---|---|
| *COX-2* | |
| Forward | F: 5` TACTGGAAGCCAAGCACTTT 3` |
| Revers | R: 5` GGACAGCCCTTCACGTTATT 3` |
| *TNF-alpha* | |
| Forward | F: 5` CCAGGGACCTCTCTCTAATCA 3` |
| Reverse | R: 5` TCAGCTTGAGGGTTTGCTAC 3` |
| *IL-1b* | |
| Forward | F: 5`CCTTAGGGTAGTGCTAAGAGGA 3` |
| Reverse | R:5`AAGTGAGTAGGAGAGGTGAGAG3` |

### 3.3.9. Statistics

The collected data was subjected to statistical analysis using one-way analysis of variance (ANOVA), followed by Dunnett's test for comparing mean values. GraphPad Prism 6 software was used to perform the analysis and prepare the graphics.

## 3.4. Results

### 3.4.1. Eugenol Increases Cellular Viability in Untreated and HGHL-Treated Samples

A pilot study was conducted to assess cell viability across eugenol concentrations ranging from 5 to 20 µM. The findings revealed that concentrations between 5 and 15 µM enhanced cell viability, whereas 20 µM decreased it, (Supplementary figure 1). Thus, for the co- and post-treatment experiments, we limited the eugenol dose to 15 µM.

Replication test results confirmed the supplementary figure and data regarding the effects of various eugenol concentrations (1 to 15 µM), both under stress and non-stress conditions.

Significant improvements in cell viability were observed compared to controls, with 15 µM eugenol (E15) demonstrating the most pronounced effect among all other eugenol concentrations.

Analysis showed that exposure of untreated fibroblasts to E15 resulted in significant increase in cell viability in both post-treatment and co-treatment experiment (Figure 1). In the post-treatment experiment, E15 showed the highest cell viability under conditions altered by HGHL (Figure 1A). Similarly, in the co-treatment experiment, E15 was also the most effective (Figure 1B). In both cases, we observed dose-dependent increase in cell viability when eugenol was applied to HGHL-treated cells. Therefore, E15 was selected for all subsequent experiments, both for post-treatment and co-treatment protocols.

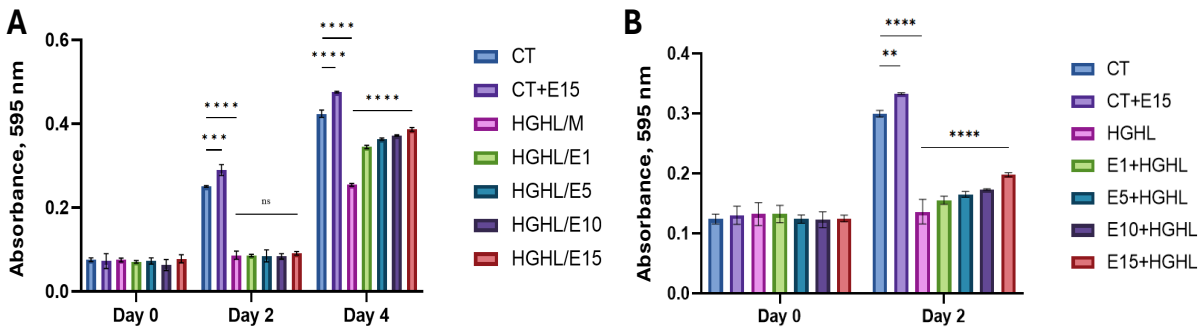



**Figure 1. Effects of different concentrations of eugenol on the cell viability in post-treatment (A) and co-treatment (B) of HGHL-induced fibroblasts.** Data shows the cell viability (MTT assay). Y axis shows absorbance at 595 nm in fibroblasts measured at days 0, 2 and 4 after eugenol application in HGHL post-treatment (A) and day 0 and day 2 of co-treatment of eugenol and HGHL (B). CT - Control (untreated fibroblasts); CT+E15 - eugenol 15 µM added to untreated fibroblasts; E1, E5, E10, E15 - eugenol at 1, 5, 10, 15 µM; HGHL - high glucose (25 mM) and high lipid (400 µM) treatment; HGHL/M – media added to HGHL treatment sample; HGHL/E1, E5, E10, E15 - eugenol at various doses added post HGHL induction; E1, E5, E10, E15+HGHL- eugenol at different doses added together with HGHL as a co-treatment. Data are presented as mean ± SD. N=3. The collected data was subjected to statistical analysis using one-way analysis of variance (ANOVA), followed by Dunnett's test for comparing mean values. GraphPad Prism 6 software was used to perform this analysis. Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns - not significant.

### 3.4.2. Eugenol Demonstrates a Reduction in Beta-Galactosidase Activity in Both Untreated and HGHL-Treated Fibroblasts

Beta-galactosidase levels, a marker of cellular senescence, increase with aging. Analysis showed the highest beta-galactosidase activity in a HGHL-treated sample in co-treatment (Figure 2B), and in a HGHL-treated sample followed addition of culture media in post-treatment (Figure 2A). Beta-galactosidase activity of other samples was significantly lower than HGHL-treated samples in both co- and post-treatments. This experiment showed that eugenol is able to prevent the increase in cellular senescence in response to HGHL in co-treatment experiment (Figure 2B) as well as decreases the cellular senescence caused by HGHL in post-treatment experiment (Figure 2A).

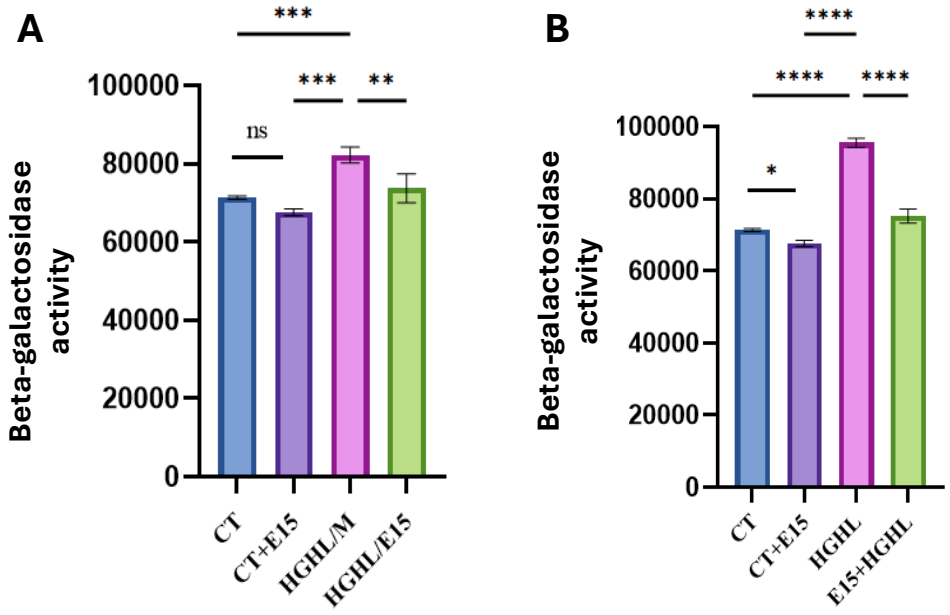



**Figure 2. Effects of eugenol on beta-galactosidase activity in fibroblasts treated with HGHL.** Data shows the beta-galactosidase activity (measured in fluorescence intensity, arbitrary units, Y axis) after HGHL treatment in post- (A) and co-treatment experiments (B) with eugenol. CT - Control (untreated fibroblasts); CT+E15 - eugenol 15 µM added to untreated fibroblasts; HGHL - high glucose (25 mM) and high lipid (400 µM) treatment; HGHL/M – media added to HGHL treatment sample; HGHL/E15 - 15 µM eugenol added post HGHL induction; E15+HGHL - 15 µM eugenol added together with HGHL as a co-treatment. Data are presented as mean ± SD. N=3. Statistical analysis was performed using one-way ANOVA, followed by Dunnett's test for mean comparisons, with GraphPad Prism 6 software. Significance levels: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; ns - not significant.

### 3.4.3. Eugenol Reduces Apoptosis in HGHL-Treated Fibroblasts

The apoptosis test is designed to detect and measure the occurrence of apoptosis in fibroblasts, providing important insights into cellular health, treatment effects, or disease progression. The primary goal of this experiment was to assess the extent and rate at which fibroblasts undergo apoptosis. For both co- and post-treatment with eugenol, the results showed that HGHL and HGHL/Media significantly induced apoptosis compared to the CT (Figure 3). Exposure of untreated fibroblasts to E15 resulted in a nonsignificant increase in apoptosis (Figure 3A, B). Both E15 treatments significantly reduced apoptosis under the stress (Figure 3A and B).

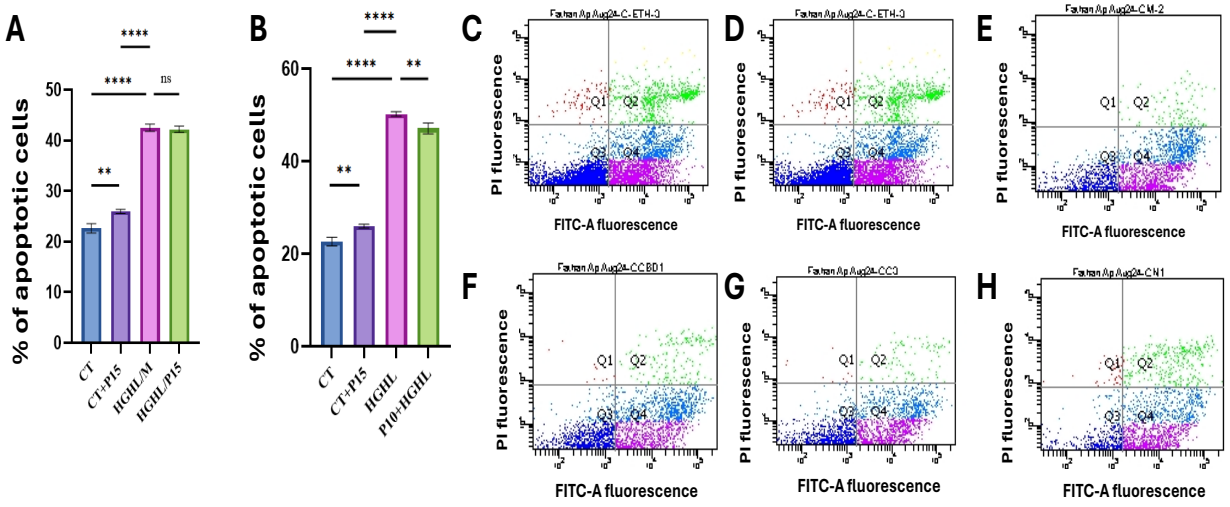



**Figure 3. Effects of eugenol on apoptosis in untreated and HGHL-treated fibroblasts.** Data shows the percentage of apoptotic cells after HGHL treatment in post- (A) and co-treatment (B) experiments with eugenol. Representative images of apoptosis in CT (C), CT+E15 (D), HGHL (E), HGHL/M (F), HGHL/E15 (G), E15+HGHL (H). CT - control (untreated fibroblasts); CT+E15 - eugenol 15 µM added to untreated fibroblasts; HGHL - high glucose (25 mM) and high lipid (400 µM) treatment; HGHL/M – media added to HGHL treatment sample; HGHL/E15 - 15 µM eugenol added post HGHL induction; E15+HGHL - 15 µM eugenol added together with HGHL as a co-treatment. Data are presented as mean ± SD. N=3. Statistical analysis was performed using one-way ANOVA, followed by Dunnett's test for mean comparisons, with GraphPad Prism 6 software. In panels C to H, the **X-axis** represents FITC-A (Annexin V–FITC fluorescence, indicating phosphatidylserine exposure), and the **Y-axis** represents Propidium Iodide (PI) fluorescence, which measures membrane permeability associated with late apoptosis

or necrosis. Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns - not significant.

