Abstract

Antiretroviral therapy (ART) is highly effective in suppressing human immunodeficiency virus (HIV) replication. Still, it is frequently associated with adverse side effects with long-term use of the drugs, including oxidative stress from mitochondrial dysfunction. This study aimed to investigate whether *Thymus vulgaris* (garden thyme) could serve as a viable source of antioxidants to reduce ART-induced oxidative stress. Antioxidant capacity assays were conducted to quantify and verify phenolic content and free radical-neutralizing potential of extracts. Hepatocytes were used to assess the cytotoxicity of thyme and to determine the effect of the combined treatment with thyme and tenofovir disoproxil fumarate (TDF), a commonly prescribed nucleoside reverse transcriptase inhibitor (NRTI) for HIV that is associated with mitochondrial toxicity. Cell viability and oxidative stress assays were performed to evaluate whether thyme could reduce the adverse effects of TDF. Results demonstrate that thyme extracts possess antioxidant capacity and affect cellular viability in a concentration-dependent manner, suggesting the potential use of *Thymus vulgaris* in mitigating oxidative stress associated with ART treatment. The combined treatment of thyme and TDF yields inconclusive results and needs further testing. While further investigation is needed, these findings suggest that antioxidants such as *Thymus vulgaris* may be useful to combat ART-related oxidative stress. This work contributes to ongoing efforts to identify supportive interventions that improve treatment tolerance and adherence, particularly for populations disproportionately affected by HIV.

POTENTIAL MITIGATION OF ADVERSE EFFECTS OF ANTIRETROVIRAL THERAPY WITH ANTIOXIDANTS

Team Toxins Gemstone Thesis 2026

Cameron White[1,2,4], Eesha Reddy[1,2,3], Graham Ferguson[1,2,3], Hannah LaPadula[1,2,3,4], Kenna Costello[1,2,3], Madeline Semler[1,2,3,5], Margaret Kato[1,2,3], Megan Chen[1,2,3], Nivetha Rajapandi[1,2,3], Sofia Orezzoli[1,2,3], Tami Mumuney[1,2,3,5] | Yanjin Zhang[1,2,6]

[1]University of Maryland, College Park, MD | [2]Gemstone Honors College, University of Maryland, College Park, MD | [3]College of Computer, Mathematical & Natural Sciences | [4]A. James Clark School of Engineering | [5]College of Arts & Humanities | [6]College of Agriculture & Natural Resources

Thesis submitted in partial fulfillment of the requirements of the Gemstone Honors Program, University of Maryland, 2026

Dedication

In memory of Johannes John.

Acknowledgements

We are deeply grateful to our team mentor, Dr. Yanjin Zhang, for his mentorship, guidance, and support throughout the entire research process. We thank the members of Dr. Zhang's lab, Dr. Peixi Chang, and Bhargava Teja Sallapalli, for their generous knowledge and assistance in conducting our experimental work, as well as Dr. Liangli Yu and Ethan Young-Hyun Lee, for providing extensive support and resources towards our initial experiments. We also thank the Do Good Institute, UMD Libraries, and LaunchUMD donors for the financial support that made this research possible. Finally, we sincerely appreciate the Gemstone Honors Program, Dr. David Lovell, Dr. Allison Lansverk, and our team librarian, Matthew Cain, for providing us with the opportunity and resources to do this research.

Table of Contents

Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AIDS	acquired immunodeficiency syndrome
ART	antiretroviral therapy
ATP	adenosine triphosphate
AUC	area under the curve
CD4	CD4+ T-lymphocytes
DI	deionized [water]
DMEM	Dulbecco's Modified Eagle Medium
DPPH	2,2-diphenyl-1-picrylhydrazyl
ETC	electron transport chain
FBS	fetal bovine serum
GAE	gallic acid equivalent
GSH	reduced glutathione
HAT	hydrogen atom transfer
HIV	human immunodeficiency virus
II	integrase inhibitor
LOX	lipoxygenase
MRC	mitochondrial respiratory chain
MSM	men who have sex with men
mtDNA	mitochondrial DNA
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside/nucleotide reverse transcriptase inhibitor
ORAC	Oxygen Radical Absorption Capacity
PBS	phosphate-buffered solution
PI	protease inhibitor
Pol-γ	polymerase gamma
PrEP	preventative pre-exposure prophylaxis
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species
RT	reverse transcriptase
SOD	superoxide dismutase
TDF	tenofovir disoproxil fumarate
TE	Trolox equivalents
TEAC	Trolox equivalent antioxidant capacity
TPC	total phenolic content

Chapter 1: Introduction

Antiretroviral drugs are used to combat retroviral infections by disrupting viral replication. A key feature of retroviral infection is viral reverse transcription and genomic integration into the host chromosome (Ganguly, 2025). Treatment with antiretroviral drugs does not entirely eradicate the virus from the host, but suppresses it to an undetectable, nontransmissible level (Arts & Hazuda, 2012). One of the most prevalent applications of antiretroviral drugs is against human immunodeficiency virus (HIV) infection. The HIV epidemic is one of the world's largest public health challenges, resulting in the infection of 91.4 million and the death of 44.1 million people since 1981, with 40.8 million people living with HIV (PLWH) worldwide as of 2024 (WHO, 2025). Advancements in antiretroviral therapy (ART) have reduced mortality and viral transmission. However, the cytotoxicity of these drugs may also increase the concentration of free radicals in the body, known as reactive oxygen species (ROS), leading to oxidative stress, with resulting damage to proteins, DNA, and cell membranes (Pham-Huy et al., 2008; Saki et al., 2023). These effects may manifest in PLWH using ART through a weakened immune system, renal failure, liver disease, lipodystrophy, diabetes, and insulin resistance (Chen et al., 2013).

To address concerns about ROS-induced damage from these drugs, antioxidants derived from natural sources and plant products have been tested for their ability to reduce ART-related side effects (Sharma, 2014). Several studies have demonstrated that oral antioxidant supplements are effective in reducing oxidative stress in ART patients and in other conditions associated with high free radical concentrations (Allard et al., 1998; Gambo et al., 2021; Kumar et al., 2013). However, there have been limited or conflicting findings in studies on whether these

supplements improve quality of life for PLWH treated with ART (Bepe et al., 2011; Saki et al., 2023). Therefore, further research is necessary to explore the relationship between antioxidants and the adverse effects of ART, and to assess whether antioxidant supplementation is a viable approach to reduce oxidative stress induced by ART.

This study hypothesizes that antioxidant-rich plants, such as *Thymus vulgaris,* reduce ROS levels in ART-treated cells, thereby mitigating the adverse effects of antiretroviral treatment. Although previous literature shows a correlation between antioxidant use and reduced ROS in the body, *Thymus vulgaris* is a novel antioxidant that has not been tested for its ability to reduce oxidative stress induced by ART regimens (Proskurnina et al., 2020). This research therefore aims to analyze the antioxidant effect of *Thymus vulgaris* on ART-related adverse effects. The broader implications of the benefits of antioxidant supplementation in drug therapy may extend to other illnesses and treatments, underscoring the importance of further research on antioxidant use. Overall, this research will contribute to the current knowledge of antioxidants and their therapeutic uses.

Chapter 2: Literature Review

2.1: HIV Infection and Treatment

2.1.1 HIV Infection

HIV-1 is a retrovirus that attacks the body's immune system and stays with hosts for the duration of their lives. If left untreated, it can lead to acquired immunodeficiency syndrome (AIDS). Although HIV-1 infection is no longer considered a fatal diagnosis with treatment, it remains a chronic and life-long illness as HIV establishes latent viral reservoirs. Current treatment options can reduce the level of the virus in the blood to an undetectable, nontransmissible level, but cannot eliminate it. Most PLWH acquire it through anal or vaginal sex, or by sharing drug injection equipment such as needles or syringes. HIV can also be transmitted from mother to child during pregnancy, birth, or breastfeeding (CDC, 2022).

HIV infection begins when it fuses with the host cell's membrane. The main receptor for HIV is CD4+ T-lymphocytes (CD4), found on the surface of many immune cells, including helper T-lymphocytes, monocytes, macrophages, and dendritic cells. The membrane surrounding the virus consists of surface and transmembrane glycoproteins. The viral glycoprotein on the HIV surface, gp120, binds to the host CD4 receptor. Attachment of gp120 to CD4 induces conformational changes in the glycoprotein surface, creating a bridge between the HIV particle and the host cell. Binding to a co-receptor (CCR5 or CXCR4 chemokine receptor) consequently triggers conformational changes in gp41, a subunit of the viral envelope glycoprotein complex, allowing fusion of the viral envelope with the host cell membrane (Volberding & Deeks, 2010).

HIV variants use either CCR5 or CXCR4, or sometimes both; these are termed R5, X4, or R5X4 viruses (Deeks et al., 2015). CCR5 and CXCR4 are expressed differently in T-cell

subsets: CCR5 is expressed in memory T-lymphocytes but not in naive ones, whereas CXCR4 is present in both. Macrophages and dendritic cells also express CCR5. The virus primarily targets activated T-lymphocytes, which are more susceptible to infection than resting cells, although dendritic cells can capture the virus and spread it to nearby T-lymphocytes. HIV can also attach to the follicular dendritic cells, concentrating infectious virus within B-cell follicles in lymph nodes. Additionally, HIV induces lymphoid tissue fibrosis by increasing chronic immune activation, regulatory T-cells, and transforming growth factor-β signaling (Deeks et al., 2015).

Following fusion of the viral envelope with the host cell membrane, the viral capsid enters the cytoplasm. HIV's viral RNA genome is then reverse transcribed into complementary DNA by viral reverse transcriptase (RT), followed by conversion to proviral double-stranded DNA. This proviral DNA, along with viral proteins, forms the preintegration complex that enters the nucleus and is inserted into the host chromosome by viral integrase. After being inserted into the host cell genome, the viral genetic information can be transcribed by the host cell's RNA polymerase and then converted to proteins (Sierra et al., 2005). The newly synthesized viral RNA and proteins assemble into new virions that bud from the cell. HIV protease cleaves viral polyproteins into functional proteins, producing mature and infectious virions that can infect other cells (NIH, 2025a).

Overall, HIV infection is harmful for host cells, efficiently replicates within the body, and is easily transmitted to other individuals. If left untreated, HIV infection can affect more cells in the body and lead to critical conditions such as AIDS, which is typically shown by a CD4 T-cell count of less than 200 cells/mm^3 (Battistini Garcia et al., 2025). The attack on CD4 T-lymphocytes weakens the immune system's ability to defend against infections, illnesses, and cancers, further worsening the disease. Thus, ART is a crucial treatment strategy (NIH, 2025a).

2.1.2: Current Controls & Treatments

HIV “cures” do not yet exist for several reasons. HIV’s high mutation rate, ability to infect immune cells, integration into the host genome, and capacity to remain dormant in cells for extended periods of time all make permanent cures difficult to achieve (Richman et al., 2009). However, ART reduces infectious HIV RNA to undetectable levels and has been advancing over several decades, greatly improving the prognosis and quality of life of PLWH. The standard treatment regimen consists of a combination of two to three antiretroviral drugs that target different stages of the HIV life cycle (Eggleton & Nagalli, 2023). There are eight distinct categories of antiretroviral drugs based on their mechanism of action: nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, integrase inhibitors (II), CCR5 antagonists, post-attachment inhibitors, and capsid inhibitors. The most common treatments include two NRTIs, one NNRTI, and one PI or II (Kemnic & Gulick, 2023).

For each class of drugs, structure plays a key role in how HIV replication is inhibited. For example, NRTIs and NNRTIs target viral RT to inhibit replication. NRTIs act as chain-terminating nucleoside analogs that are incorporated into viral DNA to terminate elongation during reverse transcription. In contrast, NNRTIs are small, hydrophobic compounds that bind allosterically to the p66 subunit of the RT p66/p51 heterodimer. This interaction induces conformational changes that inhibit the polymerase activity and indirectly affect RNase H function, reducing viral replication in HIV-1 (Sluis-Cremer & Tachedjian, 2008). PIs target a later stage of the viral life cycle by inhibiting HIV protease, the enzyme required for cleavage of viral precursor for maturation into functional viral proteins (Sluis-Cremer & Tachedjian, 2008).

Building on this mechanism, NRTIs require intracellular activation to terminate retro-transcribed DNA. Once inside the host cell, NRTIs are phosphorylated by cellular kinases into their active triphosphate forms, NRTI-TPs, which then compete with natural nucleotides during DNA synthesis and terminate chain elongation due to the absence of a 3' hydroxyl group and lack of phosphodiester bond formation (Patel & Zulfiqar, 2023). Despite their effectiveness, NRTIs are associated with mitochondrial toxicity, particularly due to off-target effects on mitochondrial DNA (mtDNA) polymerase (White, 2001). Regardless, NRTIs remain an essential part of most ART regimens, making it a challenge to find a balance between viral suppression and side effects. This dilemma has led to the exploration of antioxidant supplementation as a potential strategy to mitigate ROS production and mitochondrial damage, specifically associated with NRTI treatment for PLWH.

2.2: Adverse Effects of Antiretroviral Therapies

2.2.1 NRTIs & Mitochondrial Toxicity

Drug development in recent decades has brought increased ART efficacy and reduced toxicity. Still, the treatments remain associated with a range of adverse effects that reduce quality of life for PLWH (NIH, 2024b). These side effects can include hepatitis and hepatic failure due to hepatotoxicity, insulin resistance, reduced bone density, lipodystrophy, chronic kidney disease, lactic acidosis, and cardiovascular disease (NIH, 2024b, 2025b; Pedro et al., 2018; Wei et al., 2025). Many can be attributed to ART interruptions of mitochondrial function and subsequent mitochondrial toxicity (White, 2001).

Mitochondria are essential for adenosine triphosphate (ATP) production. The reduction of mitochondrial activity caused by NRTIs leads to increased electron leakage and the generation of

ROS, including superoxide, peroxide, and hydroxyl radicals (Apostolova et al., 2011). Although low levels of ROS are produced during normal mitochondrial function and play roles in cellular signaling, excess ROS leads to damage of vital biological molecules, including lipids, proteins, and DNA (Pizzino et al., 2017). These free radicals can interact with these components through oxidation, thereby disrupting biological processes. Specifically, excess ROS can impair mitochondrial respiratory chain (MRC) function and lead to observable clinical symptoms, including myopathy, neuropathy, hepatic failure, and lactic acidosis. Additionally, mitochondrial toxicity is especially apparent in cells that require high energy levels, such as muscle cells, neurons, and liver cells, which helps explain the prevalence of these specific symptoms and underscores the overall detrimental effects of mitochondrial toxicity and excess ROS on cellular functions (Apostolova et al., 2011).

The major contributor of ARTs leading to mitochondrial toxicity is the mechanism of NRTIs, as aforementioned (Muñoz-Muela et al., 2025; White, 2001). mtDNA polymerase gamma (Pol-γ) activity is highly affected by NRTIs (Bienstock & Copeland, 2004), leading to reduced mtDNA replication, and consequently, the production of dysfunctional mitochondrial proteins. Because the mitochondrial genome encodes proteins necessary for mitochondrial function, this interruption prevents the formation of enzyme complexes essential for mitochondrial function (Taanman, 1999). The accumulation of dysfunctional proteins affects the MRC, increasing ROS production (Margolis et al., 2014), impairing MRC function, decreasing ATP production, and causing further damage to cellular components, resulting in insufficient energy production and the manifestation of clinical symptoms (White, 2001).

2.2.2 ROS Production

ROS are produced as a byproduct of oxidative phosphorylation when electrons prematurely react with oxygen, particularly at complexes I and III, in the electron transport chain (ETC) (Shadel & Horvath, 2015). The imbalance between ROS and cellular antioxidant mechanisms may lead to oxidative stress (Pizzino et al., 2017). This stress may result from the overproduction of ROS under hyperoxia, when electron concentration in the ETC is elevated. This stress can also occur under hypoxic conditions, in which ROS increase due to altered electron flow and partial reduction of oxygen, leading to an accumulation of electrons (Burton & Jauniaux, 2011). ROS production can become self-amplifying, meaning that environments with excess ROS encourage further ROS production, making the side effects of mitochondrial toxicity even more dangerous (Kudin et al., 2004).

The most commonly produced ROS in mitochondria is superoxide, and its production has been linked to two specific complexes in the ETC: complex I and complex III (Kudin et al., 2004). Though the transfer of electrons to O_2 is highly regulated, a small percentage of electrons leak out to react with O_2 to form superoxide (Mittal et al., 2014). In a correctly functioning cell, superoxide dismutase (SOD) enzymes detoxify the superoxide, converting it into hydrogen peroxide. The function of these enzymes is significant due to a shift in the concentrations of these two species, which can form via reactions involving hydrogen peroxide and transition metals, to cause the formation of a much more dangerous ROS: a hydroxyl radical. Under conditions of excess superoxide, it can react with nitric oxide to form peroxynitrite, which is significantly more reactive than superoxide (Kudin et al., 2004).

2.2.3 Impact of ROS on Cells

ROS are important signaling molecules in a variety of cellular processes, including cell proliferation and differentiation, as well as homeostatic and oxidation signaling pathways (De Almeida et al., 2022). However, they can also activate inflammatory, apoptotic, and necrotic pathways, leading to cell death and physical symptoms. ROS modulate signaling cascades that regulate redox-sensitive transcription factors, including p53, NF-κB, and AP-1, which regulate pro-inflammatory cytokines. Activation of these pathways leads to increased levels of pro-inflammatory enzymes and tumor necrosis factors such as TNF-α, and activation of the apoptotic cascade. This condition can ultimately lead to cell death through necrosis or apoptosis (Burton & Jauniaux, 2011). Necrosis causes cellular degradation through swelling and loss of membrane integrity, triggering an inflammatory response from the body (Kanzler & Galle, 2000), while apoptosis plays a significant role in liver injury and, along with necrosis, can lead to liver failure due to the susceptibility of certain liver cells, specifically hepatocytes and cholangiocytes, to death receptor-mediated apoptosis (Guicciardi et al., 2013). Apoptosis is also induced by the activation of ASK1, which activates the mitogen-activated protein kinase p38 and stress-activated protein kinase–c-Jun N-terminal kinases (Burton & Jauniaux, 2011). These pathways are adaptive at physiological ROS levels and help restore homeostasis, but high levels can trigger apoptosis and necrosis, ultimately causing greater damage.

Cell and organelle membranes are more susceptible to damage by ROS because they contain polyunsaturated fatty acids (PUFAs) (Su et al., 2019). This process is referred to as lipid peroxidation, which involves free radical species removing hydrogen atoms from lipids, resulting in more reactive intermediates that destabilize the membrane. This peroxidation directly damages these phospholipids and can induce cell death, including ferroptosis, a type of cell death

dependent on iron. Lipid peroxidation can be induced via two mechanisms, one being non-enzymatic and the other being enzymatic. In the non-enzymatic mechanism, PUFA-containing phospholipids undergo a free-radical chain reaction involving oxygen, in which a compound spontaneously loses electrons to the surrounding air (Su et al., 2019). This process forms many reactive electrophilic products that can disrupt cellular processes. In the enzymatic mechanism, membrane peroxidation is catalyzed by lipoxygenase (LOX), which forms hydroperoxides (R-OOH). This process acts on specific PUFAs within certain phospholipids, which serve as substrates for the LOX enzyme, which uses molecular oxygen to create hydroperoxyl groups at varying positions along the acyl chain.

In the mitochondrial membrane, ROS can cause damage to the cells through a positive feedback mechanism (Zorov et al., 2014). ROS can induce the opening of the mitochondrial permeability transition pore, leading to further ROS generation and mitochondrial release, sometimes resulting in mitochondrial and cellular damage. This process of mitochondrial ROS inducing the further release and formation of ROS is referred to as ROS-induced ROS release (Zorov et al., 2014). ROS production and antioxidant defenses can become imbalanced, in some cases from therapies like ART, which creates oxidative stress on the cells. Generally, the effects of ROS can be differentiated by classifying them as either physiological signaling ROS, which are beneficial to the cell, or pathological ROS, which are detrimental to the cell.

ROS interactions with proteins result in changes in folding, hydrophobicity, and charge, which affect catalytic, structural, and communication functions. While nucleic acids have robust repair mechanisms and proteins can sometimes be repaired or refolded to counteract oxidative damage, severely damaged proteins are often marked and targeted for degradation (Stringfellow et al., 2014). This process can cause significant damage to the cell, as proteins are responsible for

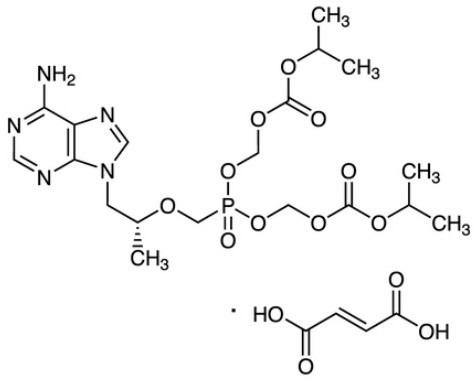
the majority of cellular work. ROS affect proteins in three main ways: oxidation of amino acid residues, cleavage of peptide bonds, and protein aggregation. There are many functional groups present on amino acid residues susceptible to oxidation. One significant target of ROS is sulfur-containing groups in amino acids like methionine and cysteine. The oxidation of these groups can yield reversible or irreversible products, including sulfenic, sulfinic, or sulfonic acids, which can impair function and, if severe, target the protein for permanent degradation by the proteasome. Another is aromatic groups, especially in tyrosine which can alter protein function and promote aggregation or degradation (Kehm et al., 2021).

As described, highly damaging ROS can cause direct effects to nucleic acids in mtDNA, as well as attack chromosomal DNA bases, alter DNA double helix structure, and alter corresponding bonds between nitrogenous base pairs (Sakai et al., 2006). The attack by hydroxyl radicals, one of the most reactive and damaging ROS, causes oxidative stress to both purine and pyrimidine bases. ROS interaction can, for example, change a guanine base in DNA to an 8-oxo-7,8-dihydroguanine (8-oxoG lesion) base, which has the mutational ability to mispair with adenine instead of a cytosine (Hebert & Schlegel, 2019). Other ROS can also interact with guanine and other bases to cause different oxidative products. 8-oxoG lesion is one of the most common oxidative DNA lesions in cells and is caused by the oxidation of guanine via a hydroxyl radical, which modifies it so that it can mispair with adenine at transcription forks, replication bubbles, and DNA double helix strands (Fleming et al., 2012). Mutations by ROS can cause these spontaneous mutations, including transversions, base substitutions, and small frameshift deletions (Sakai et al., 2006). Overall, the presence of ROS can interact with DNA base pairs, causing structural mutations in bases and increasing the likelihood of defects, cellular dysfunction, and cell death. This further exacerbates the effect of increased ROS concentration

from mitochondrial toxicity of ART drugs as seen through the structural changes to DNA, a vital component of every cell.

2.2.4 Potential NRTI Drug of Interest

A common NRTI used for ART treatment of HIV is tenofovir disoproxil fumarate (TDF). Although to a lesser extent than some older NRTIs, TDF has been associated with mitochondrial toxicity. This antiretroviral drug is often used as part of a first-line treatment regimen for PLWH (Fioroti et al., 2022). In addition, TDF is one of two drugs in the combination anti-HIV medicine Truvada, a widely used combination ART according to Gilead Sciences, a pharmaceutical company leading in the research and development of HIV drugs (Contract Pharma, n.d.). Despite its efficacy in reducing the viral load of HIV, TDF has been associated with nephrotoxicity (i.e. renal toxicity) as well as bone toxicity, leading to bone density issues (Hashwin Singh et al., 2024). In some cases, studies have revealed severe hepatic adverse effects associated with long term use of NRTIs such as TDF, rarely associated with lactic acidosis and liver failure (NIH, 2020). In severe cases, TDF has been identified as a significant contributor to cases of proximal tubulopathy (a form of renal disease) found in PLWH taking ART treatment (Fioroti et al., 2022).



Scheme 1. Chemical structure of tenofovir disoproxil fumarate. (Image source: TCI Chemicals)

The mechanisms of these adverse side effects are not fully understood at this time. One explanation for TDF-induced nephrotoxicity is that mitochondrial toxicity through inhibition of mtDNA Pol-γ in proximal tubular cells may cause oxidative stress, apoptosis, and other negative effects on the kidney (Fioroti et al., 2022). This is supported by another study, which indicated that damage to proximal tubular mitochondria in rats led to antioxidant depletion and oxidative stress, causing renal damage (Abraham et al., 2013). However, an *in vitro* study contradicted this theory and reported relatively low levels of oxidative stress in different types of TDF-treated cells (Cihlar et al., 2002). Therefore, more research is needed to elucidate these conflicting results. In addition, the causes of TDF-related bone density loss are less clear, with some potential explanations including imbalances of growth factors, alteration of gene expression, and various factors influencing the complex interactions of osteoblast and osteoclast activity (Hashwin Singh et al., 2024).

Given that this is such a widely used drug with adverse effects that may be caused by mitochondrial toxicity, TDF is a strong ART candidate for investigation of the impact of antioxidants on the resulting oxidative stress, which may provide insight into mechanisms of this drug's adverse effects and a potential strategy to counter them to improve drug adherence and improve the quality of life for PLWH.

2.3: Mitigating ART Toxicity

2.3.1: Drug Structure-Activity Relationships

One method used in past studies to reduce the cytotoxicity of ART is altering drug structure to modulate resulting activity (Kaur et al., 2020). NNRTIs have been an important class of anti-HIV therapies due to their strong antiviral activity and high selectivity. The NNRTI

compound 4ab has been shown to exhibit similar antiviral activity with reduced cytotoxicity compared to original JX-7 (Jin et al., 2022). This method provides one possible starting point for improving current therapies. Unfortunately, it is also known that these NNRTIs can exhibit cytotoxicity in certain contexts in addition to rapid drug resistance and can limit long-term effectiveness in some cases. This issue highlights the need for further exploration, as it calls for the development of improved ARTs with simultaneous high potency and low toxicity.

In efforts to take an existing ART and manipulate its structure, it could be incredibly helpful to incorporate structural elements from natural compounds that have previously been shown to demonstrate antiviral activity. As mentioned, bulky substituents at the C4 position in calanolides promote the ability of calanolide molecules to inhibit RT. It is therefore considered to add bulky substituents at a similar molecular position in a selected antiretroviral drug structure as a methodology. Regardless, understanding the activity of naturally derived agents against HIV will serve as a solid foundation for future endeavors investigating synthetically derived compounds.

2.3.2: Antioxidant Supplements

Another potential method to reduce or prevent ART-induced cytotoxicity is antioxidant supplementation. Antioxidants are molecules that neutralize reactive molecules such as ROS, thereby protecting cellular components from oxidative stress. These compounds can be classified into three groups: endogenous enzymatic antioxidants, endogenous non-enzymatic antioxidants, and exogenous non-enzymatic antioxidants (Losada-Barreiro et al., 2022).

Endogenous antioxidants are naturally produced by the body. They include enzymatic antioxidants such as SOD, glutathione peroxidase, and glutathione reductase, as well as

non-enzymatic antioxidants like melatonin and coenzyme Q10 (Losada-Barreiro et al., 2022). These molecules work through a variety of mechanisms, including limiting ROS formation, scavenging ROS, or supporting antioxidant systems. Endogenous compounds are assisted by exogenous non-enzymatic antioxidants, which originate from outside the body (Munteanu & Apetrei, 2021). They act cooperatively with endogenous antioxidants to maintain the balance of ROS in cells, either scavenging free radicals or supporting endogenous antioxidant systems (Bouayed & Bohn, 2010). Some examples of exogenous antioxidants include vitamins C and E, carotenoids, and polyphenols. Naturally found in plants, they are obtained through dietary intake and contribute to cellular antioxidant defenses.

Many antioxidant-rich plants are already used in traditional medicine for their wide range of bioactive components, and have been investigated both *in vitro* and clinically for potential therapeutic effects against cancer, microbial infection, respiratory conditions, heart disease, and a variety of viruses. For example, antioxidants derived from *P. hysterophorus* leaves have demonstrated anti-HIV and cytoprotective effects *in vitro*, as well as moderate success when assayed in relation to breast cancer and leukemia (Parham et al., 2020). Another antioxidant-rich leaf, *Moringa oliefera*, has been studied for its antioxidant and immunomodulatory properties, with some studies suggesting potential improvements in immune markers such as CD4 counts in a clinical trial with PLWH (Gambo et al., 2021; Nworu et al., 2013). Overall, plant-based antioxidant supplementation may be an effective way to reduce the negative effects of oxidative stress induced by HIV and ART.