### 3.4.4. Eugenol Alleviates Stress and Enhanced the Cell Cycle in HGHL-Induced Fibroblasts

In the cell cycle assay, fibroblasts were categorized into distinct phases based on DNA content: G0/G1 (resting or initial growth phase), S (DNA synthesis phase), and G2/M (second growth and mitosis phase), allowing for an analysis of the fibroblast population's distribution across these stages. Both co-treatment and post-treatment with HGHL and HGHL/M induced significant cellular stress, leading to a shift of many fibroblasts into the S phases and disrupting normal cell cycle progression (Figure 4).

When treated with E15 alone, there was a notable increase and alteration in the S phase of the cell cycle compared to control conditions (Figure 4A, B). However, post-treatment with eugenol significantly alleviated the stress caused by the HGHL environment, preserving a high proportion of fibroblasts in the G1 phase and reducing the number of cells in the S phases (Figure 4A).

Similarly, co-treatment with eugenol significantly mitigated the stress induced by the HGHL environment (Figure 4B). Eugenol demonstrated protective and restorative effects on the cell cycle under HGHL conditions, highlighting its potential to counteract stress-induced disruptions in cellular function.

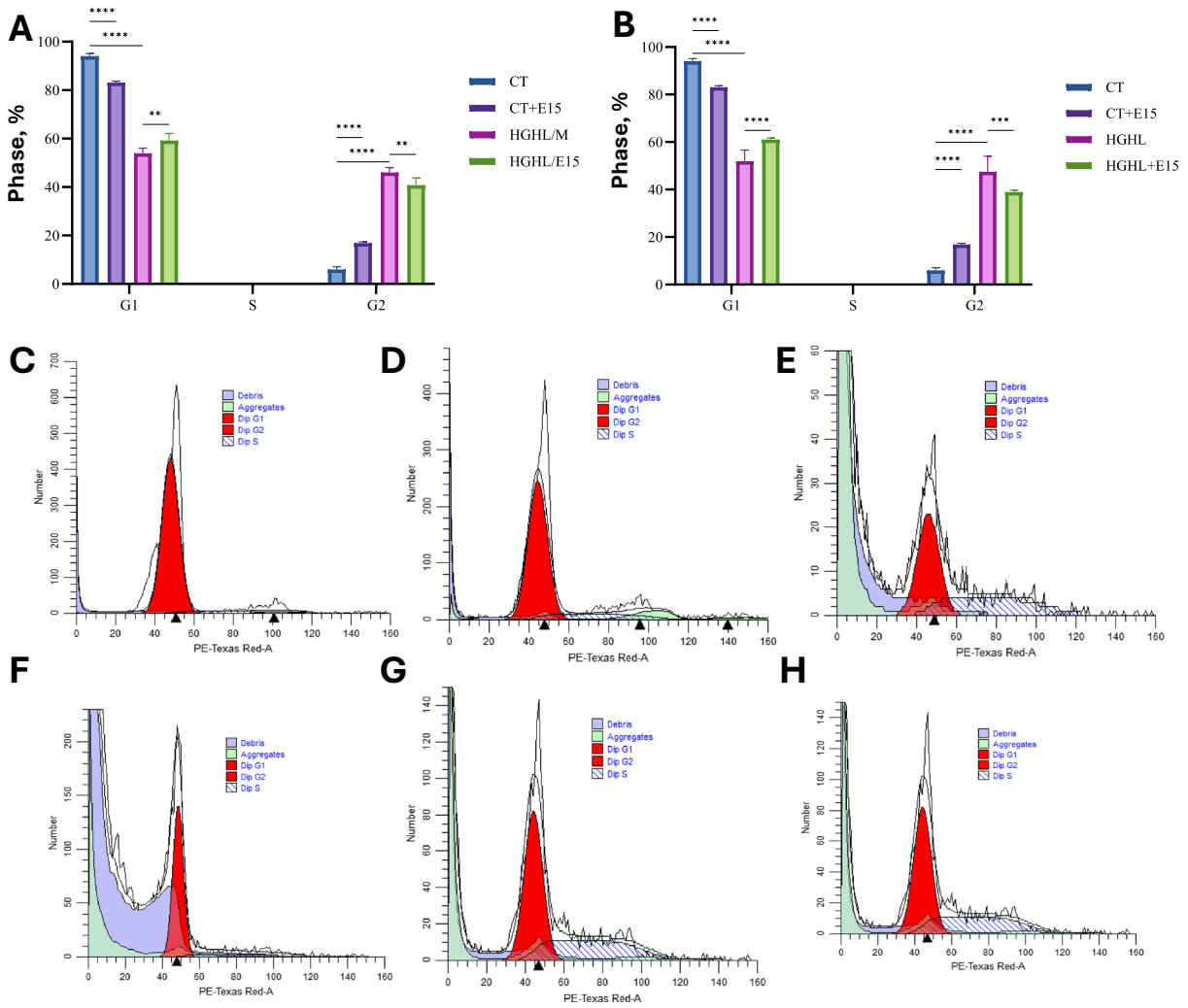


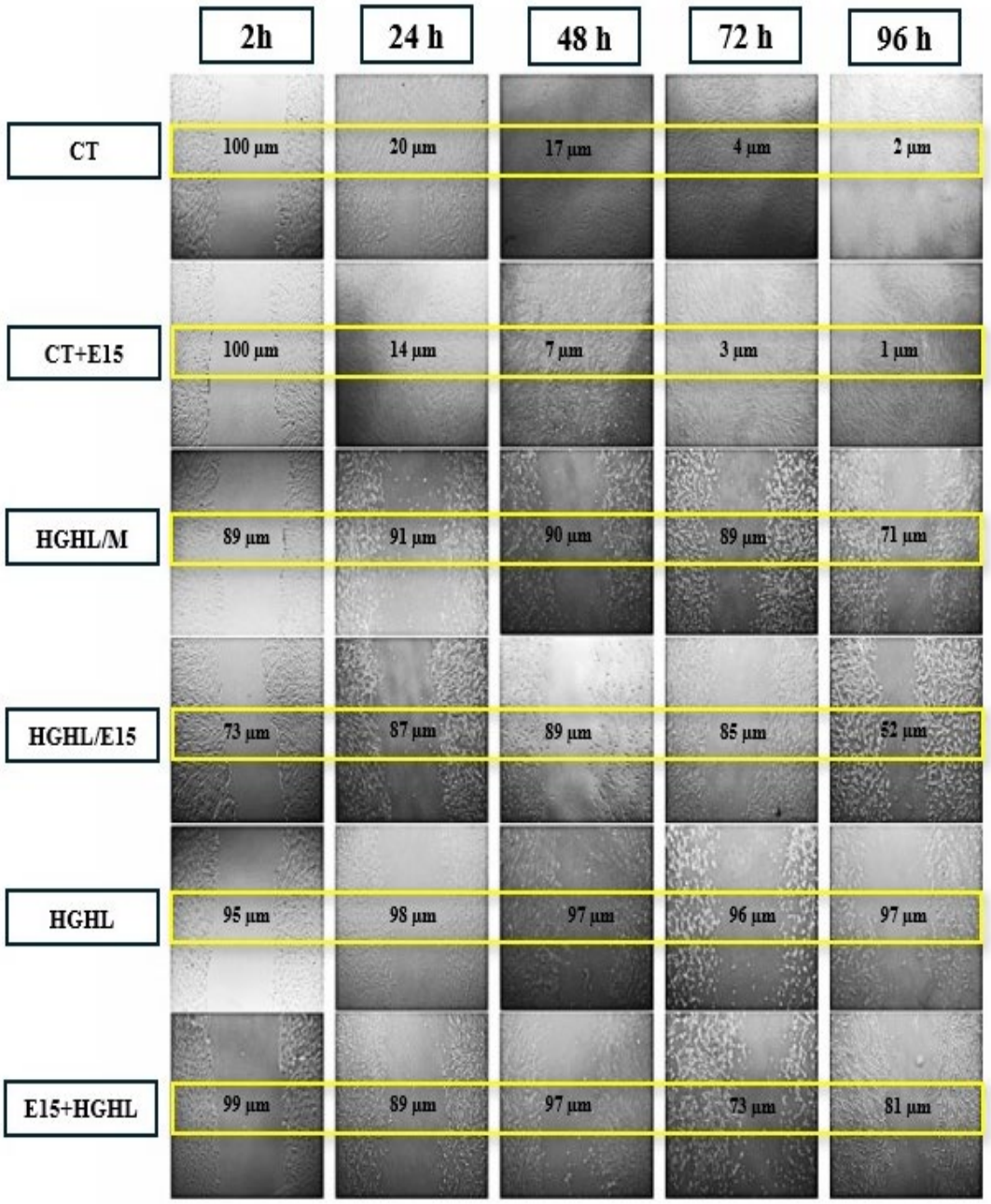

**Figure 4. Impact of eugenol on the cell cycle in untreated and HGHL-treated fibroblasts**

Data shows the percentage of cells in specific cell cycle phase after HGHL treatment in post- (A) and co-treatment (B) experiments with eugenol. Representative images of cell cycle in CT(C), CT+E15 (D), HGHL (E), HGHL/M (F), HGHL/E15 (G), E15+HGHL (H). CT - control (untreated fibroblasts); CT+E15 – eugenol 15 µM added to untreated fibroblasts; HGHL - high glucose (25 mM) and high lipid (400 µM) treatment; HGHL/M – media added to HGHL treatment sample; HGHL/E15 - 15 µM eugenol added post HGHL induction; E15+HGHL - 15 µM eugenol added together with HGHL as a co-treatment. Data are presented as mean ± SD. N=3. Statistical analysis was conducted using one-way ANOVA, followed by Dunnett's test for mean comparisons, using GraphPad Prism 6 software. Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns - not significant.