However, while some studies regarding the use of antioxidants in managing HIV have yielded encouraging results, others provide conflicting or insufficient evidence. In a review of trials using zinc, vitamins, and other micronutrient supplements to combat HIV, it was concluded

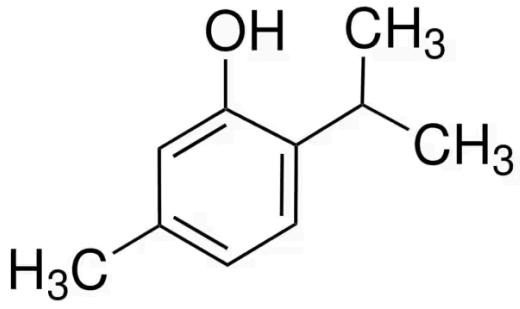
that these studies show inconsistent outcomes and instead rely on an individual's micronutrient status (Wilkinson et al., 2018). Additionally, a survey of 151 ART patients found that those taking herbal supplementation were more likely to develop adverse side effects such as rashes and abdominal pain, likely due to unforeseen interactions between the drug and natural supplement (Bepe et al., 2011). These findings suggest that naturally derived antioxidants may influence health outcomes of PLWH, but further research is necessary to understand the role and the entire effects of antioxidant supplementation.

While clinical studies investigating the impact of antioxidants on diseases such as HIV have yielded mixed or inconclusive results, a number of studies have suggested that antioxidants can mitigate drug toxicity caused by oxidative stress. Antioxidant compounds such as vitamin E have been shown to reduce the neurotoxic effects in certain chemotherapy contexts (Stankovic et al., 2020). However, the results of antioxidant supplementation on health outcomes in cancer patients have been inconsistent, as antioxidants have also been found to reduce the efficacy of these drugs (Khurana et al., 2018). Therefore, when investigating antioxidant sources as a means to reduce drug toxicity, it is essential to consider the involvement of the antioxidant in both the mechanisms of side effect development and those of drug function.

Thymus vulgaris, commonly known as garden thyme, is a rich source of antioxidants with high radical scavenging activity (Assiri et al., 2016). It contains many phenolic compounds, such as thymol, carvacrol, and glycosides, which have been studied and characterized not only for their antioxidant capabilities but also for their therapeutic and antimicrobial properties (Hammoudi Halat et al., 2022; Nagoor Meeran et al., 2017). One study examined the effect of *Thymus vulgaris* essential oil on the function of HIV-Tat, a transcriptional regulatory protein, and found that the essential oil reduced Tat-mediated transcription (Feriotto et al., 2018). Some

antioxidant-rich plants such as *Arctium lappa*, *Syzygium cumini*, and *Tribulus terrestris* have similarly been previously studied and have demonstrated anti-HIV activity *in vitro* (Parham et al., 2020). In addition to its potential anti-HIV activity demonstrated by these studies, the high concentration of antioxidant compounds present in *Thymus vulgaris* makes it a promising candidate for investigating the mitigation of oxidative stress induced by HIV treatment.

Given that thymol, one of the main phenolic compounds of *Thymus vulgaris,* has shown exceptional antioxidant abilities (Nagoor Meeran et al., 2017), it is therefore reasonable to also investigate whether its other individual compounds also contribute to these antioxidant effects. Thymol has shown anti-inflammatory effects by modulating inflammatory signaling, reducing cytokine and chemokine production, and also through free radical scavenging and enhancement of endogenous enzymes as well as chelation of metal ions. Reviews of *in vitro* and *in vivo* data suggest potential therapeutic benefits of thymol as a dietary supplement to be used concurrently with agents to treat various diseases (Nagoor Meeran et al., 2017). The properties of thymol are specifically tied to the phenolic hydroxyl group, which transiently forms a resonance-stabilized phenoxyl radical after thymol donates a hydrogen atom. This pathway protects against free radicals by neutralizing them, thereby preventing deleterious effects.



Scheme 2. Chemical structure of thymol. (Image source: Sigma-Aldrich)

The scavenging of free radicals can be assessed through measurements of endogenous antioxidant enzyme activity, such as SOD, glutathione-S-transferase, and through non-enzymatic antioxidant levels like reduced glutathione (GSH) (Nagoor Meeran & Stanely Mainzen Prince, 2012). Studies have shown thymol to have an exceptional ability to scavenge superoxide anion and hydroxyl radicals in a concentration-dependent manner, as demonstrated using assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Nagoor Meeran et al., 2015; Nagoor Meeran & Stanely Mainzen Prince, 2012).

Through evaluating the effect of thymol treatment on hepatotoxicity, studies have suggested thymol as a protective agent against oxidative stress. A previous study showed 30 mg/kg thymol dosage co-administered with hydrocortisone in rats led to a decrease in important liver enzymes such as aspartate aminotransferase and alanine transaminase, tumor necrosis factor-α in the serum and liver, indicative of a significant reduction in liver damage, inflammation, and oxidative stress (Aboelwafa & Yousef, 2015). Another study showed decreased cytotoxicity, apoptosis, and ROS, resulting in reduced oxidative damage in Chang liver cells after treatment with 50 μg/ml thymol (Kim et al., 2014). Given the established antioxidant properties of thymol, this study evaluates whether these effects can mitigate oxidative stress associated with TDF treatment, a clinically relevant source of drug-induced oxidative damage.

This study aims to mitigate ART-induced oxidative stress, which remains underexplored in conjunction with thyme supplementation. Antioxidant supplementation represents a promising strategy to reduce treatment-associated adverse effects without directly altering the pharmacological activity of ART. In addition, these antioxidants can be naturally derived from plants, providing a potentially more accessible and cost-effective approach for reducing

ART-induced oxidative stress for under-resourced populations with high HIV prevalence (Parham et al., 2020). Further investigation of thyme and its active compound, thymol, may advance understanding of their antioxidant properties and their potential use in pharmaceutical applications.

2.4 Assessing Antioxidant Capacity & Oxidative Stress

There are numerous methods for quantifying antioxidant compounds and measuring antioxidant activity in plant extracts. The antioxidant potential of plant extracts is often correlated with the levels of phenolic compounds (such as phenolic acids, lignin-related compounds, tannins, lignins, and flavonoids), which act as exogenous antioxidants. Phenolic compounds can be quantified by gas chromatography or high-performance liquid chromatography, as well as by spectrophotometric assays such as the Folin-Ciocalteu total phenolic content (TPC) assay (Khoddami et al., 2013).

Additional assays are needed to directly measure the antioxidant activity of a sample. Many of these assays measure the ability of the antioxidant to perform either hydrogen atom transfer (HAT) or single-electron transfer to neutralize ROS that contain unpaired electrons (Munteanu & Apetrei, 2021). Oxygen Radical Absorption Capacity (ORAC) is a commonly used and physiologically relevant assay that measures the ability of a sample to neutralize a peroxyl radical via HAT by monitoring fluorescence decay relative to a standard such as the vitamin E analog, Trolox. Another commonly used assay is the DPPH radical-scavenging assay, which measures antioxidant capacity by assessing the ability of compounds to donate electrons or hydrogen atoms to reduce the stable free radical DPPH, resulting in a measurable color change. The 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay functions similarly,

measuring the transfer of both hydrogen atoms and electrons (Munteanu & Apetrei, 2021). These analytical methods have been widely used to evaluate plant extracts, including *Thymus vulgaris*. Studies have shown that extracts of *Thymus vulgaris* contain high concentrations of phenolic compounds and exhibit strong antioxidant potential in these assays (Assiri et al., 2016).

Oxidative stress can also be evaluated in cellular systems using biological assays that assess cell health and redox balance. One indirect method of assessing oxidative-stress-induced damage is through cell viability assays, which measure the proportion of living cells after exposure to a stressor. Oxidative stress can impair mitochondrial function, damage cellular molecules, and ultimately reduce cell survival, making decreased cell viability a commonly used indicator of oxidative toxicity (Katerji et al., 2019). However, while cell viability assays can provide some information about the overall impact of damage, they do not directly measure antioxidant activity or intracellular redox status. For this reason, more specific biochemical assays are often used in conjunction with viability measures.

In cellular systems, oxidative stress can also be assessed using assays that measure redox balance and cell viability. One important indicator of oxidative status is glutathione, a key intracellular antioxidant that plays a central role in maintaining cellular redox homeostasis. Glutathione-based assays measure the relative levels of reduced and oxidized glutathione within cells, providing insight into oxidative stress and mitochondrial function. Because glutathione is directly involved in detoxifying reactive oxygen species and maintaining redox balance, changes in its levels serve as indicators of cellular health and oxidative damage (Owen & Butterfield, 2010). As a result, assays measuring the ratio of reduced to oxidized glutathione are frequently used in studies investigating antioxidant interventions and plant-derived compounds to assess their ability to mitigate oxidative stress in biological systems (Ndlovu et al., 2023)**.**

2.5: Social Impact

Due to the transmission mechanism of HIV, social context and human behavior are extremely significant in the spread of HIV. Since the start of the epidemic in 1981, HIV has disproportionately impacted certain populations, including men who have sex with men (MSM), transgender individuals, people who inject drugs, incarcerated individuals, and sex workers. In 2019, 66% of new HIV diagnoses were the result of MSM contact despite gay and bisexual men making up less than 5% of the US population (NIH, 2026). Fear and lack of knowledge, especially at the beginning of the epidemic, contributed to stigma and discrimination against people living with HIV, or PLWH, as well as against groups that were perceived as being associated with HIV transmission (De Cock et al., 2011). In 1983, the CDC defined the high-risk populations as the four H's: homosexuals, hemophiliacs, heroin addicts, and Haitians, which further influenced the public's perceptions of HIV (Gonsalves & Staley, 2014). Homophobia and stigma against PLWH have created a barrier in the prevention and treatment of HIV among MSM. Fear of being perceived as sexually promiscuous, as well as family and social rejection, may cause MSM to avoid testing and hide their HIV status, increasing risk of transmission and limiting their care (Lott et al., 2022).

In the United States, the general population has relatively low HIV rates. However, there are areas with higher HIV prevalence, particularly in populations involving marginalized demographics. Communities with lower socioeconomic status not only experience higher HIV rates but also experience worse health outcomes. Specifically, higher rates of HIV are prevalent among individuals at or below the poverty line, individuals with less than a high school diploma, and unemployed individuals (Pellowski et al., 2013). Socioeconomic factors also intersect with race, contributing to the significant racial disparities seen, such that Black/African American and

Hispanic/Latino populations are disproportionately affected. In 2022, Black/African American people made up 12% of the population and 37% of people newly infected with HIV (CDC, 2024a) and Hispanic/Latino people made up 19% of the population and 32% of people newly infected with HIV (CDC, 2024b). This is in contrast to the white population, which made up 60.1% of the U.S. population and 28.5% of PLWH (CDC, 2024a). Further, the most affected populations are Black/African American MSM, and Hispanic/Latino MSM. Additionally, Black/African-American women are significantly more impacted than other groups of women, as they account for a disproportionate share of 54% of U.S. women with HIV while only accounting for 13% of the country's female population (NIH, 2024a).

This inequity is shaped by structural determinants of health, including systemic disparities and discrimination in housing, education, the criminal justice system, and immigration policy. Lack of resources and environmental stressors have made it more difficult for Americans living in low-income, predominantly minority communities to receive HIV testing and treatment. Additionally, disadvantaged communities often face higher incarceration rates, which increases the rate of HIV transmission, as prisons create a high risk for HIV contraction through unprotected sexual activity, needle sharing, and tattooing (Robinson & Moodie-Mills, 2012).

There are also significant gaps in the quality of treatment between racial groups. One study found that only 11% of African Americans and 21% of Hispanic/Latinos eligible for HIV preventative pre-exposure prophylaxis (PrEP) were prescribed, compared to 78% of white people (Kamitani et al., 2024). Viral suppression is observed at lower rates in African American populations, with 38.9% of African American PLWH and 47.3% of white PLWH reaching viral suppression. This may be in part due to lower ART persistence in Black populations. Studies have shown that young people, women, Black and Latino individuals, and people who had spent

less time living with HIV were more likely to self-discontinue treatment (Gwads et al., 2021). Additionally, a study comparing ART regimens of those with Medicare and Medicaid (which accounts for over half of PLWH receiving treatment) found that only 45% of African American patients had sufficient supplies to maintain an ART regimen compared to 52% of white patients (Landovitz et al., 2017).

These disparities may be due to discrimination and microaggressions linked to worse health outcomes and maladaptive health behaviors, lack of trust in providers, and low health literacy (Simoni et al., 2012). Studies have found inequities in African American populations due to reduced engagement in care and nonadherence influenced by medical mistrust and perceived discrimination. Racial discrimination within and outside the medical system is known to reduce trust in healthcare among African Americans, and a combination of lack of trust in healthcare systems, individual physicians, and HIV-specific doubts plays a significant role in the treatment and outcomes of PLWH. In addition to discrimination in the medical field, other issues such as financial insecurity (such as lack of adequate housing), the social stigma surrounding HIV diagnosis, and negative perceptions of ART also influenced people's ability and willingness to pursue ART (Gwads et al., 2021). In one study, a quarter of eligible patients refused treatment, and a third had low adherence after 12 months of treatment. Based on patient questionnaires, this was due to both fear of side effects and disbelief that the drug was necessary (Horne et al., 2007). Furthermore, another study found that the longer it took people to receive their HIV test results, the less likely they were to begin ART (Wood et al., 2006). The combination of these physical and mental barriers that individuals face, particularly marginalized communities, makes the consistent use of ART very difficult and demonstrates a need for change.

These disparities in HIV care demonstrate a clear requirement for consideration of socioeconomic status in HIV research. One important barrier this research may address is the increase in adverse effects associated with cheaper treatments. The general cost of HIV care is a significant barrier to well-being and virus suppression, as the lifetime median cost of HIV care was estimated to be $420,285 discounted and $1,079,999 undiscounted in the US in 2019 (Bingham et al., 2021). While there are state and federal programs (such as ADAP and Medicaid) that assist people with paying for HIV care, these programs are often restricted to people with very low incomes and limited or no health insurance, leaving others to pay high out-of-pocket costs. The price of ART drugs is often associated with newer drug development and formulation complexity, which makes effective medications more expensive and difficult to access. Recent advancements in HIV treatment include long-acting injections administered every two months. This treatment offers many advantages, including lower side effect burden and increased adherence. However, being a new treatment, it is typically less accessible than current common treatments due to limited healthcare infrastructure (Łupina et al., 2025). Because this treatment must be administered by a medical provider, it can place additional burdens on patients such as clinic and administration fees, insurance benefit determination and prior authorization requirements, inadequate insurance reimbursement, and additional medication costs incurred by clinics (NIH, 2021). In addition, these long-acting treatments can only be used in individuals who have already reached viral suppression, meaning those who face barriers to current HIV treatments may not be able to benefit from these advancements.

Aside from financial limitations, groups disproportionately affected by HIV may face other barriers to treatment. As discussed previously, one of the barriers to effective HIV care is a lack of trust in medication and healthcare providers due to factors such as discrimination and

lack of representation in drug trials. Natural supplements may have the potential to improve the acceptability of ART regimens to those who have doubts in its safety. While this research project will not utilize human clinical trials, it is essential that future research uses a diverse testing population to understand reactions to antioxidant treatment across multiple groups.

Although HIV treatments are continuously advancing to improve ease of adherence and reduce side effects, there are many existing barriers that prevent equitable access to these treatments. By investigating natural supplements to offset adverse effects rather than contributing to the development of a new treatment with fewer adverse effects, this study aims to find a more affordable and accessible way to improve the quality of life of those taking ART.

2.6: Research Objective

The objective of this research is to investigate whether a common antioxidant-rich plant, *Thymus vulgaris*, can mitigate the adverse effects associated with the widely prescribed ART, tenofovir disoproxil fumarate (TDF), for the treatment of HIV infection. This study analyzes the effects of *Thymus vulgaris* and TDF on cellular oxidative stress and cell death in a hepatocyte cell model. It is hypothesized that dual treatment with TDF and *Thymus vulgaris* will improve cell viability compared with TDF alone, thereby reducing the adverse effects of ART. This research intends to provide a social benefit to PLWH by reducing adverse side effects and improving medication compliance, therefore reducing the viral load and spread of HIV through a non-invasive, covert treatment method. Specifically, this research explores the use of accessible supplements for medicinal purposes to assist communities disproportionately affected by HIV. The outcome of this research can inspire further research into antioxidant supplementation to mitigate the adverse effects of various medications.

Chapter 3: Materials & Methods

3.1: Thyme Extraction

Thyme extracts were prepared by using finely powdered dried thyme, sourced from Frontier Co-op (Norway, IA, USA). 2.5 g of powdered thyme, weighed on an analytical balance, were suspended in 25 mL of deionized (DI) water to achieve a 1:10 solid-to-solvent ratio. Water was the solvent of choice for the extracts used in subsequent *in vitro* testing due to the potential cytotoxicity of ethanol extractions. For antioxidant capacity assays only, an additional extract was prepared using 2.5 g of thyme and 25 mL of 30% ethanol solution. The solution was then vortexed for 2 minutes, and the resulting mixture was stored at 4℃ overnight.

The prepared solution was then centrifuged at 1055 × g for 10 minutes at 4℃. The supernatant was decanted into a new 50 mL centrifuge tube and the pellet was discarded. The new solution was centrifuged at 2700 × g for 10 minutes at 4℃. Gravity filtration was subsequently performed using filter paper and a plastic funnel.

To sterilize the thyme extract and prevent contamination during cell treatment, the solution was syringe-filtered with a 0.22 μm filter under sterile conditions. The sterilized extract was aliquoted into 1.5 mL microfuge tubes, and subsequently stored at -20℃ for no more than four months at a time, as informed by longevity studies of other thyme extract compositions (Usai et. al, 2011). Antioxidant capacity assays not involving cells used freshly prepared thyme extract, stored at 4℃ for no more than one week.

3.2: Assessing the Antioxidant Capacity of Thyme Extracts

All chemical reagents used in the following assays were generously provided by Dr. Liangli Yu.

3.2.1: TPC Assay

The TPC of the thyme extracts was determined using the Folin–Ciocalteu method (Moore et al., 2005) adapted by Dr. Yu's lab. A standard curve was generated using gallic acid dilutions ranging from 50 to 800 μg/mL. Gallic acid is a known antioxidant with a high concentration of phenolic hydroxyl groups. Thyme extracts in water and ethanol were diluted in seven 10-fold dilutions. The Folin–Ciocalteu reagent and ultrapure water were mixed with the gallic acid standards or thyme extract samples and vortexed. After one minute, a 20% Na_2CO_3 solution was added and the samples rested for two hours in the dark. Absorbance was then read at 765 nm using a spectrophotometer.

3.2.2: ABTS Assay

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) cation radical scavenging capacity assay (Miller et al., 1996) was performed using a protocol adapted by Dr. Yu's lab. An ABTS solution was made by dissolving MnO_2 and ABTS salt in ultrapure water. A working solution was then prepared by diluting the ABTS solution in phosphate buffer (PB) to an absorbance value of 0.700 at 734 nm. Five 10-fold thyme extract dilutions in water and ethanol were tested and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) dilutions between 0 and 300 μM were used as a standard. The ABTS working solution was added to the samples and immediately vortexed for 30 seconds. Absorbance was read after an additional 60 seconds at 734 nm.

3.2.3: ORAC Assay

The oxygen radical absorbance capacity (ORAC) assay (Moore et al., 2005) was also conducted using a protocol adapted by Dr. Yu's lab. The relative ability of thyme extract to neutralize peroxyl radicals and prevent fluorescein quenching compared to a Trolox standard was determined. A working fluorescein solution of 81.63 nM was prepared in PB and pipetted into each utilized well of a 96-well plate, along with either a thyme extract sample, blank, or standard. Five 10-fold thyme extract dilutions in water and ethanol were tested and Trolox dilutions between 0 and 100 μM were used as a standard. The plate fluorescence was then read using an excitation wavelength of 485 nm and an emission wavelength of 528 nm. 0.36 M 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) in PB was added to each well, mixed by pipetting, and plate readings were taken using the same protocol every minute for 45 minutes.

3.3: *In Vitro* Assessment of Thyme, Tenofovir, & Combined Treatments

3.3.1: Cell Culturing

Huh7.5.1 cells (and later Vero cells) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% Penicillin-Streptomycin, 2 mM L-Glutamine, and 1 mM sodium pyruvate. Cells were incubated at 37°C with 5% CO_2 and passaged after reaching 80-90% confluency using Trypsin EDTA.

For each experiment, an opaque-walled 96-well plate was seeded with 30,000 cells per well. The plate was then placed in the incubator to grow to a cell confluency of at least 70% before treatment.

3.3.2: Thyme Treatment

An aliquot of previously prepared thyme extract was removed from a -20℃ freezer and thawed to room temperature using a 25℃ water bath. Once thawed, the tube was disinfected and brought into the biosafety cabinet. The extract (serving as a 100,000 ug/mL stock solution) was then serially diluted with DMEM to make solutions of thyme ranging from 50 to 10,000 µg/mL. Volumes of 96-well plate wells previously seeded with Huh7.5.1 cells were aspirated, and 200 µL of the treatment solution was pipetted into each well. Each plate included a group of untreated controls and a group of vehicle controls containing the same percentage of DI water as the treatment group with the highest concentration of thyme extract. The plate was then incubated for either 24 or 48 hours before a CellTiter-Glo® 2.0 Assay (purchased from Promega) was performed to assess cytotoxicity and cell death.

3.3.3: Thymol Treatment

Thymol crystals (supplied by Ward's Science) were dissolved in a small volume of 99% ethanol and vortexed for 5 seconds or until completely dissolved. Ethanol was used as the initial solvent because thymol is more soluble in ethanol than in water. This amount of ethanol was not expected to produce cytotoxic effects during subsequent cell treatment. This solution was sterilized by syringe-filtering using a 0.2 µm filter, and then subsequently diluted with DMEM to generate a 10,000 µg/mL stock solution. The stock solution was then serially diluted with DMEM to make solutions of thymol ranging from 10-250 µg/mL. Volumes of 96-well plate wells previously seeded with Huh7.5.1 cells were aspirated, and 200 µL of treatment solution was pipetted into each well. Each plate included a group of untreated controls and a group of vehicle controls containing the same percentage of ethanol as the treatment group with the

highest thymol concentration. The plate was then incubated for 48 hours before a CellTiter-Glo® 2.0 Assay was performed to assess cytotoxicity and cell death.

3.3.4: Tenofovir Treatment

TDF (supplied by Ambeed) was dissolved in phosphate-buffered saline (PBS, pH 7.2) to create a 5000 μg/mL stock solution. This solution was vortexed for 5 seconds or until completely dissolved and syringe-filtered using a 0.2 μm filter to sterilize. A small volume of the stock solution was diluted with DMEM to generate a new solution of 480 μg/mL TDF. This solution was then serially diluted with DMEM to prepare solutions of 360, 240, 120, 60, and 30 μg/mL TDF. These specific concentrations are multiples of the C_{max} of TDF after oral administration (0.3 μg/mL), scaled up to induce oxidative stress and/or cytotoxicity in a cell culture model (Ndlovu et al., 2023). Volumes of 96-well plate wells previously seeded with Huh7.5.1 cells were aspirated, and 200 μL of treatment solution was pipetted into each well. Each plate included a group of untreated controls and a group of vehicle controls containing the same percentage of PBS as the treatment group with the highest TDF concentration. The plate was then incubated for 48 hours before a CellTiter-Glo® 2.0 Assay was performed to assess cytotoxicity and cell death.

3.3.5: Dual Treatment with Thyme & Tenofovir

Stock solutions of thyme extract and tenofovir were prepared in the same manner as indicated in Sections 3.3.2 and 3.3.4, and subsequently diluted with DMEM to the appropriate concentrations required for each dual treatment experiment. Volumes of 96-well plate wells previously seeded with Huh7.5.1 cells were aspirated, and 200 μL of treatment solution was pipetted into each well. For dual treatment groups, this included 100 μL of thyme solution and

100 µL of TDF solution (each original solution was doubled in concentration to achieve the intended dual-treatment concentrations upon mixing in the plate). Each plate also included a group of untreated controls and a group of vehicle controls containing the same percentage of DI water and PBS as the treatment group with the highest concentration of thyme extract and TDF. The plate was then incubated for 48 hours before a CellTiter-Glo® 2.0 Assay was performed to assess cytotoxicity and cell death.

3.3.6: Dual Treatment with Thymol & Tenofovir

Stock solutions of thymol and tenofovir were prepared in the same manner as indicated in Sections 3.3.3 and 3.3.4, and subsequently diluted with DMEM to the appropriate concentrations required for each dual treatment experiment. Volumes of 96-well plate wells previously seeded with Huh7.5.1 cells were aspirated, and 200 µL of treatment solution was pipetted into each well. For dual treatment groups, this included 100 µL of thymol solution and 100 µL of TDF solution (each original solution was doubled in concentration to achieve the intended dual-treatment concentrations upon mixing in the plate). Each plate also included a group of untreated controls and a group of vehicle controls containing the same percentage of EtOH and PBS as the treatment group with the highest concentrations of thymol and TDF. The plate was then incubated for 48 hours before a CellTiter-Glo® 2.0 Assay was performed to assess cytotoxicity and cell death.

3.4: *In Vitro* Assays of Cultured Cells

3.4.1: CellTiter-Glo Viability Assay

Cell viability was quantified using the CellTiter-Glo® 2.0 Assay, which generates a luminescent signal proportional to the ATP concentration in each well. Because cells were seeded and treated in opaque-walled 96-well plates, this assay could be performed and read in the same plate at the end of each treatment period. A volume of the CellTiter-Glo reagent equivalent to volume of cell culture media in each well was added to each well. Wells with just DMEM alone (no cells) were included and served as a blank to measure background luminescence. The plate was mixed on an orbital shaker for 2 minutes, then incubated at room temperature for 10 minutes to induce lysis. The plate was then read using a luminescence microplate reader to determine cell viability.

3.4.2: Glutathione (GSH) Assay

Dual treatment of Huh7.5.1 cells with thyme and TDF (or thymol and TDF) was carried out as detailed in Section 3.3.6. After 48 hours of treatment, a GSH-Glo™ Glutathione Assay (purchased from Promega) was performed. This assay produces a luminescent signal based on the amount of reduced glutathione (GSH) present in each well. Because cells were seeded and treated in an opaque-walled 96-well plate, the GSH-Glo assay could be performed and read in the same plate at the end of the treatment period. Solutions of known glutathione concentrations were prepared and added to empty wells on the plate, later used to generate a standard curve.

GSH-Glo and reconstituted luciferin detection reagents were prepared using solutions provided in the assay kit. After aspirating all cell culture media from the treated plate, 100 µL of

GSH-Glo reagent was added to each well (including those designated for the glutathione standard curve). This also included a set of empty wells, which served as a blank to measure background luminescence. The plate was briefly mixed with an orbital shaker and then incubated for 30 minutes at room temperature. At the end of the incubation period, 100 µL of reconstituted luciferin detection reagent was added to all of the wells that had also received the GSH-Glo reagent. The plate was briefly mixed with an orbital shaker and then incubated for 15 minutes at room temperature. Finally, the plate was read using a luminescence microplate reader to determine intracellular GSH levels. The luminescence of each well after GSH reaction was measured, along with that of the glutathione standards. Using the GSH standard curve, the concentration of GSH remaining in each well post-reaction was calculated to determine relative levels of oxidative stress among treatment groups.

3.5: Data & Statistical Analyses

3.5.1: TPC Assay Analysis

Absorbance values from the TPC assay were recorded for both gallic acid standards and thyme extract samples. Absorbance values of gallic acid at 765 nm were plotted against their concentrations to generate a linear standard curve. The regression equation of this curve was used to convert the average absorbance value (± SD) calculated for each thyme extract sample to the concentration of thyme (expressed as µg gallic acid equivalent (GAE)/mL). This value was then converted to mg GAE/mL and multiplied by the volume (in mL) of solvent used for sample extraction/grams of sample (where the mass of thyme is based on the dry weight used for extraction) to produce a TPC value in mg GAE/g thyme.

3.5.2: ABTS Assay Analysis

Absorbance values from the ABTS radical scavenging assay were recorded for both Trolox standards and thyme extract samples. Absorbance values of Trolox were plotted against their concentrations to generate a linear standard curve. The regression equation of this curve was then used to convert the average absorbance value (± SD) calculated for each thyme extract sample to Trolox equivalent antioxidant capacity (TEAC) values, expressed as μmol Trolox equivalent (TE)/g thyme (where the mass of thyme is based on the dry weight used for extraction). These TEAC values assess the radical scavenging ability of *Thymus vulgaris* extracts relative to a known antioxidant reference standard.