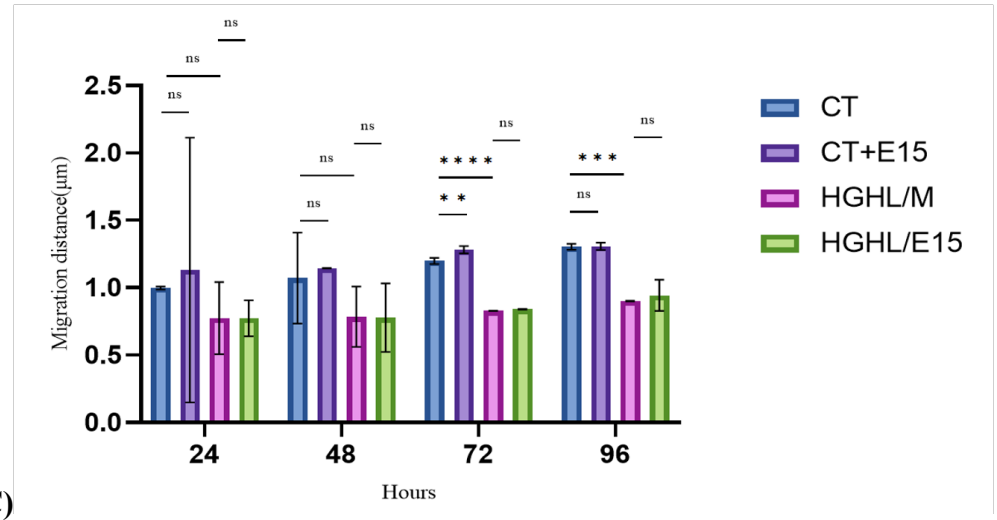
### 3.4.5. Eugenol Does not Improve the Wound Healing in Untreated and HGHL-Treated Fibroblasts

The wound healing assay is commonly used to assess the anti-aging effects of various compounds by evaluating fibroblast proliferation and migration. We observed that E15 applied to untreated fibroblasts showed a trend toward improved cell movement and wound healing compared to the control, although the differences were not statistically significant (Figure 5). Exposure to HGHL significantly impaired wound healing compared to the control group and adding media (HGHL/M group) did not lead to any improvement (Figure 5). Co-treatment with E15 and HGHL showed some improvement in cell movement and wound healing, though the differences were not significant. Similarly, post-treatment with E15 did not result in any significant enhancement of wound healing and cell movement.

**A)**


2h
24 h
48 h
72 h
96 h
CT
100 µm
20 µm
17 µm
4 µm
2 µm
CT+E15
100 µm
14 µm
7 µm
3 µm
1 µm
HGHL/M
89 µm
91 µm
90 µm
89 µm
71 µm
HGHL/E15
73 µm
87 µm
89 µm
85 µm
82 µm
HGHL
95 µm
98 µm
97 µm
96 µm
97 µm
E15+HGHL
99 µm
89 µm
97 µm
73 µm
81 µm

**B)**




**C)**

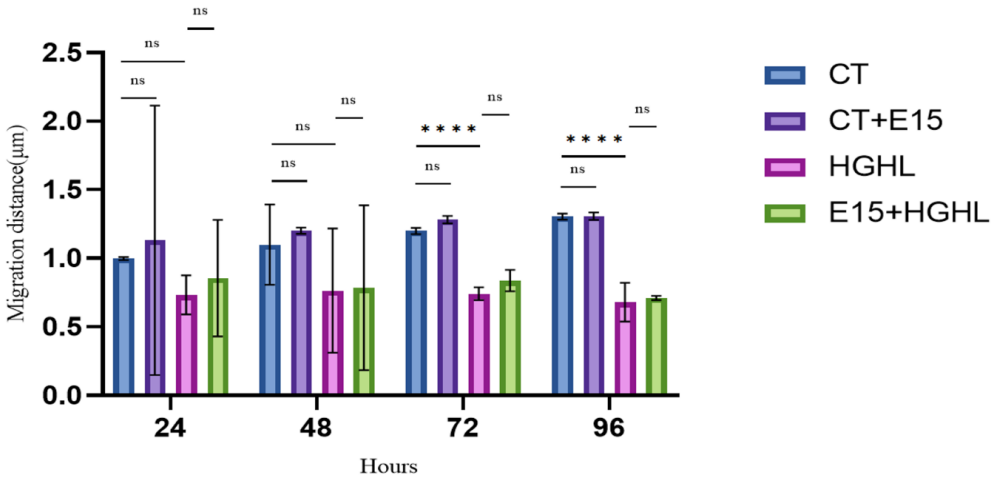



**Figure 5. Effects of eugenol on wound healing progress in untreated and HGHL-treated fibroblasts**

A. Microscope images depict the wound healing process in untreated and HGHL-treated fibroblasts under various treatment conditions. For post-treatments, HGHL was applied 2 hours after wound creation and continued for 48 hours. After 48 hours, HGHL was removed, fibroblasts were washed with 1X PBS, and treatments with E15 and media were applied for an additional 48 hours (HGHL/E15). Additional conditions included: untreated fibroblasts treated with E15 (Ct+E15), where E15 was added 2 hours after wound creation; fibroblasts co-treated with HGHL and E15 simultaneously 2 hours post-wound creation (E15+HGHL); fibroblasts treated with HGHL alone 2 hours after wound creation (HGHL), and CT.

Images were taken at specific intervals: 2 hours post-wound creation, and at 24-, 48-, 72- and 96-hours following treatment. Wound closure was quantified using ImageJ software. The 2-hour images for each well were intended to be consistent; however, manual processing with 100-µL pipette tips introduced variability. Thus, images at subsequent time points were normalized using their respective 2-hour images as references.

B. Quantitative analysis of wound healing progression in HGHL-induced fibroblasts post-treated with E15.
C. Quantitative analysis of wound healing progression in fibroblasts co-treated with HGHL and E15.
Data are shown as mean ± SD. N=3. Statistical significance was determined using one-way ANOVA, followed by Dunnett's post hoc test in GraphPad Prism 6 software. Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns - not significant.

### 3.4.6. Protective Effects of Eugenol on the Expression of Genes Involved in ECM Maintenance and Inflammatory Response in Untreated and HGHL-Treated Fibroblasts

Here, we first tested the expression of two collagen encoding genes, *Collagen type I alpha 1* (*COL1A1*), *Collagen type III alpha 1* (*COL3A1*), and *elastin* (*ELN*) gene, as these represent the most important and abundant ECM proteins that define the structural integrity of the skin.

Exposure to E15 in untreated fibroblasts significantly reduced the expression of *COL1A1* and *ELN* genes (Figures 6A, 6B, 6E, 6F), while it significantly increased the expression of *COL3A1* (Figures 6C, 6D). HGHL treatment led to a decrease in the expression of all three genes, both in co-treatment and post-treatment scenarios. When E15 was added to HGHL treated cells during post-treatment, it had no significant effect on *COL1A1* and *COL3A1* expression levels, but it further downregulated *ELN* expression (Figures 6A, 6C, 6E). In contrast, co-treatment of E15 with HGHL did not significantly increase *COL1A1* and *COL3A1* expression. However, it did result in a significant increase in *ELN* expression compared to HGHL treatment (Figures 6B, 6D, 6F).

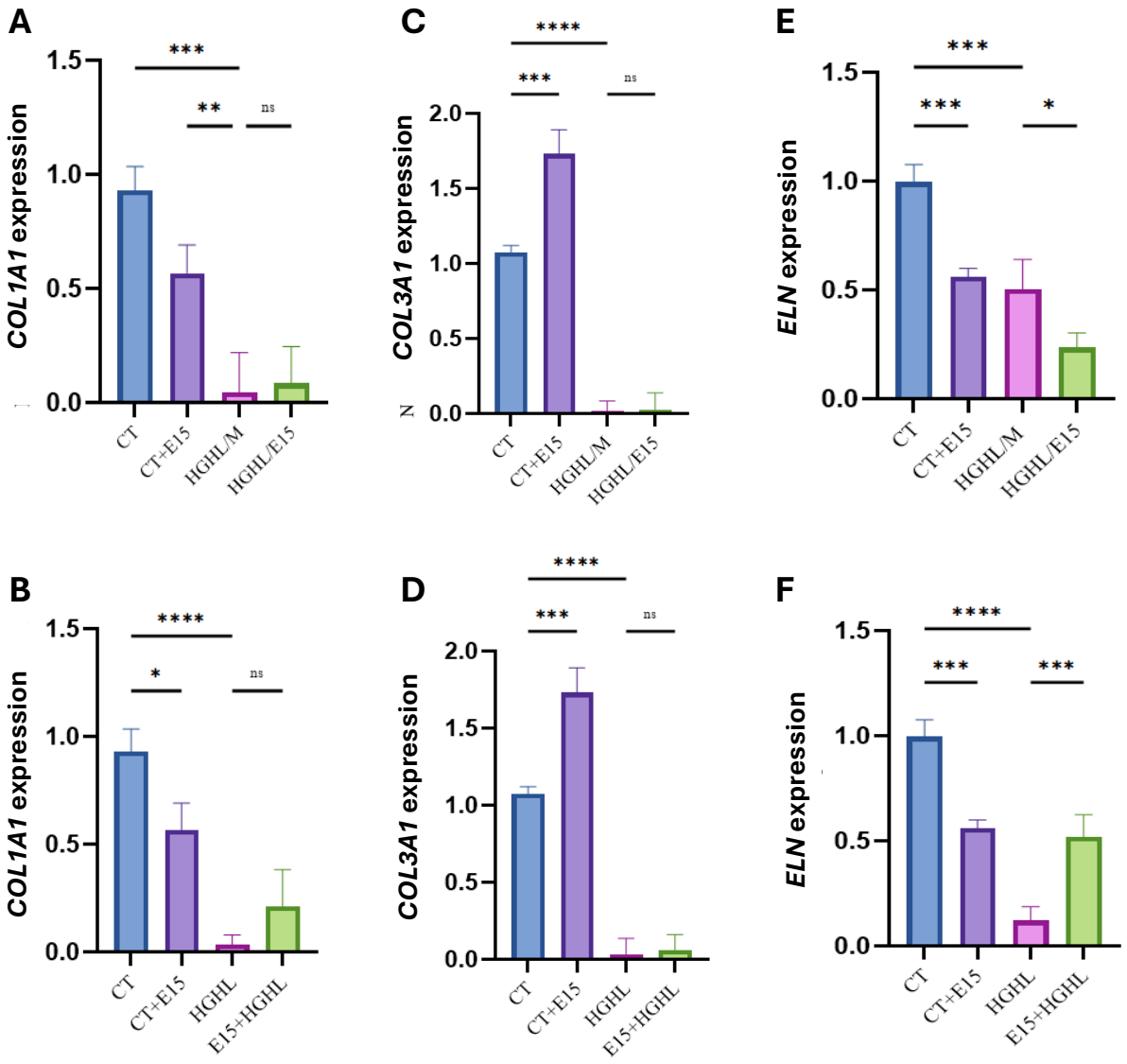



**Figure 6. Effect of eugenol on the expression of *COL1A1*, *COL3A1* and *ELN* genes in untreated and HGHL-treated fibroblasts.** *COL1A1* expression in post-treatment (A) and co-treatment (B). *COL3A1* expression in post-treatment (C) and co-treatment (D). *ELN* expression in post-treatment (E) and co-treatment (F). CT - control (untreated fibroblasts); CT+E15 – eugenol 15 µM added to untreated fibroblasts; HGHL - high glucose (25 mM) and high lipid (400 µM) treatment; HGHL/M – media added to HGHL treatment sample; HGHL/E15 - 15 µM eugenol added post HGHL induction; E15+HGHL - 15 µM eugenol added together with HGHL as a co-treatment. Data are presented as mean ± SD. N=3. Statistical analysis was performed using one-way ANOVA, followed by Dunnett's test for mean comparisons, using GraphPad Prism 6 software. Significance levels: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; ns - not significant.

In the analysis of inflammatory cytokine gene expression, the addition of E15 to untreated fibroblasts significantly decreased the expression of *IL-1β, IL-6,* and *COX-2* genes, with a non-

significant decrease in *TNF-α* expression (Figure 7). HGHL treatment, on the other hand, significantly increased the expression of all tested genes in both co-treatment and post-treatment experiments. In the post-treatment experiment, the addition of E15 after HGHL treatment significantly reduced the expression of all genes, except for *TNF-α* (Figures 7A, 7C, 7E, 7G). In the co-treatment experiment, E15 significantly reduced the expression of all genes, except *IL-6,* which was increased significantly, when compared to HGHL treatment alone (Figures 7B, 7D, 7F, 7H).

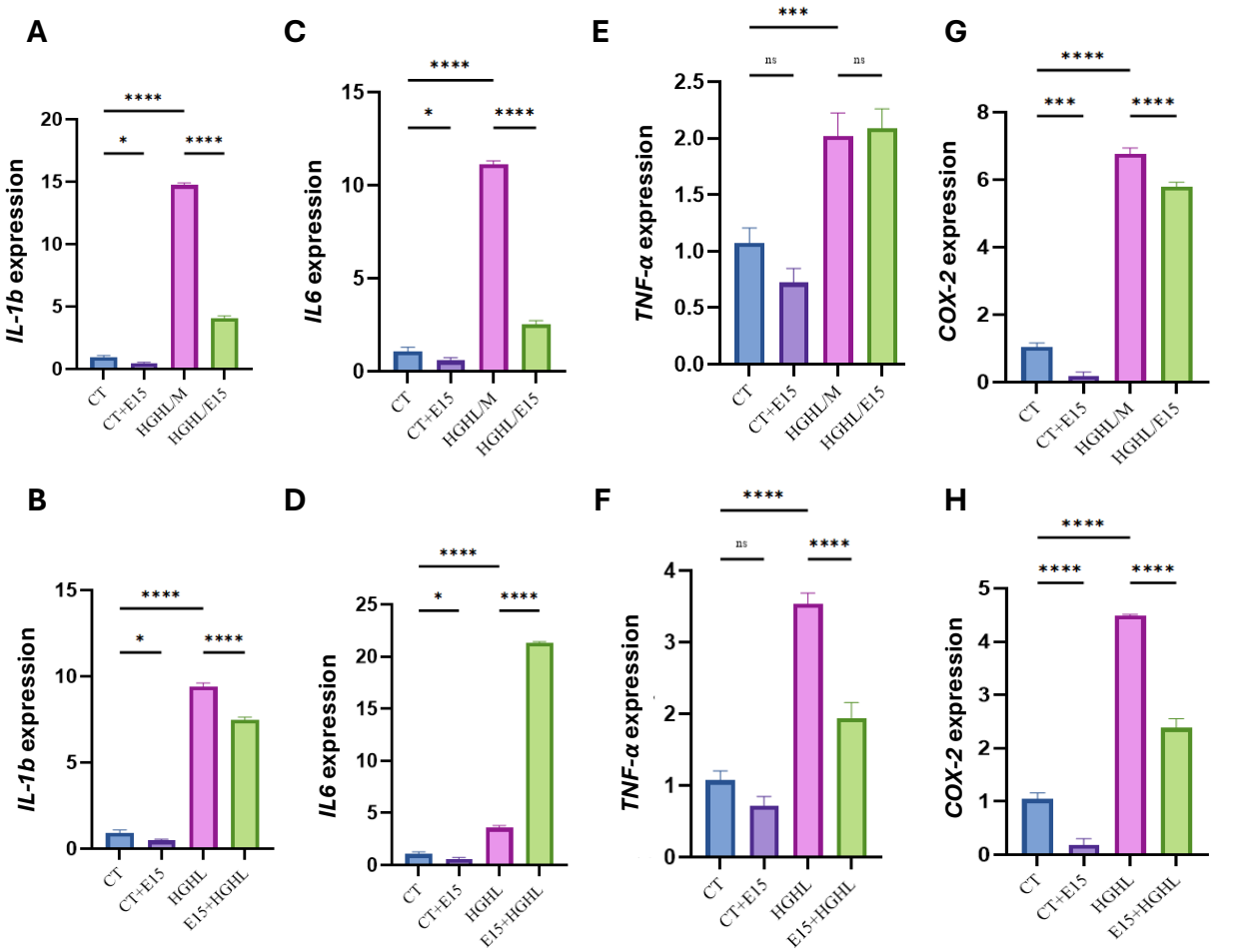



**Figure 7. Effect of eugenol on the expression of inflammatory cytokines in untreated and HGHL-treated fibroblasts**

*IL-1β* expression in post-treatment (A) and co-treatment (B). *IL-6* expression in post-treatment (C) and co-treatment (D). *TNF-α* expression in post-treatment (E) and co-treatment (F). *COX-2* expression in post-treatment (G) and co-treatment (H). CT - control (untreated fibroblasts); CT+E15 – eugenol 15 µM added to untreated fibroblasts; HGHL - high glucose (25 mM) and

high lipid (400 µM) treatment; HGHL/M – media added to HGHL treatment sample; HGHL/E15 - 15 µM eugenol added post HGHL induction; E15+HGHL - 15 µM eugenol added together with HGHL as a co-treatment. Data are presented as mean ± SD. N=3. Statistical analysis was performed using one-way ANOVA, followed by Dunnett's test for mean comparisons, using GraphPad Prism 6 software. Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns - not significant.

### 3.5. Discussion

In this study, we induced cellular aging in fibroblasts by exposing them to a combination of high glucose (25 mM) and high lipid (palmitic acid 400 µM) conditions. This approach was designed to mimic an environment associated with metabolic stress, commonly observed in conditions like obesity and type 2 diabetes.

However, prior to our investigation, the combined effect of HGHL conditions on dermal fibroblasts had not been studied in-depth. Additionally, most studies have primarily focused on UV radiation or $H_2O_2$-induced aging in skin fibroblasts, while metabolic aging has received less attention, despite its growing relevance (Pole et al., 2016; Song et al., 2024).

In this study, we aimed to fill this gap by providing clear evidence that the combination of these two metabolic stressors not only induces cellular senescence but also results in alterations in cell viability (Figure 1), cell cycle progression (Figure 4), and apoptosis (Figure 3). Additionally, we observed increased senescence-associated β-galactosidase (SA-β-gal) activity (Figure 2), enhanced oxidative stress markers, and significant changes in the expression of inflammatory (Figure 7) and ECM-related genes, such as *collagen* and *elastin* (Figure 6). Furthermore, wound healing assays (Figure 5) demonstrated a decline in fibroblast migration under these conditions.

Eugenol, a phenolic compound from clove oil, has gained interest in anti-aging skincare due to its strong antioxidant, anti-inflammatory, and antimicrobial properties (Ouadi et al., 2022; Hirose et

al., 2024). Its biocompatibility and biosynthesis via the phenylpropanoid pathway make it a promising natural agent in dermatology. Recent nano-encapsulation techniques have improved its bioavailability and reduced cytotoxicity, enhancing its therapeutic potential (Sahlan et al., 2021). Eugenol's ability to reduce oxidative stress and AGEs is particularly beneficial under HGHL conditions (Nisar et al., 2021; Damasceno et al., 2024). It activates the Nrf2/ARE pathway, boosting antioxidant defenses and collagen synthesis in fibroblasts (Ma et al., 2021; Wang et al., 2022), while also increasing catalase activity and scavenging free radicals (Barboza et al., 2018).

### 3.5.1. Effect of HGHL and Eugenol on Cell Viability and Senescence

In our study, the 15 μM eugenol treatment (E15) significantly enhanced fibroblast viability under both normal and HGHL conditions. E15 showed no cytotoxicity in untreated cells and, notably, produced the highest viability compared to the control group (CT) and other concentrations tested (Figure 1). Moreover, E15 was the most effective dose when used as a post-treatment or co-treatment with HGHL, outperforming HGHL alone, HGHL/M, and other eugenol concentrations (Figure 1).

Several studies have demonstrated that high glucose conditions significantly reduce cell viability across various cell types, including fibroblasts. For instance, Zhang et al. (2019) showed that exposure to high glucose (50 mM) in retinal pigment epithelial (RPE) cells led to increased ROS generation, apoptosis, and reduced proliferation and mitophagy. Similarly, Gojani et al. (2023) reported that exposure to high glucose (25 mM) and palmitic acid (400 μM) for 48 hours in beta cells decreased viability and induced apoptosis, highlighting the compounded effects of metabolic stressors.

In the context of lipid metabolism, studies like Zheng et al. (2023) found that elevated dietary intake of saturated fatty acids is associated with increased mortality and aging-related diseases in the elderly. Furthermore, Ford (2010) reported that aging and high lipid levels are linked through p53 activation, which alters fatty acid metabolism, increases intracellular palmitate, and promotes apoptosis or senescence via mitochondrial dysfunction (Ford, 2010; Zhang et al., 2019; Zheng et al., 2023).

Consistent with these findings, our MTT assay revealed a significant reduction in fibroblast viability after 48 hours of HGHL exposure (25 mM glucose + 400 μM palmitate), confirming the detrimental effects of these metabolic conditions on cell survival (Figure 1A, 1B).