3.5.3: ORAC Assay Analysis

Fluorescence decay curves were recorded for both Trolox standards and thyme extract samples over the 45-minute assay period and used to calculate the area under the curve (AUC) for each well. Net AUC values were determined by subtracting the blank AUC from each sample and standard measurement. A linear standard curve was generated by plotting the net AUC values of Trolox standards against their concentrations, and the resulting regression equation was used to convert the average net AUC value (± SD) calculated for each thyme extract sample to TEAC values, expressed as μmol TE/g thyme (where the mass of thyme is based on the dry weight used for extraction). These TEAC values assess the peroxyl radical scavenging ability of *Thymus vulgaris* extracts relative to a known antioxidant reference standard.

3.5.4: Cell Viability Assay Analysis

Each experiment's treatment groups were performed in triplicate. Average luminescence readings (as measured by the CellTiter-Glo® 2.0 Assay) and standard errors were calculated per treatment group for each experiment, with adjustment for background luminescence. These values were then normalized to represent a percentage of the control group's average luminescence (expressed as % control cell viability). These percent control values were then averaged across multiple independent experiments for each experimental design and graphed as the mean ± SE. A one-way ANOVA test was run on each set of averaged data, with $p \leq 0.05$ considered statistically significant. If the ANOVA produced a p-value less than 0.05, Dunnett's post hoc test was subsequently performed to determine which specific treatment groups differed significantly from the control. An additional one-way ANOVA test was also used to verify that the viability of the vehicle control(s) was not significantly different from the viability of the untreated control.

3.4.5: GSH Assay Analysis

Each treatment group produced four replicates. Average luminescence readings (as measured by the GSH-Glo® Glutathione Assay) and standard errors were calculated per treatment group, with adjustment for background luminescence. Luminescence values of glutathione standard dilutions (ranging from 0-5 μM) were plotted against their concentrations to construct a linear standard curve. The equation for the standard curve was used to convert the average luminescence and standard error for each treatment group to the average intracellular glutathione concentration and standard error (expressed in μM GSH). A one-way ANOVA test was run on the dataset, with $p \leq 0.05$ considered statistically significant. If the ANOVA produced

a p-value less than 0.05, Dunnett's post hoc test was subsequently performed to determine which specific treatment groups differed significantly from the control. An additional one-way ANOVA test was also used to verify that the viability of the vehicle control(s) was not significantly different from the viability of the untreated control.

Chapter 4: Results

4.1: Assessing Antioxidant Capacity of Thyme

Phenolics are major bioactive compounds in plants that act as antioxidants. Therefore, the TPC and antioxidant capacity of powdered *Thymus vulgaris* in water and ethanol extracts (1:10 solid:solvent ratio) were assessed with three *in chemico* assays: TPC, ABTS, and ORAC. One trial of triplicate for pure water and ethanol thyme extracts (0.1 g/mL) and five serial dilutions ranging from 1:10 to 1:100000 were performed for TPC and ORAC, while one trial of duplicates was performed for ABTS.

Table 1. *Assessing total phenolic and antioxidant capacity of Thymus vulgaris with TPC, ABTS, and ORAC assays.*

Extract & Dilution	TPC (mg GAE/g Thyme)	ABTS (µmol TE/g Thyme)	ORAC (µmol TE/g Thyme)
Water (pure extract)	7.54 ± 0.18[a]	-[b]	-
Water (1:10)	0.96 ± 0.12	-	-
Water (1:100)	-	193.98 ± 6.34	199.50 ± 2.96
Ethanol (pure extract)	7.73 ± 0.07	-	-
Ethanol (1:10)	1.08 ± 0.13	-	-
Ethanol (1:100)	0.28 ± 0.01	365.76 ± 3.41	169.37 ± 13.56

[a] Values are presented as mean ± SD.

[b] Dilutions with absorbances that fell outside of the standard curve were omitted (-), including all dilutions greater than 1:100. Values are presented as mean ± SD.

A TPC assay was used to assess the total phenolic content of the extracts. Due to the limited range of dilutions tested, several values fell outside of the detection or calibration range. However, within the range of detection, thyme extracted with water (pure extract) and ethanol

(pure extract) exhibited GAE values of 7.54 ± 0.18 and 7.73 ± 0.12 mg GAE/g thyme, respectively (Table 1). An ABTS assay was used to assess the radical-scavenging capacity of the thyme extracts. As with TPC, most dilutions fell outside the detectable range. Within the range of detection, 1:100 dilutions of the water extract provided a TEAC value of 193.98 ± 6.34 µmol TE/g thyme, whereas 1:100 dilutions of the ethanol extract provided a TEAC value of 365.76 ± 3.41 µmol TE/g thyme. Finally, an ORAC assay was used to assess the peroxyl radical scavenging capacity of the thyme extracts. TEAC values of 199.50 ± 2.96 and 169.37 ± 13.56 µmol TE/g thyme extract were found for the water and ethanol extracts (1:100 dilution), respectively. To avoid potential negative side effects of ethanol in a cell model, the thyme extract prepared with water was used in subsequent *in vitro* testing.

4.2: Cytotoxicity Induced by Thyme Extract, Thymol, & Tenofovir

4.2.1: Cell Viability After Thyme Extract Treatment

Herbal extractions may exhibit cytotoxic effects in high doses. Therefore, a series of *in vitro* assays was performed to determine the maximum possible concentration of thyme extract that could be administered to Huh7.5.1 cells without inducing cell death. Effects on cell viability were assessed at 24 and 48 hours with concentrations ranging from 0 to 10,000 µg/mL.

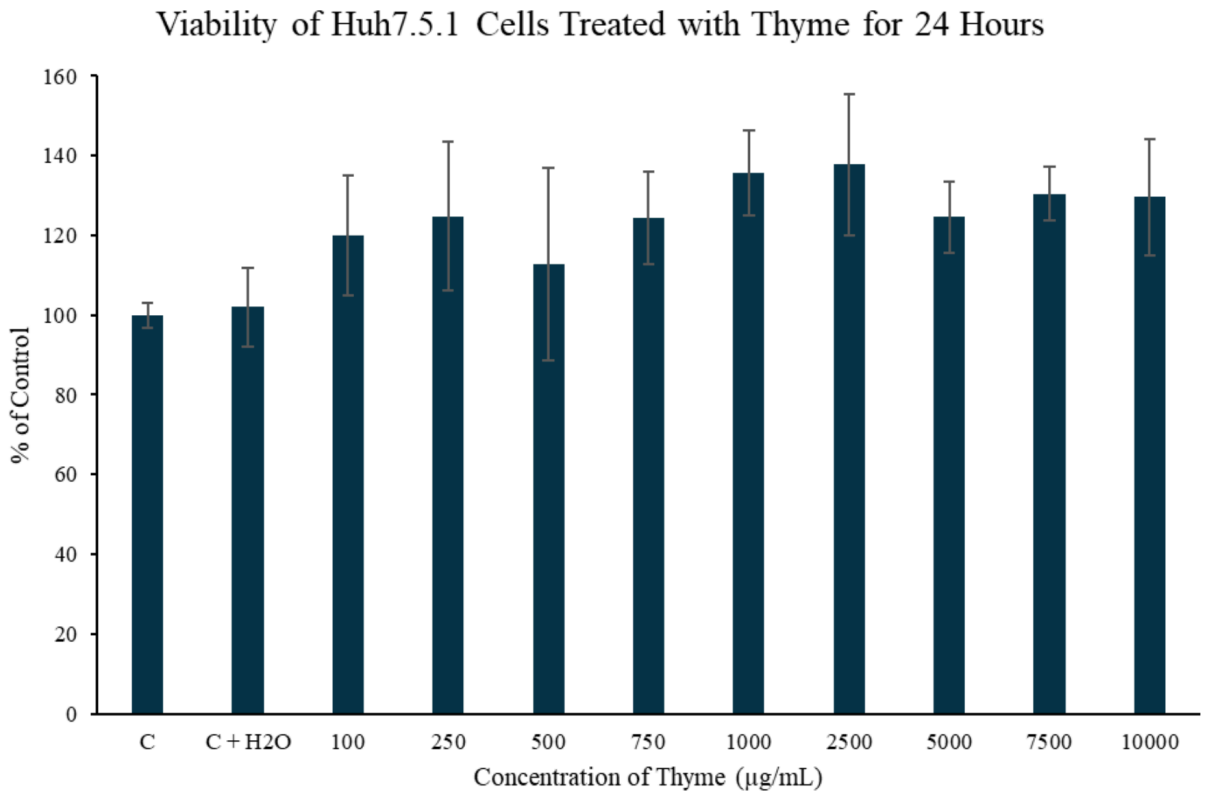


Figure 1. *Thyme treatment for 24 hours maintains cell viability in Huh7.5.1 cells.* Huh7.5.1 cells were treated with increasing concentrations of thyme extract (0–10,000 µg/mL) for 24 hours (n = 3). Control groups (0 µg/mL thyme) are labeled as C and C + H_2O, where C is an untreated control and C + H_2O serves as the vehicle control. Cell viability is quantified and expressed as a percentage relative to C. Data are shown as mean ± SE, and statistical differences were assessed by a one-way ANOVA. No significant differences were found between the tested concentrations ($p = 0.6345$).

Across three independent trials, thyme extract treatment for 24 hours did not induce significant cytotoxicity in the cell model. As shown in Figure 1, cell viability remained consistently above 100% of the untreated control across the majority of tested concentrations. Although some fluctuations were observed, there is no apparent dose-dependent decline in viability, as confirmed by a one-way ANOVA analysis revealing no significant difference between treatment groups. These results suggest that thyme extract maintains cell viability even at high concentrations over a 24-hour period.

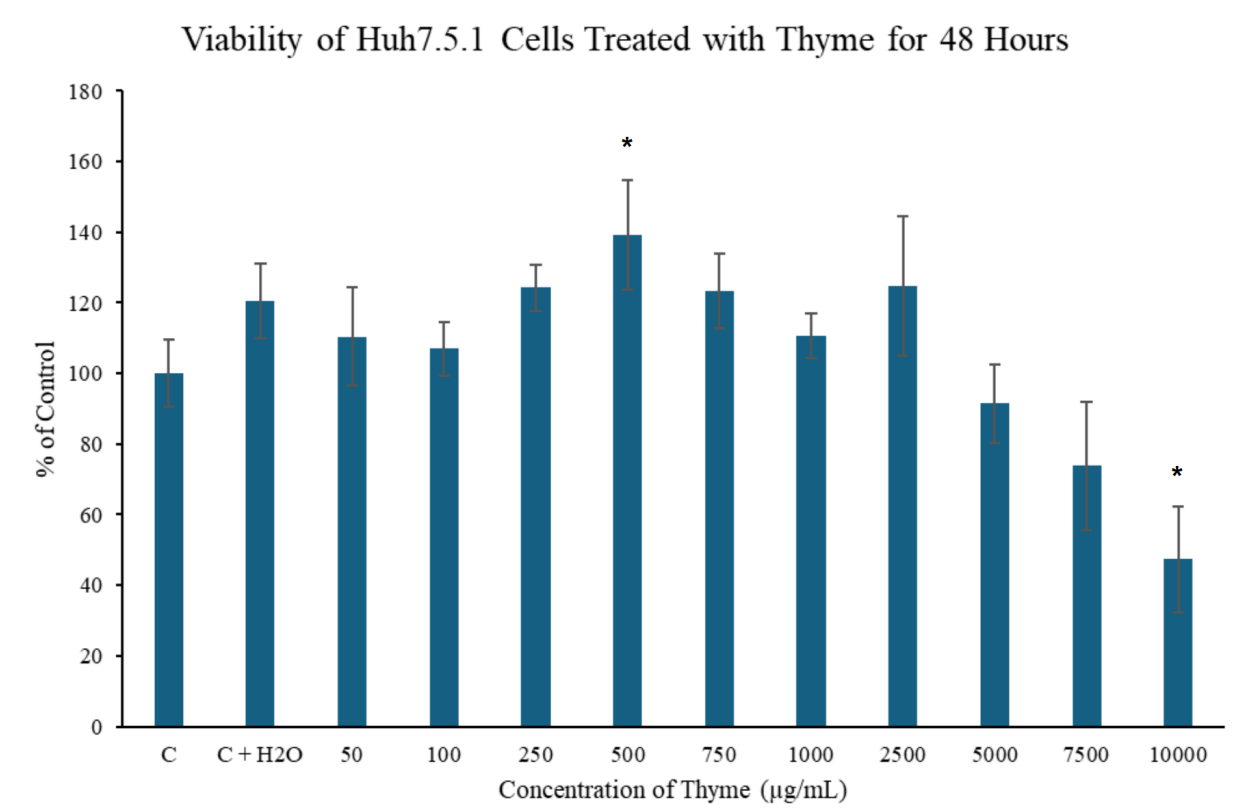


Figure 2. *Low to moderate concentrations of thyme extract maintain cell viability in Huh7.5.1 cells following 48-hour treatment.* Huh7.5.1 cells were treated with increasing concentrations of thyme extract (0–10,000 µg/mL) for 48 hours (n = 6). Control groups (0 µg/mL thyme) are labeled as C and C + H_2O, where C is an untreated control and C + H_2O serves as the vehicle control. Cell viability is quantified and expressed as a percentage relative to C. Data are shown as mean ± SE, and statistical differences were assessed by a one-way ANOVA ($p = 0.0005$) and subsequent Dunnett's test. * Represents a significant difference between the viability of the thyme concentration and the viability of C ($p \leq 0.05$).

After 48 hours of treatment, Huh7.5.1 cells displayed a concentration-dependent decline in viability in response to treatment with the thyme extract (Figure 2). Treatments between 50 and 2500 µg/mL concentrations consistently resulted in cell viability above 100% of the control, indicating preserved cellular health and potential efficacy at these doses. In contrast, higher concentrations resulted in a gradual reduction in viability, with a statistically significant difference between the control and 10000 µg/mL ($p = 0.0057$). These findings suggest that low to moderate thyme concentrations between 50 and 2500 µg/mL represent a safe range that balances biological activity with minimal toxicity, making them suitable for further studies. Given that viability at 500 µg/mL was significantly higher than the control ($p = 0.0285$), an

optimal range of 500-2000 µg/mL of thyme was selected for subsequent experiments on dual treatment with TDF.

4.2.2: Cell Viability After Thymol Treatment

Thymol is a major bioactive phenolic compound in *Thymus vulgaris* that may contribute to the alleviation of TDF-induced oxidative stress. Therefore, this isolated compound was selected for *in vitro* dual treatment with TDF. To identify thymol concentrations that do not induce cell death, thymol solutions ranging from 50 to 250 µg/mL were prepared and used to treat Huh7.5.1 cells. Cell viability was then measured after a 48-hour period, and the results were used to determine the concentrations to be used in the dual treatment.