Interestingly, eugenol has shown promise in promoting cell viability. Absalan et al. (2017) demonstrated that eugenol improved viability and reduced aging markers in human adipose-derived stem cells.

The reduction in the cell viability in response to HGHL is not only indicative of cytotoxicity but also suggest a progression toward cellular senescence, a central feature of aging —which is supported by the observed increase in β-galactosidase activity (Figure 2).

Increased activity of β-galactosidase (SA-β-gal) is a widely recognized marker of senescence (Dodig et al., 2019). For instance, Rouault et al. (2021) demonstrated that high glucose exposure increases SA-β-gal activity, further linking metabolic stress to the onset of cellular aging and metabolic disease.

Building on this, we examined the effects of eugenol on cellular senescence. As expected, HGHL exposure elevated β-gal activity, indicating enhanced senescence. However, treatment with 15 μM

eugenol (E15) reduced β-galactosidase activity in untreated fibroblasts compared to all other groups, including the control (CT), suggesting a potential anti-senescence effect even under basal conditions (Figure 2). Notably, both co-treatment and post-treatment with E15 effectively significantly lowered β-gal activity compared to HGHL and HGHL/M treatments, demonstrating eugenol's ability to attenuate senescence even under metabolic stress (Figure 2). These findings support the hypothesis that eugenol mitigates the accumulation of senescent cells, contributing to its protective role in skin aging (Tong et al., 2024).

### 3.5.2. Effect of HGHL and Eugenol on Cell Cycle

In addition to promoting senescence, HGHL conditions have also been associated with dysregulated cell cycle progression. Our findings align with previous studies highlighting the impact of metabolic stress on cell cycle regulation. Kim et al. (2019) demonstrated that high glucose and insulin levels promote cell cycle progression in bladder epithelial cells by upregulating cyclins (e.g., cyclin D) and cyclin-dependent kinases (Cdk4, Cdk2), facilitating S phase entry. Similarly, Xu et al. (2023) reported that elevated lipid levels activate lipid biosynthesis through squalene epoxidase (SQLE), promoting proliferation and advancing cell cycle progression. Together, these findings suggest that metabolic stressors such as HGHL levels can disrupt cellular homeostasis and contribute to aging-related diseases or cancer.

As a consequence of HGHL stress, cells experience oxidative damage, which activates DNA damage checkpoints during the S and G2 phases to pause the cycle and allow for repair. The accumulation of cells in these phases reflects an adaptive response aimed at preventing the propagation of damaged DNA, making S/G2 arrest a well-known hallmark of cellular stress (Hamdulay et al., 2010). In line with this, our cell cycle analysis showed a significant increase in

the proportion of fibroblasts in the S phase following HGHL exposure, indicating disrupted regulatory control and elevated cellular stress (Figure 4).

Eugenol, on the other hand, has been shown to arrest cell cycle progression in the S phase and inhibit E2F1 activity in melanoma cells, resulting in growth suppression (Ghosh et al., 2005). In our study, treatment with 15 μM eugenol (E15) maintained normal cell cycle progression under control conditions by retaining a higher proportion of cells in the G1 phase. Moreover, E15 effectively countered the cell cycle disruption caused by HGHL, reducing the percentage of cells in the S phase and restoring cell cycle balance (Figure 4). These results suggest that eugenol supports cellular homeostasis and fibroblast function by modulating the cell cycle under both normal and stress-induced conditions.

### 3.5.3. Effect of HGHL and Eugenol on Apoptosis

In this study, the apoptosis assay revealed that HGHL conditions induced significant apoptosis in fibroblasts, reflecting the detrimental effects of these stressors. Treatment with 15 μM eugenol did not significantly induce apoptosis under normal conditions. However, in post-treatment and co-treatment scenarios, E15 significantly reduced apoptosis induced by HGHL (Figure 3), suggesting that eugenol can significantly protect against stress-induced cell death in some conditions. These findings align with existing research demonstrating eugenol's potential in modulating apoptosis, particularly under stress conditions (Wang et al., 2022).

Cell cycle progression and apoptosis are distinct processes, but disturbances in either can lead to significant effects on cellular function and survival. Apoptosis assays revealed that HGHL conditions induce apoptosis compared to other treatments (Figure 3), which aligns with previous

studies. For instance, research on the role of TSPAN8 in regulating autophagy and apoptosis in HK-2 cells exposed to high glucose conditions demonstrated that high glucose could trigger apoptosis in various cell types, mimicking the environment of diabetic nephropathy (Zhuang et al., 2022). Additionally, other study showed that HGHL conditions induce significant apoptosis in pancreatic β-cells, impairing insulin secretion. Treatment with telmisartan effectively reduced this HGHL-induced apoptosis, further emphasizing the detrimental impact of HGHL levels on β-cell function (Wang et al., 2019).

Eugenol has also been shown to offer protective effects in other contexts, such as against transmissible gastroenteritis virus (TGEV)-induced intestinal damage. It achieves this by reducing oxidative stress and inhibiting apoptosis in intestinal epithelial cells. Studies have demonstrated that eugenol supplementation lowers ROS levels, thereby decreasing oxidative stress and preventing apoptosis in both *in vitro* and *in vivo* models of TGEV infection. This suggests that eugenol could be a promising therapeutic strategy for managing apoptosis (Wang et al., 2022).

### 3.5.4. Effect of HGHL and Eugenol on the Expression of Proinflammatory Cytokines

Elevated glucose and lipid levels are known to impair wound healing by increasing the expression of proinflammatory cytokines, reducing collagen and elastin gene expression, and disrupting cell migration. These metabolic imbalances activate intracellular signaling pathways that trigger inflammatory responses (Glass et al., 2012; Zhang et al., 2019). In fibroblasts, high glucose conditions have been shown to upregulate cytokines such as *IL-6* and *TNF-α*, primarily through activation of the nuclear factor kappa B (NF-κB) pathway, as demonstrated by Du et al. (2016) and Shi et al. (2019). Similarly, Hasan et al. (2019) reported that palmitate, a saturated fatty acid,

stimulates *IL-6* and *TNF-α* expression in fibroblasts via the Toll-like receptor 4 (TLR4) signaling pathway.

In our study using dermal fibroblasts, HGHL conditions led to a marked increase in inflammatory cytokine expression, consistent with previous findings (Figure 7).

Eugenol, known for its antioxidant properties, also exhibits significant anti-inflammatory activity, enhancing its potential as an anti-aging compound. It inhibits NF-κB activation, thereby reducing the expression of proinflammatory mediators such as *IL-1β, TNF-α*, *IL-6*, and *COX-2* (Harb et al., 2019; Wang et al., 2022; Damasceno et al., 2024), which supports our results with some exceptions. Additionally, eugenol acts as a TRPV1 receptor agonist, helping to mitigate cellular stress and inflammation, both of which contribute to skin aging.

In the case of *IL-6*, the highest expression was observed in fibroblasts treated with E15 and HGHL. Post-treatment with E15 led to a significant reduction in *IL-6* levels, whereas co-treatment with HGHL surprisingly increased *IL-6* expression compared to HGHL alone. This suggests that eugenol's regulation of *IL-6* may be context-dependent and influenced by the timing and nature of exposure (Figure 7).

While the consistent downregulation of *IL-1β* and *COX-2* across different treatment strategies supports eugenol's broad anti-inflammatory potential, its effects on *IL-6* and *TNF-α* appear more variable. The increase in *IL-6* during co-treatment highlights the need for a tailored therapeutic approach, considering the specific inflammatory environment. Similarly, the efficacy of eugenol in reducing *TNF-α* through co-treatment—but not post-treatment—emphasizes the importance of treatment timing in maximizing its therapeutic benefits.

### 3.5.5. Effect of HGHL and Eugenol on the Expression of ECM Components

Proinflammatory cytokines such as IL-6 and TNF-α negatively impact ECM synthesis by downregulating the production of key structural proteins like collagen and elastin, which are essential for maintaining skin integrity and elasticity. Kuk (2014) demonstrated that TNF-α suppresses type I collagen expression in human dermal fibroblasts by inhibiting the transforming growth factor-beta (TGF-β)/Smad signaling pathway. Similarly, Anwar et al. (2012) reported that IL-6 reduced elastin expression in vascular smooth muscle cells by modulating elastin gene promoter activity. Although this finding pertains to smooth muscle cells, similar regulatory mechanisms are likely to occur in dermal fibroblasts.

The combined effects of elevated cytokine levels and diminished ECM protein expression can also hinder fibroblast migration, a key step in wound repair. Potekaev et al. (2021) emphasized the importance of ECM in supporting fibroblast motility, while Pastar et al. (2024) found that high TNF-α levels impaired fibroblast migration and proliferation by disrupting cytoskeletal organization and focal adhesion formation, ultimately delaying wound closure.

Consistent with these findings, our study showed that HGHL conditions significantly reduced the gene expression of *COL1A1*, *COL3A1*, and *ELN* in fibroblasts (Figure 6). Additionally, wound healing assays revealed impaired fibroblast migration under these metabolic stress conditions (Figure 5). These results reinforce the idea that hyperglycemia and hyperlipidemia compromise ECM production and fibroblast function, thereby limiting tissue repair capacity.

Eugenol contributes to ECM preservation and skin regeneration through multiple mechanisms. It inhibits the formation of AGEs, which are detrimental sugar–protein or lipid complexes that stiffen

the ECM, reduce elasticity, and accelerate skin aging. Eugenol lowers blood glucose levels and competes with sugars for binding to serum albumin, thereby protecting collagen integrity (Singh et al., 2016; Gojani et al., 2023). Furthermore, eugenol shields ECM components from both glycation and oxidative stress, supporting structural stability (Barboza et al., 2018). It also modulates pathways involved in ECM remodeling and cytokine–cytokine receptor interactions, which are crucial for tissue regeneration (Damasceno et al., 2024).

In our experiments, treatment with 15 μM eugenol (E15), both as a post-treatment and co-treatment, led to a modest, though non-significant, increase in *COL1A1* and *COL3A1* expression relative to HGHL/M and HGHL groups. Notably, E15 treatment in untreated fibroblasts significantly elevated *COL3A1* expression compared to all other groups, including controls. Co-treatment with E15 significantly increased ELN gene expression compared to HGHL treatment alone (Figure 6), indicating a protective effect on ECM components. This enhancement suggests that E15 may be beneficial when used as a co-treatment, especially in comparison to the positive control condition represented by HGHL.

The wound healing assay further evaluated eugenol's regenerative potential. Fibroblasts treated with 15 μM eugenol (E15), both alone and under HGHL conditions, exhibited a slight improvement in wound closure rates compared to their respective controls. However, none of these improvements reached statistical significance, indicating a limited but observable trend toward enhanced healing (Figure 5).