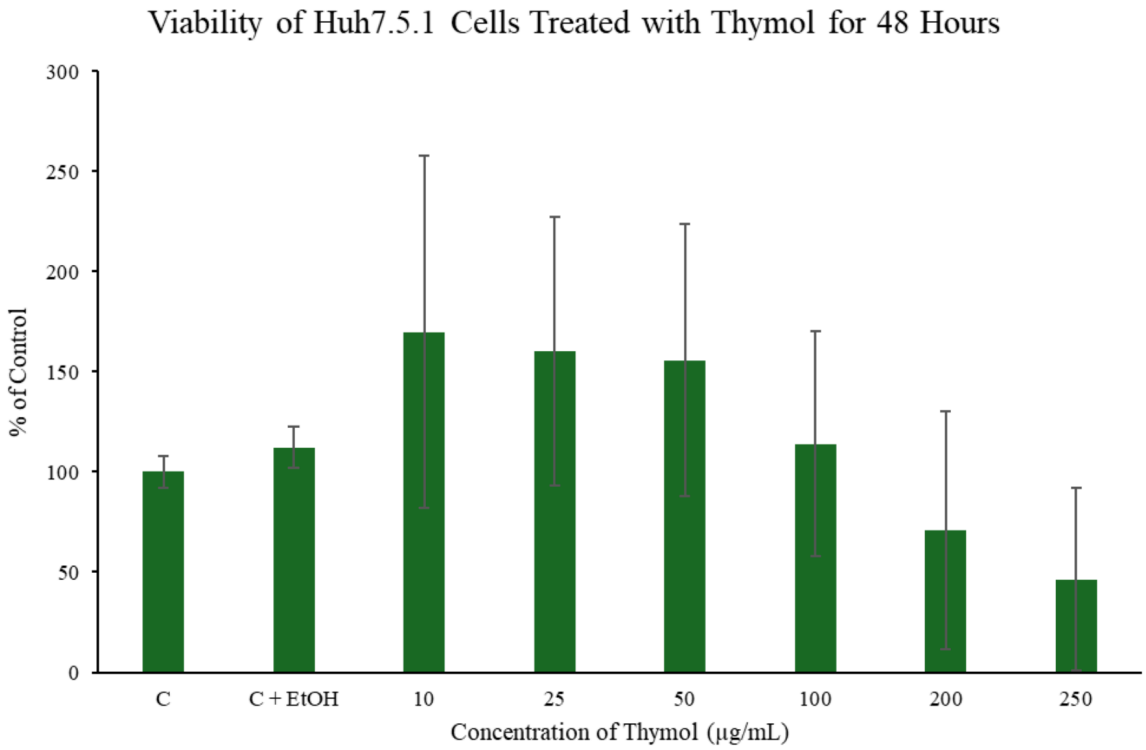


Figure 3. *Thymol induces no significant negative effects on cell viability after 48 hours of treatment.* Huh7.5.1 cells were treated with increasing concentrations of thymol (0–250 µg/mL) for 48 hours (n = 3). Control groups (0 µg/mL thymol) are labeled as C and C + EtOH, where C is an untreated control and C + EtOH serves as the vehicle control. Cell viability is quantified and expressed as a percentage relative to C. Data are shown as mean ± SE, and statistical differences were assessed by a one-way ANOVA. No significant differences were found between the tested concentrations ($p = 0.7709$).

Across three independent trials, Huh7.5.1 cells treated with thymol at concentrations between 0 and 100 µg/mL had, on average, higher cell viability than untreated controls after 48 hours of treatment (Figure 3). As with thyme, cell viability declined in a concentration-dependent manner, with concentrations >200 µg/mL showing reduced viability. However, given the lack of statistical significance between the control and the tested thymol concentrations, concentrations ranging from 0 to 250 µg/mL were still used in subsequent dual treatments with TDF and thymol.

4.2.3: Cell Viability After Tenofovir Treatment

Cells were treated with varying concentrations of TDF to determine a dose that would induce enough cytotoxicity without completely killing the tested population. Doses ranging from 0 to 480 µg/mL were tested over 48 hours.

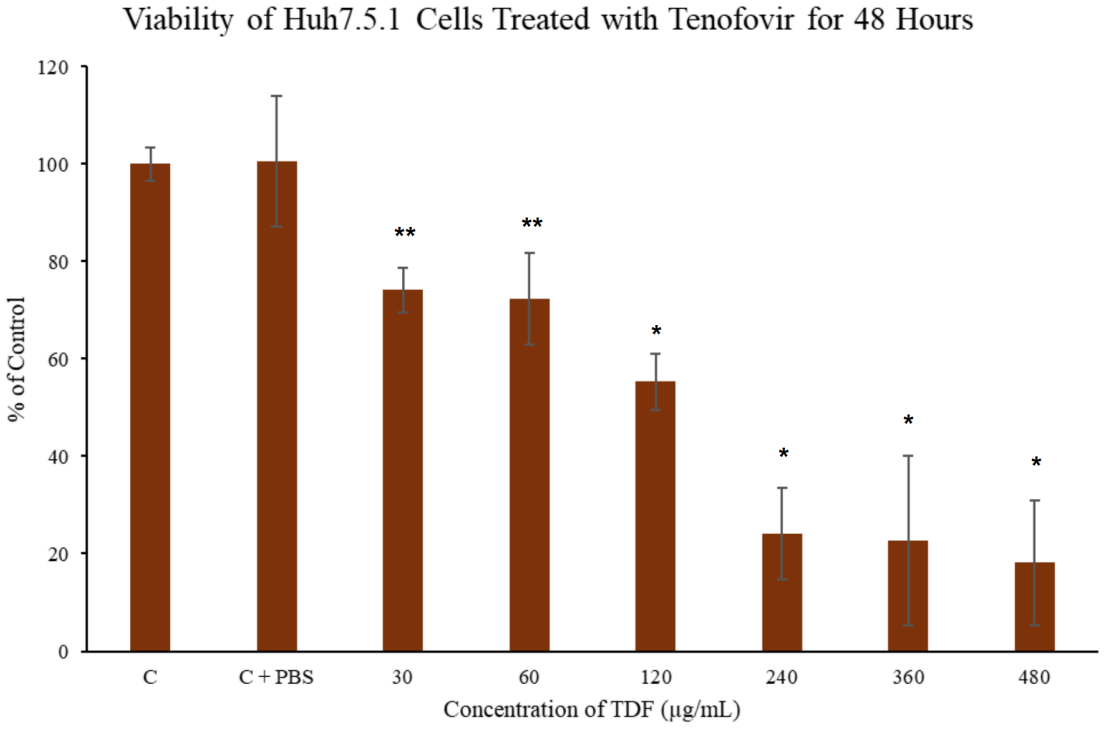


Figure 4. *TDF induces dose-dependent cytotoxicity in Huh7.5.1 cells after 48 hours of treatment.* Huh7.5.1 cells were treated with increasing concentrations of TDF (0-480 µg/mL) for 48 hours (n = 3). Control groups (0 µg/mL

TDF) are labeled as C and C + PBS, where C is an untreated control and C + PBS serves as the vehicle control. Cell viability is quantified and expressed as a percentage relative to C. Data are shown as mean ± SE, and statistical differences were assessed by a one-way ANOVA ($p = 0.00006$) and subsequent Dunnett's test. * Represents a significant difference between the viability of the TDF concentration and the viability of C ($p \leq 0.05$). ** Represents a significant difference between the viability of the TDF concentration and the viability of C ($p \leq 0.1$).

As shown in Figure 4, TDF exposure for 48 hours produced a clear concentration-dependent cytotoxic effect in the cell model. Cell viability gradually decreases across concentrations of 30 ($p = 0.0998$), 60 ($p = 0.0768$), and 120 µg/mL TDF ($p = 0.0082$) before dropping to much lower levels at 240 ($p = 0.0001$), 360 ($p = 0.0001$), and 480 ($p \approx 0.0000$) µg/mL. The consistent decrease in viability at elevated doses confirms that TDF exerts measurable toxicity that can act as a drug control for dual treatments in this cell model. The toxic effect observed at 30, 60, and 120 µg/mL suggests it could serve as a model of moderate cell injury while preserving partial cell survival. Consequently, subsequent dual-treatment experiments use some or all of those doses to investigate the recovery effects of thyme. Some experiments also included testing at 240 µg/mL to evaluate recovery at much more cytotoxic levels.

4.3: Thyme & Thymol Influence on Cell Viability in TDF-Treated Cells

4.3.1: Cell Viability After Dual Thyme & Tenofovir Treatment

Huh7.5.1 cells were treated with three concentrations of TDF: 60, 120, and 240 µg/mL, and non-toxic concentrations of thyme (determined from the optimal range in Figure 2) to identify the optimal conditions for mitigating TDF toxicity. Cell viability was assessed after 48 hours of exposure.

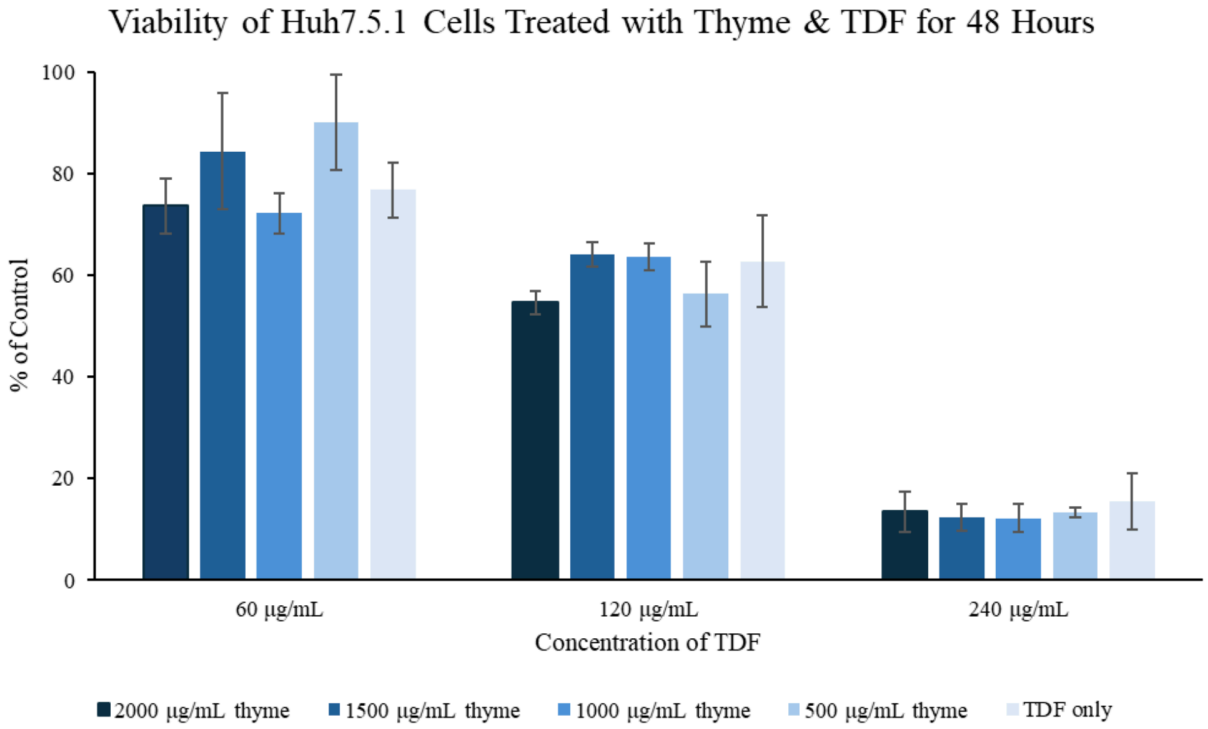


Figure 5. *Effects of thyme on TDF-induced cytotoxicity.* Huh7.5.1 cells were treated with 60, 120, and 240 µg/mL TDF and varying concentrations of thyme extract (500–2,000 µg/mL) for 48 hours (n =1 for 60 µg/mL TDF, n = 3 for 120 µg/mL TDF, and n = 2 for 240 µg/mL TDF). The drug control (0 µg/mL thyme) is labeled as TDF only. Cell viability is quantified and expressed as a percentage relative to an untreated control without thyme or TDF. Data are shown as mean ± SE, and statistical differences were assessed by a one-way ANOVA. No significant differences were found between the dual treatment groups for 60 ($p = 0.4570$), 120 ($p = 0.6595$), or 240 ($p = 0.9628$) µg/mL TDF.

Following dual-treatment trials with 60, 120, and 240 µg/mL TDF, it was observed that 120 µg/mL and 60 µg/mL consistently produced the moderate level of toxicity desired for testing. Averaging the three independent trials using 60 µg/mL TDF, there was no clear visible trend in viability across the tested concentrations of thyme, with 2000 µg/mL and 1000 µg/mL displaying decreased viability and 1500 µg/mL and 500 µg/mL displaying increased viability. The 120 µg/mL trials also showed no clear trend across thyme concentrations, with 2000 µg/mL and 500 µg/mL showing decreased viability and 1500 µg/mL and 1000 µg/mL showing increased viability. All 240 µg/mL thyme treatments showed decreased viability compared with the TDF-only control. Considering these averaged values of the three independent runs, no

concentration of thyme exhibits a significant increase or decrease in cell viability for any concentration of TDF.

However, some significant differences were observed within individual runs (evaluated first by a one-way ANOVA, and subsequently with Dunnett's test if $p \leq 0.05$). The first experiment tested dual treatment groups for 60 and 120 μg/mL TDF. While no significant differences were found for any concentrations of thyme combined with 60 μg/mL TDF ($p = 0.4570$), cells treated with 120 μg/mL of TDF produced a significant increase in viability at 2000 μg/mL of thyme ($p = 0.0015$) and a significant decrease in viability at 1500 ($p = 0.0041$) and 1000 μg/mL of thyme ($p = 0.0343$). The second experiment tested dual-treatment groups at 120 and 240 μg/mL TDF. While no significant differences were found for any concentrations of thyme combined with 120 μg/mL TDF ($p = 0.0669$), cells treated with 240 μg/mL of TDF produced a significant increase in viability at 500 μg/mL of thyme ($p = 0.0193$). The third experiment again tested dual-treatment groups at 120 and 240 μg/mL TDF. While no significant differences were found for any concentrations of thyme combined with 240 μg/mL TDF ($p = 0.6040$), cells treated with 120 μg/mL of TDF produced a significant decrease in viability at 500 μg/mL of thyme ($p = 0.0030$). All of these points to a high degree of variability in the cell culture model, or to the need for further study of the mechanism underlying these interactions.

In order to preliminarily assess the ability of thyme treatment to mitigate ART-induced nephrotoxicity, a lineage of kidney epithelial cells from the African green monkey, known as Vero cells, were dually treated with thyme and TDF for 48 hours (Ammerman et al., 2008). Cells were treated with concentrations of 30, 60, 120, and 240 μg/mL of TDF in order to gauge the range in which this new cell line would react to the drug. Data collected from cells treated with

120 µg/mL TDF has been omitted due to contamination of the dual treatment groups during the experiment.

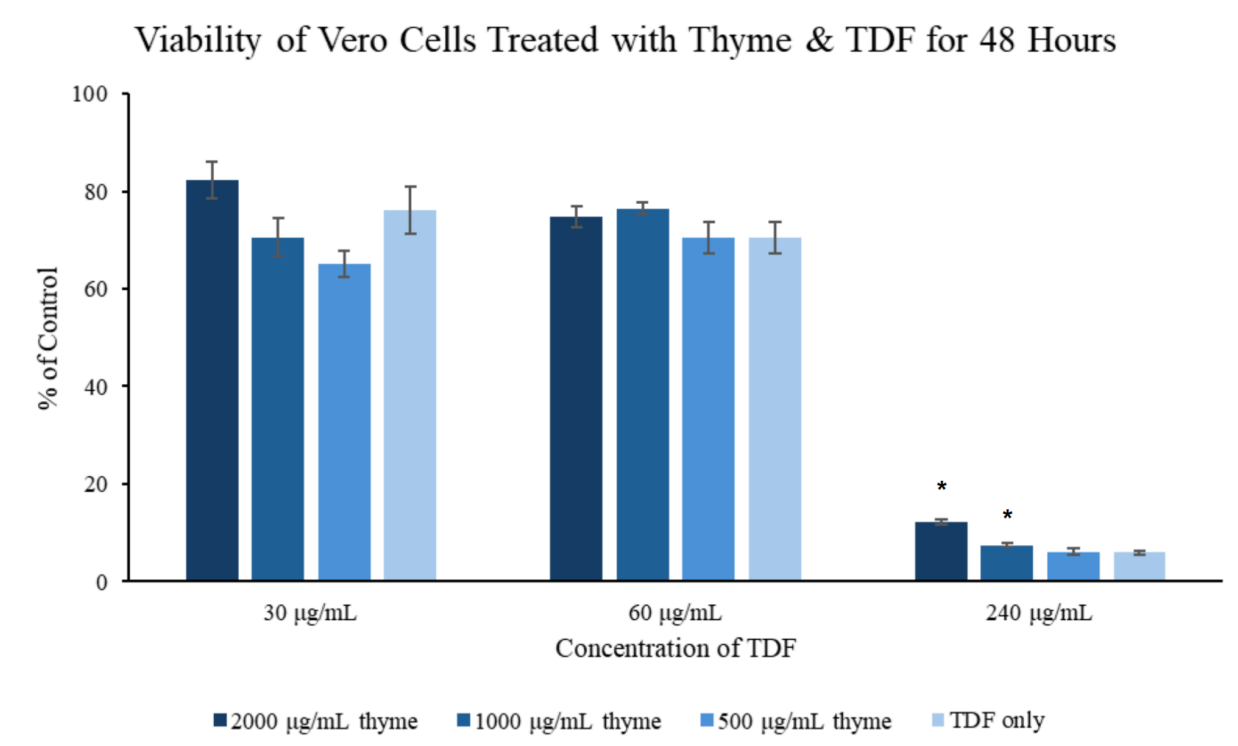


Figure 6. *Effects of thyme on TDF-induced cytotoxicity in Vero cells.* Vero cells were treated with 30, 60, and 240 µg/mL TDF and varying concentrations of thyme extract (500–2,000 µg/mL) for 48 hours (n = 1). The drug control (0 µg/mL thyme) is labeled as TDF only. Cell viability is quantified and expressed as a percentage relative to an untreated control without thyme or TDF. Data are shown as mean ± SE, and statistical differences were assessed by a one-way ANOVA ($p = 0.0277$ for 30 µg/mL TDF, $p = 0.2743$ for 60 µg/mL TDF, $p = 3 \times 10^{-9}$ for 240 µg/mL TDF) and subsequent Dunnett's test if $p \leq 0.05$. * Represents a significant difference between the viability of the dual treatment group and its corresponding TDF-only group ($p \leq 0.05$).

As shown in Figure 6, Vero cells treated with TDF alone exhibit the same pattern as Huh7.5.1 cells in Figure 4: increasing concentrations of TDF reduce cell viability relative to the control, with 240 µg/mL producing the most cytotoxicity. However, within this concentration, both 2000 µg/mL ($p \approx 0.0000$) and 1000 µg/mL ($p = 0.0472$) of thyme produced significantly higher viability than the TDF-only control. No concentration of thyme exhibited a significant difference in cell viability for 30 or 60 µg/mL TDF (although $p = 0.0277$ for 30 µg/mL TDF, Dunnett's test did not yield any p-values ≤ 0.05), but a visible increase in viability was observed

with increasing concentrations of thyme for the 60 µg/mL thyme treatment, similar to the 240 µg/mL treatment.

4.3.2: Cell Viability After Dual Thymol & Tenofovir Treatment

Thymol was tested for its ability to reduce cytotoxicity in TDF-treated Huh7.5.1 cells through an experiment similar to dual treatment with thyme and TDF in section 4.3.1. As no thymol concentration was found to significantly harm cell viability in Figure 3, the thymol concentrations used in dual treatment ranged from 25 to 200 µg/mL. The TDF concentrations tested were 60 and 120 µg/mL, and cell viability was assessed after 48 hours.

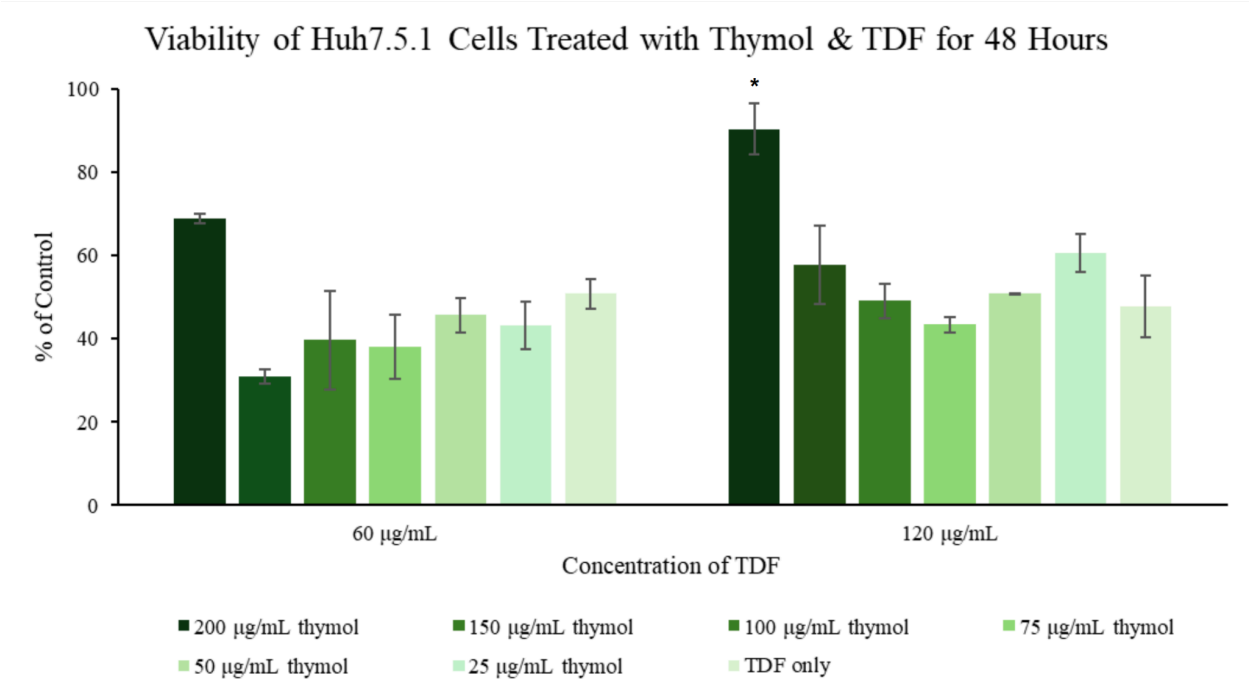


Figure 7. *Effects of thymol on TDF-induced cytotoxicity.* Huh7.5.1 cells were treated with 60 and 120 µg/mL TDF and varying concentrations of thymol (25-200 µg/mL) for 48 hours (n = 1). The drug control (0 µg/mL thymol) is labeled as TDF only. Cell viability is quantified and expressed as a percentage relative to an untreated control without thymol or TDF. Data are shown as mean ± SE, and statistical differences were assessed by a one-way ANOVA ($p = 0.0544$ for 60 µg/mL TDF, $p = 0.0073$ for 120 µg/mL TDF) and subsequent Dunnett's test if $p \leq 0.05$. * Represents a significant difference between the viability of the dual treatment group and its corresponding TDF-only group ($p \leq 0.05$).

As shown in Figure 7, thymol significantly increased viability at 200 µg/mL for cells treated with 120 µg/mL TDF ($p = 0.0007$). This TDF treatment also exhibited a general increase

in viability at higher thymol concentrations (excluding 50 and 75 µg/mL). However, the 60 µg/mL treatment group showed the opposite visual trend, with viability decreasing as thymol concentration increased (except for the 200 µg/mL treatment), and no significant differences among treatments. More trials are needed to confirm the reliability of the results observed in this experiment.

4.4: Thyme's Influence on TDF Treatment via Glutathione (GSH) Assay

To gain a deeper understanding of the intracellular oxidative changes taking place during dual treatment with thyme and TDF, a GSH assay was performed. Huh7.5.1 cells were treated with concentrations of thyme ranging from 0-2000 µg/mL (within the optimal range established by Figure 2) alongside three concentrations of TDF: 30, 60, and 120 µg/mL. Intracellular GSH levels were assessed after 48 hours of exposure.

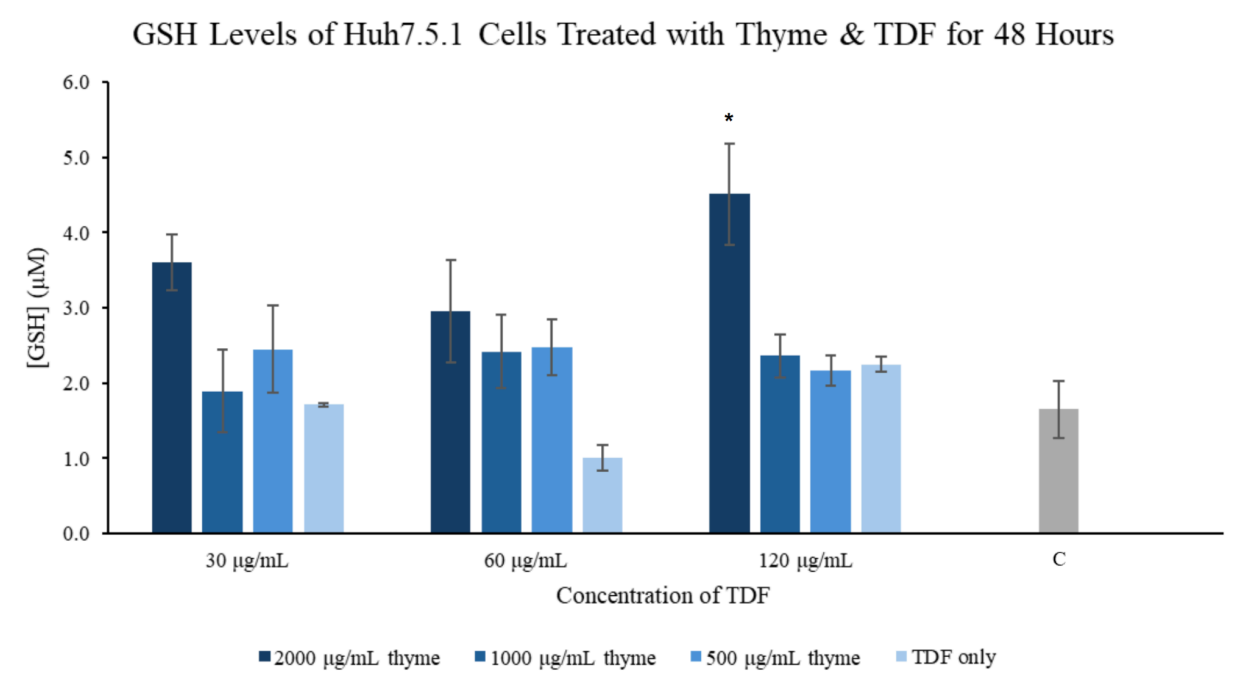


Figure 8. *Effects of thyme on oxidative stress levels of TDF-treated cells.* Huh7.5.1 cells were treated with varying concentrations of thyme extract (0–2,000 µg/mL) and TDF (30-120 µg/mL) for 48 hours (n = 1). The drug control (0 µg/mL thyme) is labeled as TDF only, and the untreated control is labeled as C. Intracellular glutathione levels are

quantified and expressed in units of μM. Data are shown as mean ± SE, and statistical differences were assessed by a one-way ANOVA ($p = 0.0802$ for 30 μg/mL TDF, $p = 0.0959$ for 60 μg/mL TDF, $p = 0.0013$ for 120 μg/mL TDF) and subsequent Dunnett's test if $p \leq 0.05$. * Represents a significant difference between the GSH levels of the dual treatment group and its corresponding TDF-only group ($p \leq 0.05$).

Higher GSH concentrations are indicative of lower oxidative stress levels. As shown in Figure 8, increasing thyme concentrations led to higher GSH concentrations in the 60 μg/mL TDF and 120 μg/mL TDF treatments. Confirming this trend, intracellular glutathione levels were significantly greater in the presence of 2000 μg/mL thyme and 120 μg/mL TDF than in the presence of 120 μg/mL TDF alone ($p = 0.0005$). However, no thyme concentration showed a significant difference in GSH levels at 30 or 60 μg/mL TDF. These results suggest that higher thyme concentrations may reduce oxidative stress levels, but further trials are necessary. These results also support the idea that changes in oxidation may be better assessed at the molecular level rather than through a broad endpoint such as cell viability, though future study could assess the exact mechanism by which antiretroviral drugs such as TDF produce cytotoxicity, and if interventions such as antioxidants can mitigate the toxicity through this mechanism.