These findings align with a study by Ashjazadeh et al. (2019), which demonstrated that eugenol-loaded nanofibers enhanced granulation tissue formation and collagen production in Wistar rats, thereby accelerating wound healing (Ashjazadeh et al., 2019; Tahlia et al., 2019). Their results

support the hypothesis that eugenol promotes tissue regeneration by stimulating collagen synthesis and repair processes.

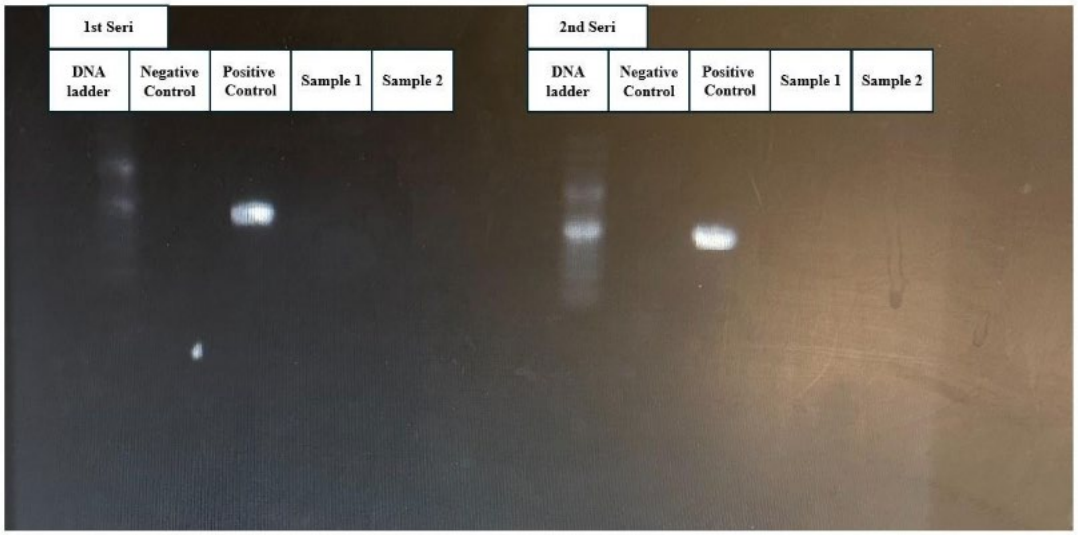
### 3.6. Conclusion

In conclusion, this study provides compelling evidence that eugenol, particularly at a concentration of 15 μM in some conditions, offers significant protective effects against skin aging and damage induced by HGHL conditions. Eugenol mitigates cellular senescence, apoptosis, oxidative stress, and inflammation through its antioxidant, anti-inflammatory, and AGE-reducing properties, while also promoting ECM synthesis. These findings support the potential of eugenol as a therapeutic agent for skin aging, offering a promising avenue for anti-aging skincare treatments. Further research is necessary to optimize the dosage and explore the long-term effects of eugenol in clinical settings.

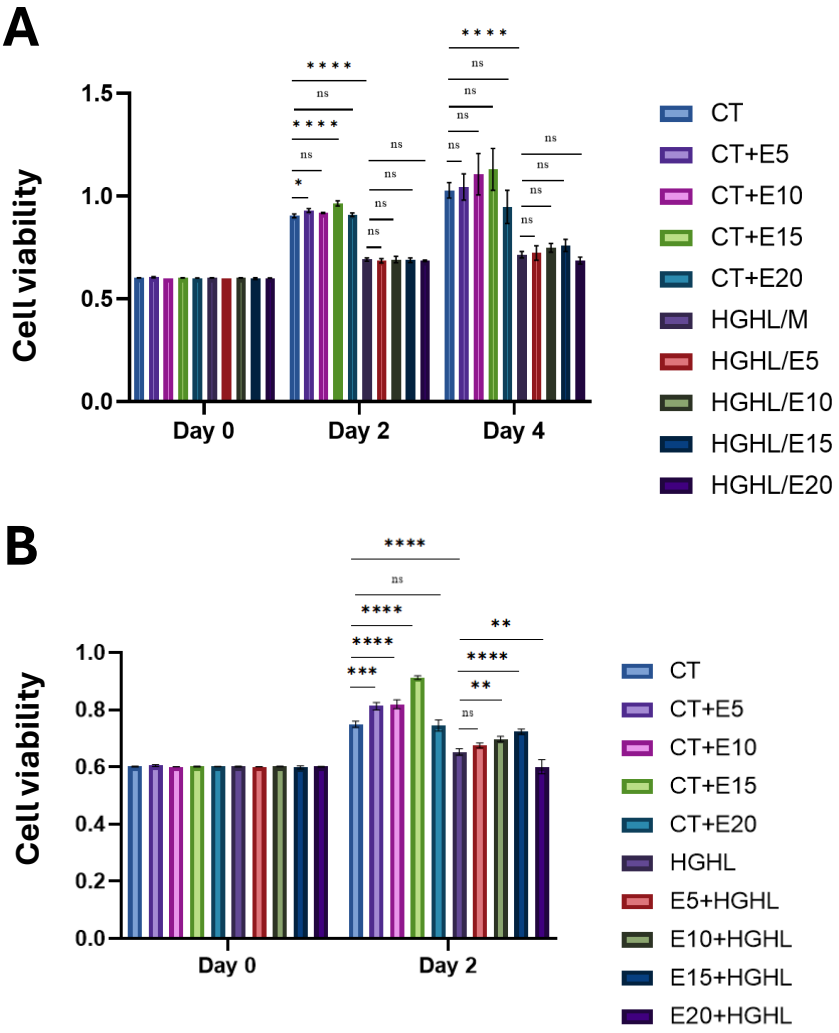
### 3.7. Future Direction

Future research should focus on optimizing eugenol concentrations for different skin types and exploring its underlying molecular mechanisms, particularly related to oxidative stress, inflammation, and ECM remodeling. Long-term safety and efficacy need to be evaluated through animal models and clinical trials to assess its potential for topical skincare products. Investigating eugenol's synergy with other anti-aging compounds and its effects on various skin conditions, such as wound healing and inflammatory diseases, would expand its therapeutic applications. Additionally, exploring eugenol in advanced drug delivery systems could enhance its bioavailability and effectiveness in skin treatments.

### 3.8. Supplementary Results




**Supplementary Figure 1.** PCR-based mycoplasma detection in BJ-5TA fibroblast cultures. *Left gel:* DNA ladder (lane 1), negative control (lane 2), positive control (lane 3), BJ-5TA samples (lanes 4–5) — all samples tested negative. *Right gel:* Replicate assay showing identical lane arrangement and confirming negative results for both BJ-5TA samples.




**Supplementary Figure 2. Effects of different concentrations of eugenol on the cell viability in post-treatment (A) and co-treatment (B) of untreated and HGHL-induced fibroblasts.** Data shows the cell viability (MTT assay) Y axis shows absorbance at 595 nm) of fibroblasts measured at days 0, 2 and 4 after HGHL treatment (A) and day 0 and day 2 co-treatment of eugenol and HGHL (B). CT - Control (untreated fibroblasts); CT+E5, E10, E15 and E20 - eugenol 5, 10, 15 and 20 µM added to untreated fibroblasts; E5, E10, E15, E20 - eugenol at 5, 10, 15, 20 µM; HGHL - high glucose (25 mM) and high lipid (400 µM) treatment; HGHL/M – media added to HGHL treatment sample; HGHL/E5, E10, E15, E20 - eugenol at various doses added post HGHL induction; E5, E10, E15, E20+HGHL- eugenol at different doses added together with HGHL as a co-treatment. Data are presented as mean ± SD. N=3. The collected data was subjected to statistical analysis using one-way analysis of variance (ANOVA), followed by Dunnett's test for comparing mean values. GraphPad Prism 6 software was used to perform this analysis. Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns - not significant.

### 3.9. References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# 4. Chapter 4: General Discussion, General Conclusion and General Future Directions

## 4.1. General Discussion

This thesis explored the effects of two natural compounds, psilocybin and eugenol, on dermal fibroblast aging induced by HGHL conditions. The established model effectively mimicked aging-related metabolic stress and allowed for the evaluation of multiple biological markers, including oxidative stress, cell viability, cellular senescence, inflammation, ECM remodeling, apoptosis, and cell cycle disruption. Although both compounds were tested independently in separate studies, they were investigated under identical HGHL conditions, enabling a meaningful comparison of their anti-aging efficacy.

### 4.1.1. HGHL-Induced Aging as a Metabolic Skin Aging Model

Both studies validated the HGHL model (25 mM glucose and 400 μM palmitic acid) as a reliable *in-vitro* system for inducing premature aging in dermal fibroblasts. Results were obtained, including reduced cell viability (Figures 1, in chapter 2 and 3), increased SA-β-galactosidase activity (Figure 2, in chapter 2 and 3), elevated apoptosis (Figure 3, in chapter 2 and 3), inflammation (Figure 7, in chapter 2 and 3), impaired ECM gene expression (Figure 6, in chapter 2 and 3), and altered cell cycle progression (Figure 4, in chapter 2 and 3). These findings align with previous research indicating that HGHL levels promote oxidative stress, AGEs formation, mitochondrial dysfunction, senescence, and inflammatory cytokine release in aging-related models (Bian et al., 2020; Romer et al., 2021; Lu et al., 2022).

Importantly, while most skin-aging studies focus on UV radiation or $H_2O_2$-induced models (Pole et al., 2016; Song et al., 2024), our work highlights the underappreciated but clinically relevant impact of metabolic stress on dermal fibroblast aging.

### 4.1.2. Therapeutic Potential of Eugenol and Psilocybin in Combating Metabolic Stress-Induced Skin Aging

Both eugenol and psilocybin show anti-aging potential by reducing oxidative stress and inflammation. Eugenol activates the Nrf2/ARE pathway, limits AGE formation, and enhances collagen production under HGHL-induced stress (Ma et al., 2021; Nisar et al., 2021; Ouadi et al., 2022). Psilocybin, through serotonergic signaling, decreases pro-inflammatory cytokines and promotes cellular repair (Park et al., 2021; Hecker et al., 2023; Meyer & Slot, 2023). These complementary mechanisms support their role in protecting fibroblasts and maintaining skin health under metabolic stress (Lowe et al., 2021).

### 4.1.3. Effect of Psilocybin and Eugenol on Cell Viability and Senescence under HGHL Conditions

Our results demonstrated that HGHL exposure significantly decreased fibroblast viability and increased cellular senescence, aligning with prior findings that chronic metabolic stress impairs cell survival through mitochondrial dysfunction and increased ROS generation (Zhang et al., 2019; Cialdai et al., 2022; Gojani et al., 2023). These detrimental effects were confirmed through MTT assays, where HGHL (25 mM glucose + 400 μM palmitate) reduced cell viability (Figure 1, Chapters 2 and 3), and β-galactosidase activity assays, which revealed elevated SA-β-gal levels in

HGHL-treated fibroblasts (Figure 2, Chapters 2 and 3), consistent with established aging markers (Dodig et al., 2019; Rouault et al., 2021).

To counteract these effects, we evaluated the protective potential of two natural compounds: psilocybin and eugenol. Building on previous work with beta cells (Gojani et al., 2024), we tested a range of psilocybin concentrations in fibroblasts, finding that 5–15 μM improved cell viability without inducing toxicity, while 20 μM was cytotoxic (Supplementary Figure 1, Chapter 2). Psilocybin at 15 μM did not exert any cytotoxic effects on untreated fibroblasts; in fact, it enhanced cell viability compared to all other conditions, including the CT negative control (Figure 1, Chapter 2).

In the post-treatment study, on day 4 psilocybin at 15 μM (P15) resulted in the highest cell viability compared to the HGHL/M positive control, indicating a favorable safety profile under stressed conditions. In the co-treatment study, on day 2 psilocybin at 10 μM (P10) showed the greatest cell viability relative to HGHL alone. Based on these results, these concentrations were selected for subsequent experiments involving psilocybin (Figure 1, Chapter 2).

Furthermore, both P10 (co-treatment) and P15 (post-treatment) significantly reduced SA-β-gal activity under HGHL, with P15 showed non-significant decreasing enzyme activity in untreated cells below control levels (Figure 2, Chapter 2), suggesting potential basal anti-senescence effects (Johnson et al., 2017; Smedfors et al., 2022). These results are supported by recent studies showing that psilocybin enhances mitochondrial efficiency, reduces oxidative stress, and downregulates senescence markers via serotonin-mediated pathways (Hecker et al., 2023; Fissler et al., 2023).

Similarly, eugenol 15 μM (E15) significantly enhanced fibroblast viability in both untreated and HGHL-treated cells, showing the strongest effect on day 2 under co-treatment and on day 4 under post-treatment conditions (Figure 1, Chapter 3). Unlike other concentrations, E15 improved viability beyond control levels without cytotoxic effects, supporting findings from previous work on adipose-derived stem cells (Absalan et al., 2017). In terms of senescence, E15 reduced β-galactosidase activity even in untreated fibroblasts and significantly lowered SA-β-gal levels under HGHL in both co- and post-treatment groups (Figure 2, Chapter 3), further demonstrating its protective role. These findings align with earlier reports that eugenol mitigates oxidative stress and cellular senescence, inhibits AGEs formation, and supports mitochondrial health under metabolic stress (Ford, 2010; Zheng et al., 2023; Tong et al., 2024).

Both psilocybin and eugenol enhanced fibroblast viability and reduced HGHL-induced senescence. Psilocybin at 15 μM showed the strongest effects in post-treatment and even improved viability in untreated fibroblasts, while P10 was most effective in co-treatment. E15 demonstrated consistent benefits across both untreated and HGHL-treated cells in both co- and post-treatment conditions.

### 4.1.4. Modulation of Cell Cycle Dysregulation by Psilocybin and Eugenol under HGHL Stress

Our study confirmed that exposure to HGHL conditions significantly disrupts normal cell cycle progression in dermal fibroblasts. This disruption was characterized by increased accumulation of cells in the S phases (Figure 4, Chapters 2 and 3), consistent with oxidative damage and checkpoint activation seen under metabolic stress (Hamdulay et al., 2010). HGHL overload is known to impair mitochondrial function, induce ER stress, and activate the UPR, which together trigger key

senescence-associated arrest pathways such as p53/p21 and p16/Rb (Casas-Martinez et al., 2024). This results in heightened replication stress and reduced G1-phase maintenance—hallmarks of premature senescence and genomic instability (Avelar, 2023).

Our findings align with previous literature showing that high glucose and insulin levels promote S-phase entry via upregulation of cyclins and CDKs (Kim et al., 2019), while elevated lipids drive proliferative signals through enhanced lipid biosynthesis (Xu et al., 2023). Together, these stressors push fibroblasts toward aberrant cell cycle progression, increasing vulnerability to aging-related dysfunction.

To counteract this disruption, we tested psilocybin and eugenol for their modulatory effects on cell cycle dynamics. Treatment with psilocybin—specifically 10 μM in co-treatment and 15 μM in post-treatment—helped cell cycle balance by reducing the S-phase population and increasing G1-phase retention under HGHL stress. Co-treatment with P10 fully reversed the HGHL-induced shifts, while P15 post-treatment significantly alleviated replication stress (Figure 4, Chapter 2). These results suggest that psilocybin stabilizes cell cycle regulation and promotes healthy proliferation in fibroblasts subjected to metabolic insult. Supporting this, Hecker et al. (2023) reported that psilocybin reduces cell-cycle arrest markers, enhances DNA replication, and decreases SASP-related oxidative stress in senescent human cells, positioning it as a geroprotective agent without direct senolytic effects.

In parallel, eugenol treatment at 15 μM also demonstrated the ability to correct HGHL-induced cell cycle imbalance. In unstressed conditions, E15 maintained a higher proportion of cells in the G1 phase, indicating no adverse effects on healthy cell cycling. When applied as co- or post-treatment, it significantly reduced the percentage of cells in the S phases, restoring cell cycle

progression toward a normal distribution (Figure 4, Chapter 3). While eugenol has been shown to suppress proliferation in cancer cells by inhibiting E2F1 and inducing S-phase arrest (Ghosh et al., 2005). These results suggest that eugenol supports cellular homeostasis and fibroblast function by modulating the cell cycle and mitigating oxidative stress, thereby promoting healthy proliferation under both normal and HGHL-induced stress conditions.

Psilocybin and eugenol both helped HGHL-induced cell cycle imbalance by reducing S/G2 accumulation and increasing G1-phase retention. Psilocybin was more effective in co-treatment P10, while eugenol, E15 showed modulation under both normal and stressed conditions.

### 4.1.5. Modulation of HGHL-Induced Apoptosis by Psilocybin and Eugenol

Apoptosis is a key outcome of prolonged metabolic stress in fibroblasts, particularly when oxidative damage exceeds the cell's capacity for repair. Our study demonstrated that exposure to HGHL conditions significantly increased apoptosis in dermal fibroblasts (Figure 3, Chapters 2, and 3), reflecting cellular damage and mitochondrial dysfunction triggered by HGHL accumulation. This aligns with prior studies showing that chronic glucose exposure activates the MAPK pathway and increases MMP expression, leading to ECM degradation and programmed cell death (Xuan et al., 2014). Accumulated AGEs further contribute to apoptosis by stiffening collagen and elastin, impairing wound healing, and increasing tissue fragility—effects also reflected in our ECM gene expression and wound closure results (Figures 5 and 6, Chapters 2, and 3) (Van Putte et al., 2016).

Our apoptosis assay revealed that both psilocybin and eugenol had helpful effects in modulating apoptotic responses under stress conditions. Psilocybin, when applied alone to untreated

fibroblasts, resulted in a small but significant increase in apoptosis. Moreover, it did not reduce apoptosis when applied as a post-treatment following HGHL exposure but was effective in co-treatment, where it significantly mitigated HGHL-induced cell death (Figure 3, Chapter 2). These findings suggest a context-dependent effect, where psilocybin may support cell survival under stress but induce apoptosis in healthy cells, possibly due to cell-type-specific sensitivity or dose-related mitochondrial signaling. This complexity aligns with Ghasemi Gojani et al. (2024), who reported that psilocybin reduced β-cell loss under HGHL conditions by modulating apoptotic markers and reducing phosphorylation of TXNIP, STAT-1, and STAT-3. Our findings reinforce the need for caution when applying psilocybin outside of stress contexts, as it may activate pro-apoptotic pathways in certain conditions.

In contrast, eugenol demonstrated a more consistently protective effect. HGHL-induced apoptosis was significantly reduced in both co-treatment and post-treatment with 15 μM eugenol (Figure 3, Chapter 3). E15 did non-significant induce apoptosis in untreated fibroblasts, indicating safety under normal conditions, especially when compared to psilocybin-treated fibroblasts in the same context These findings are consistent with studies showing eugenol's ability to reduce oxidative stress and inhibit apoptosis in models of intestinal epithelial damage caused by transmissible gastroenteritis virus (Wang et al., 2022). Similarly, its ROS-scavenging activity has been linked to protection against apoptosis in various stress models, including diabetic nephropathy (Zhuang et al., 2022) and HGHL-exposed β-cells (Wang et al., 2019).

These results indicate that while both compounds can mitigate HGHL-induced apoptosis, eugenol exhibits a broader safety margin, effectively reducing stress-induced cell death without affecting healthy cells. In contrast, psilocybin's effects appear to be more nuanced and context-specific,

offering protection in the presence of metabolic stress but not under normal conditions. These contrasting profiles highlight the potential for differential application of these compounds: psilocybin as a co-treatment during acute stress, and eugenol as a safer, continuous protective agent against metabolic injury.

### 4.1.6. Anti-Inflammatory Activity of Psilocybin and Eugenol under HGHL-Induced Stress

Chronic exposure to elevated glucose and lipid levels is known to initiate and sustain low-grade inflammation, which contributes to skin aging by increasing proinflammatory cytokine expression, degrading ECM components, and impairing tissue regeneration (Glass et al., 2012; Zhang et al., 2019). This inflammatory response is primarily driven by intracellular signaling through NF-κB and Toll-like receptor pathways, leading to upregulation of cytokines such as IL-6, TNF-α, IL-1β, and COX-2 (Du et al., 2016; Shi et al., 2019; Hasan et al., 2019). Consistent with this, our results showed that HGHL exposure significantly elevated cytokine expression in dermal fibroblasts (Figure 7, Chapters 2 and 3).

Treatment with eugenol effectively mitigated this inflammatory response. Both post-treatment and co-treatment with 15 μM eugenol significantly reduced *IL-1β* and *COX-2* expression compared to HGHL and HGHL/M groups, confirming its anti-inflammatory properties (Figure 7A, 7B, 7G, 7H, Chapter 3). This aligns with existing research showing that eugenol inhibits NF-κB and downregulates key inflammatory mediators (Harb et al., 2019; Wang et al., 2022; Damasceno et al., 2024). However, eugenol's effect on *IL-6* and *TNF-α* appeared context-dependent. While post-treatment reduced *IL-6* levels, co-treatment paradoxically increased *IL-6* expression compared to HGHL alone. Similarly, eugenol effectively reduced *TNF-α* expression in co-treatment, but post-treatment did not significantly change it over HGHL/M. Nonetheless, E15 reduced basal cytokine

levels in untreated fibroblasts, suggesting low-toxicity and broad anti-inflammatory capacity even under normal conditions (Figure 7C, 7D, 7E, 7F, Chapter 3).

In parallel, psilocybin demonstrated a strong anti-inflammatory effect characterized by selective modulation of individual cytokines. Psilocybin has previously been shown to modulate inflammation via NF-κB, STAT1, and STAT3, and to reduce proinflammatory mediators such as nitric oxide, prostaglandin E2, and IL-1β in LPS-stimulated macrophages (Nkadimeng et al., 2020; Nkadimeng et al., 2021; Ghasemi Gojani, 2023). In our study, P15 post-treatment and application to untreated fibroblasts significantly reduced *IL-1β* and *IL-6* levels, while P10 co-treatment reduced *IL-1β*, but it significantly increased *IL-6* gene expression compared to the HGHL-treated group (Figure 7A, 7B, 7C and 7D, chapter 2). This pattern suggests that psilocybin's anti-inflammatory effects are dose- and context-dependent, consistent with the literature (Nichols, 2020; Burmester et al., 2023).

Psilocybin's effects on *TNF-α* were more complex. In untreated fibroblasts, P15 unexpectedly increased *TNF-α* expression, and post-treatment under HGHL conditions did not reduce *TNF-α* levels. Only P10 co-treatment showed a suppressive effect on *TNF-α*, suggesting variability in psilocybin's regulatory effect depending on cell state and exposure sequence (Figure 7E, 7F, Chapter 2). Both psilocybin concentrations, however, significantly reduced *COX-2* expression, highlighting a consistent anti-inflammatory action on this key enzyme (Figure 7G, 7H, Chapter 2).

Our results, suggest that both compounds demonstrated the ability to modulate inflammation under metabolic stress, but with different profiles. Eugenol exhibited a more stable and predictable anti-inflammatory effect across most cytokines, while psilocybin showed potent but variable effects that depended on concentration, and cellular context.

### 4.1.7. Protective Roles of Psilocybin and Eugenol in ECM Preservation and Skin Structure under Metabolic Stress

The integrity of the ECM is critical for maintaining skin elasticity, tensile strength, and regenerative capacity. Inflammatory cytokines such as IL-6 and TNF-α are well-established inhibitors of ECM biosynthesis, suppressing collagen and elastin gene expression through pathways such as TGF-β/Smad inhibition and elastin promoter downregulation (Anwar et al., 2012; Kuk, 2014). These inflammatory signals, often triggered under hyperglycemic and hyperlipidemic conditions, further compromise fibroblast migration and wound repair (Potekaev et al., 2021; Pastar et al., 2024). In our model, HGHL treatment significantly decreased the expression of *COL1A1*, *COL3A1*, and *ELN* in fibroblasts and impaired wound healing, confirming the disruptive impact of metabolic stress on ECM structure and cellular mobility (Figures 5 and 6, Chapters 2 and 3).

Both eugenol and psilocybin showed protective effects on ECM components, though through distinct mechanisms. Eugenol contributed to ECM preservation by reducing AGE formation, combating oxidative stress, and shielding structural proteins from glycation-induced rigidity (Singh et al., 2016; Barboza et al., 2018; Gojani et al., 2023). It has also been shown to modulate cytokine–receptor interactions and ECM remodeling pathways, promoting collagen stability (Damasceno et al., 2024). In our study, treatment with 15 µM eugenol modestly increased *COL1A1* and *COL3A1* expression under HGHL conditions, with a notable, significant increase in *COL3A1* in untreated cells. Co-treatment with E15 significantly increased *ELN* gene expression compared to HGHL treatment alone (Figure 6, Chapter 3), indicating a protective effect on ECM components, suggesting that eugenol enhances ECM biosynthetic potential, particularly for elastic

fibers. These gene expression trends were supported by wound healing assays, where eugenol-treated fibroblasts showed faster closure rates, especially in the absence of stress (Figure 5, Chapter 3). These findings align with prior *in-vivo* studies showing that eugenol-loaded biomaterials enhance granulation tissue formation and collagen deposition (Ashjazadeh et al., 2019; Tahlia et al., 2019).

Psilocybin, while less studied in dermatological models, demonstrated promising ECM-modulating properties in our study. HGHL conditions downregulated *COL1A*, *COL 3A* and *ELN*, but treatment with psilocybin—especially P10 co-treatment—significantly restored *ELN* expression, compared to HGHL treatment alone (Figure 6, Chapter 2). While effects on collagen genes were less pronounced, the marked upregulation of elastin suggests that psilocybin may help preserve skin elasticity and resist ECM degradation under metabolic stress.

This protective effect likely stems from psilocybin's ability to reduce oxidative stress and modulate mitochondrial function (Flanagan & Nichols, 2018; Dewhirst, 2023; Rahman et al., 2024). Elevated ROS levels activate MAPK pathways, enhancing MMP activity and accelerating ECM protein breakdown (Ahuja et al., 2024), while senescence-associated inflammation further enhances this degradation (Nikolakis et al., 2016; Wang & Dreesen, 2018). Additionally, metabolic stress—via hyperglycemia and lipid overload—leads to ECM stiffening through AGE cross-linking and impairs remodeling enzymes (Lee et al., 2021; Gui et al., 2024). Psilocybin appears to buffer fibroblasts against these stressors by targeting both oxidative and metabolic disruptions.

Furthermore, serotonin receptor activation—particularly via 5-HT2A and 5-HT1B—has been shown to stimulate fibroblast proliferation, DNA synthesis, and even type IV collagen production

in mesangial cells (Barrett, 2017; Tarbit, 2019). These findings support the hypothesis that psilocybin's serotonergic signaling may enhance fibroblast function and ECM remodeling.

Compared to classical anti-aging agents such as metformin or vitamin D, which act mainly via AMP-activated protein kinase (AMPK)-related pathways, psilocybin exerts broader serotonin-driven effects—impacting mitochondrial biogenesis, inflammation, and even epigenetic markers of longevity (He et al., 2023; Shin et al., 2023; Larrea et al., 2024; Meloni, 2024).

Although its ECM-specific mechanisms warrant further investigation, our findings indicate that psilocybin supports ECM stability under metabolic stress and exhibits a modest, non-significant yet consistent trend toward improved wound healing under both normal and stress conditions (Figure 5, Chapter 2).

Regarding results in this part, both eugenol and psilocybin provide protective effects against HGHL-induced ECM degradation and functional impairment in fibroblasts. While eugenol appears to directly enhance collagen and elastin gene expression, it also showed a trend toward improved wound repair, psilocybin may promote ECM integrity through serotonin-mediated mitochondrial and anti-inflammatory pathways. Notably, psilocybin also increased elastin gene expression, further supporting its role in preserving dermal structure. Although not statistically significant, psilocybin also showed a consistent trend toward improved wound healing, similar to the effects observed with eugenol.

## 4.2. General Conclusion

This study provides comprehensive evidence that both psilocybin and eugenol exert protective, anti-aging effects on dermal fibroblasts exposed to HGHL conditions—an *invitro* model of metabolic skin aging. The findings demonstrate that these two natural compounds, while mechanistically distinct, converge in their ability to enhance fibroblast viability, reduce oxidative and inflammatory damage, restore cell cycle balance, and preserve ECM integrity.

Psilocybin exhibited a concentration-dependent profile, with 10 μM and 15 μM differentially modulating key cellular processes. P15 was most effective in improving viability, and reducing senescence, while P10 more strongly reduced apoptosis and normalized cell cycle distribution. Psilocybin also downregulated the expression of pro-inflammatory cytokines such as *IL-1β, COX-2*, and *IL-6* just in post-treatment method. contributing to ECM preservation and supporting tissue regeneration. Its capacity to restore elastin gene expression relative to HGHL treatment alone (Figure 6), along with its ability to reduce β-galactosidase activity, further highlights its potential as a promising agent in skin rejuvenation strategies.

Eugenol, particularly at 15 μM, showed broad cytoprotective effects by decreasing apoptosis, senescence, and inflammation while promoting antioxidant defenses and collagen-elastin gene expression. E15 also non-significantly improved wound closure, with a notable increase in *COL3A1* expression on un-treated fibroblast compared to CT and *ELN* expression compared to HGHL. Its actions appear to be mediated by both ROS scavenging and inhibition of inflammatory pathways such as NF-κB and TLR4, as well as suppression of AGE formation—key contributors to dermal matrix damage under metabolic stress. Additionally, E15 restored normal cell cycle

distribution by reducing S phase accumulation, further supporting its role in maintaining fibroblast homeostasis and regenerative capacity.

Together, these findings highlight eugenol and psilocybin as promising natural compounds for skin aging intervention, with overlapping benefits in reducing inflammation and oxidative stress, and complementary strengths in enhancing regeneration and maintaining ECM integrity. Importantly, their context-dependent responses underscore the need for careful dose optimization and therapeutic targeting. These compounds align well with current dermatological strategies focusing on cellular resilience, inflammation control, and ECM stabilization, offering novel avenues for integrative, bioactive anti-aging skin therapies.

### 4.3. General Future Directions

Further research is essential to comprehensively validate the anti-aging effects of psilocybin and eugenol, particularly in *invivo* and clinical settings. For eugenol, future studies should focus on optimizing dosing strategies for various skin types and ethnicities, while also characterizing its full range of molecular targets beyond Nrf2/ARE—such as NF-κB, MAPKs, and mitochondrial function. Evaluation in 3D skin equivalents and organotypic cultures will provide more physiologically relevant insight into its effects on dermal architecture, collagen remodeling, and fibroblast-keratinocyte interactions. Additionally, nanoencapsulation and transdermal delivery systems should be explored to enhance bioavailability and sustained release.

For psilocybin, rigorous investigation is required to determine its long-term safety profile, especially under chronic application or systemic administration. Its dose-response effects should be defined in animal models of skin aging, with focus on serotonin-mediated regeneration

pathways, including 5-HT2A receptor activity in dermal fibroblasts and interactions with downstream signaling such as cAMP response element-binding protein (CREB) and extracellular signal-regulated kinase (ERK). Studying its impact on stem cell activation, mitochondrial biogenesis, and senescence reversal in aged or diabetic animal models could yield valuable insights.

Moreover, exploring synergistic or additive effects of psilocybin and eugenol—both in 2D cultures and advanced 3D skin constructs—could help identify combination therapies that offer multi-target protection against oxidative damage, ECM degradation, and inflammation. Ultimately, translating these findings into controlled human trials, with dermatological endpoints such as wrinkle reduction, elasticity improvement, and biomarker analysis, will be crucial to determine their therapeutic relevance for aging and metabolically compromised skin.

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