A GSH assay was also performed to evaluate changes in oxidative stress during dual treatment with thymol and TDF. Two concentrations of thymol (100 and 200 μg/mL) were tested alongside three concentrations of TDF: 30, 60, and 120 μg/mL. Intracellular GSH levels were assessed after 48 hours of exposure.

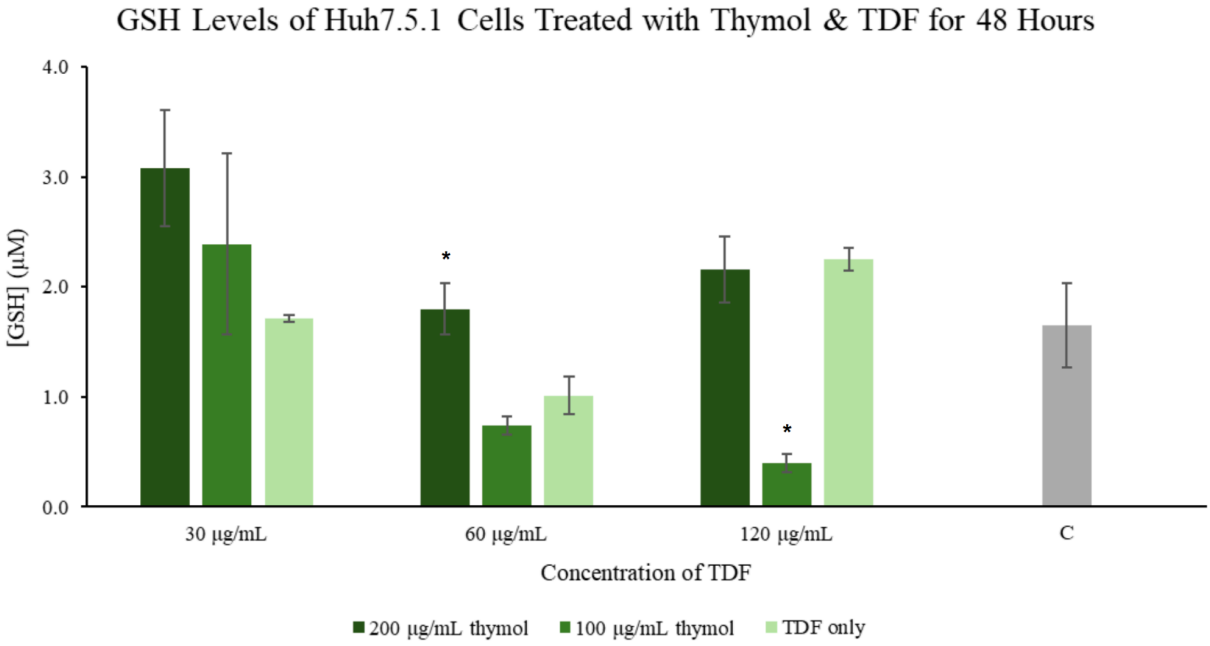


Figure 9. *Effects of thymol on oxidative stress levels of TDF-treated cells.* Huh7.5.1 cells were treated with varying concentrations of thymol (100 and 200 μg/mL) and TDF (30-120 μg/mL) for 48 hours (n = 1). The drug control (0 μg/mL thymol) is labeled as TDF only, and the untreated control is labeled as C. Intracellular glutathione levels are quantified and expressed in units of μM. Data are shown as mean ± SE, and statistical differences were assessed by a one-way ANOVA ($p = 0.4665$ for 30 μg/mL TDF, $p = 0.0060$ for 60 μg/mL TDF, $p = 0.0008$ for 120 μg/mL TDF) and subsequent Dunnett's test if $p \leq 0.05$. * Represents a significant difference between the GSH levels of the dual treatment group and its corresponding TDF-only group ($p \leq 0.05$).

Preliminary GSH assay results involving thymol and TDF dual treatment demonstrate that intracellular glutathione levels are significantly greater in the presence of 200 μg/mL thymol and 60 μg/mL TDF than in the presence of 60 μg/mL TDF alone ($p = 0.0098$). However, these results also show that GSH levels are significantly lower in the presence of 100 μg/mL thymol and 120 μg/mL TDF than in the presence of 120 μg/mL TDF alone ($p = 0.0005$). No concentration of thymol exhibits a significant difference in GSH levels for 30 μg/mL TDF. Overall, these preliminary results are contradictory and require further testing.

Chapter 5: Discussion

HIV-1 infection is a prevalent global health challenge, affecting millions of people every year. This study aimed to address some of the social, economic, and physical factors that inhibit ART adherence among PLWH by testing an accessible antioxidant source, garden thyme, to assess its ability to mitigate the harmful side effects associated with the antiretroviral medication TDF. Since oxidative stress plays a central role in the development of ART-induced toxicity, antioxidant supplementation has been proposed as a potential strategy to mitigate these adverse effects. Antioxidants function by neutralizing ROS and supporting endogenous antioxidant defense systems within the cells. Plant-derived antioxidants are of particular interest due to their accessibility and high concentrations of bioactive compounds such as polyphenols and flavonoids (Bouayed & Bohn, 2010; Munteanu & Apetrei, 2021). This study specifically aimed to evaluate whether this antioxidant capacity could translate into a measurable reduction in TDF-induced cytotoxicity.

Garden thyme was selected as an antioxidant source based on previous literature reporting high levels of phenolic compounds and strong antioxidant capability (Roby et al., 2013). In an effort to investigate these claims, the TPC of thyme extracts prepared using both water and ethanol as solvents was evaluated, in the hope of quantifying total phenolic content, since phenolic hydroxyl groups are primary contributors to plant-derived antioxidant activity. Demonstration of high phenolic content would support the mechanistic plausibility that *Thymus vulgaris* extracts could mitigate ART-induced oxidative stress via radical-scavenging pathways. TPC assay results yielded values of 7.54 mg GAE/g (water) and 7.73 mg GAE/g (ethanol) for thyme. These values were comparable to previously reported literature values of 7.30 mg GAE/g

for thyme extracted in ethanol and 8.10-8.89 mg GAE/g for thyme extracted in methanol (Mokhtari et al., 2023; Roby et al., 2013). These findings suggest that the extraction process successfully preserved at least some of the phenolic antioxidant compounds responsible for thyme's antioxidant activity. However, it is important to note that the TPC assay mentioned here was performed with n = 1 sample due to time and resource constraints.

Because ART-induced mitochondrial dysfunction increases ROS production, assays that quantify radical scavenging ability (ABTS, ORAC) were selected to evaluate whether *Thymus vulgaris* extracts could neutralize physiologically relevant oxidative species. The ABTS assay was selected since it measures both hydrogen atom transfer and single-electron transfer antioxidant mechanisms, allowing evaluation of multiple radical-neutralization pathways relevant to ROS generated during mitochondrial dysfunction. The ethanol extract of thyme was found to have an ABTS value of 366 μmol TE/g thyme, indicating high antioxidant activity consistent with previous studies (Assiri et al., 2016; Yao et al., 2023). The thyme extract in water showed a lower value of 194 μmol TE/g thyme; however, this is attributed to error from the water-diluted Trolox standard curve rather than a true reduction in antioxidant activity in the water sample. Additional trials should be conducted to more accurately assess the antioxidant potential of the thyme extracts. Literature values also demonstrate that thyme has ABTS radical-scavenging capability comparable to other known antioxidants, such as green tea (Khnissi et al., 2025; Sieniawska et al., 2017). The ORAC assay complements ABTS measurements by specifically quantifying peroxyl radical scavenging capacity, which is particularly relevant because peroxyl radicals are major contributors to lipid peroxidation during ART-induced oxidative stress. Upon antioxidant evaluation using the ORAC assay, thyme extracts in water and ethanol were found to have values of 200 and 170 μmol TE/g thyme,

respectively. Although this does indicate antioxidant activity, it does not demonstrate the very high antioxidant activity expected based on previous studies (Brewer, 2011). The limited antioxidant capacity data provided by the ORAC assay were attributed to insufficient replicates and experimental errors (e.g. errors in standard curves) rather than to a conclusive lack of high antioxidant potential in our samples.

Given support from various literature sources, *in vitro* experiments were conducted, assuming the strong antioxidant activity of the chosen sample. The thyme extracts prepared in water were selected for subsequent *in vitro* testing over the ethanol extractions, despite exhibiting similar antioxidant capacity, due to concerns of the potential cytotoxic effects of ethanol. For the cell studies, it was important to isolate the impacts of the thyme antioxidant and TDF on the cell toxicity. The use of ethanol could have introduced an additional variable affecting cell viability. Therefore, water was chosen as the extraction solvent for thyme due to its inert, non-toxic nature, ensuring minimal variability across cell treatments. For the thymol extractions, however, only a minimal amount of ethanol was used because thymol is significantly less soluble in water than in ethanol.

Following evaluation of antioxidant activity, Huh7.5.1 cell viability assays were conducted to determine the range of thyme concentrations that could be safely administered without inducing cytotoxicity. The thyme extract maintained high cellular viability across a wide range of concentrations at shorter exposure times (24 hours), while moderate decreases in viability were observed at higher concentrations (above 2500 μg/mL) after longer treatment durations (48 hours). Other literature concentrations found to be nontoxic for thyme were lower, ranging from 50-160 μg/mL for HepG2 cells treated with methanolic extract and up to ~125 μg/mL for THP-1 cells treated with ethanolic extract (Ayesh et al., 2014; Firat et al., 2025).

Higher values than those reported in other studies are observed, and the trend established by Figure 2 suggests that between 500 and 2500 µg/mL of thyme is the optimal range, most likely capable of balancing antioxidant strength with minimal cytotoxic effects.

To establish a model for ART-induced cellular stress, cells were treated with increasing concentrations of TDF. TDF treatment produced a clear concentration-dependent reduction in cell viability after prolonged exposure, demonstrating that the drug induces measurable cytotoxic death in this cell model within 48 hours. TDF concentrations of 30 and 60 µg/mL resulted in an approximately 30% decrease in cell viability and were subsequently considered appropriate for inducing moderate cellular injury while preserving a sufficient population of viable cells to evaluate potential protective effects of thyme supplementation (Figure 4). The same was true for 120 µg/mL of TDF, which demonstrated an approximately 50% decrease in cell viability. Subsequent experiments also tested thyme's recovery at 240 µg/mL TDF, which induces significantly lower cell death after 48 hours.

Following optimization of thyme and TDF treatments, dual treatment experiments assessed whether thyme extracts could mitigate cellular stress induced by TDF exposure. Cells treated with both thyme and TDF showed mixed results relative to cells treated with TDF alone, with no clear or statistically significant trend in cell viability as thyme concentration increased (Figure 5). Of the three trials conducted at 120 µg/mL TDF for 48 hours, treatment with thyme showed increased cell viability in one, decreased in another, and no effect in the third. Therefore, it is unclear from these experiments whether thyme extract may mitigate TDF-induced cytotoxicity in the Huh7.5.1 cell line. The explanation for such variability could be complex and multifaceted, including technical errors, variability in using non-pure dried thyme samples, or cell death caused by factors other than oxidative stress, as initially proposed in this paper.

To address potential confounding factors associated with the use of dried thyme extract, an additional compound was later introduced into this study: pure thymol, a major phenolic compound present in thyme and reported to have high antioxidant potential (Nagoor Meeran et al., 2015). Cells treated with varying concentrations of thymol alone for 48 hours did not display any significant reductions in viability. Still, they did exhibit a concentration-dependent decline similar to that of thyme extract (Figure 3). Thymol significantly increased viability at 200 µg/mL in cells treated with 120 µg/mL TDF, but Figure 7 shows an otherwise unclear trend between the thymol concentration in dual treatment and resulting cell viability. This observation led to an investigation of the reasons why the phenolic compound did not significantly benefit the cells as previously expected.

Upon review of the literature, the application of thymol in *in vivo* studies has shown that thymol is absorbed primarily in the upper gastrointestinal tract (Nagoor Meeran et al., 2017). The rapid metabolism and limited bioavailability may reduce its concentration and efficacy in target tissues. This finding is significant for two reasons: 1) it is possible that treatment of thymol on the selected Huh7.5.1 liver cells did not show an effect due to the lack of cellular response and interaction between the cells and thymol, and 2) given the held belief that TDF cytotoxicity affects liver function of individuals, thymol would not be applicable in a real world treatment setting due to the lack of absorption found in the liver. This finding is supported by other studies, in which oral administration of thymol showed 24-hour elimination, with low concentrations of thymol recovered in the liver, lungs, kidneys, and muscles (Nieddu et al., 2014). Another significant consideration is the possibility of thymol degradation during storage or during suspension preparation in water, leading to reduced antioxidant activity over the course of experimentation and resulting in inconsistent outcomes. Additionally, thymol has poor solubility

in aqueous solutions. While the thymol solution was initially prepared in ethanol, it was then further diluted in aqueous DMEM to reduce the ethanol concentration to a physiologically safe level, which may have caused thymol to precipitate out of solution and created variability in thymol concentration.

Vero cells, a lineage of kidney epithelial cells, were selected for further preliminary testing in order to evaluate how different cell types would react to dual treatment. Kidney cells were selected due to the prevalence of renal toxicity in ART-induced side effects. Specifically, this renal toxicity has been associated with mitochondrial dysfunction induced by TDF (Muñoz-Muela et al., 2025; White, 2001). Similar to Huh7.5.1 cells, Vero cells were treated with both thyme and TDF for 48 hours before measuring cell viability. Across all treatment groups, the only groups that were significantly higher than their TDF-only control were those treated with 240 μg/mL TDF + 2000 μg/mL thyme and 240 μg/mL TDF + 1000 μg/mL thyme. However, because this preliminary testing was n = 1, and the cell viability across the entire 240 μg/mL TDF group was below 20%, little can be concluded from this result. Further testing is needed to assess the effects of TDF and thyme treatment on kidney cells.

Given the lack of consensus from thyme and thymol dual treatments, other potential causes of our inconsistent results were considered. First, it is important to note that cell viability assays may not have fully captured biochemical changes associated with oxidative stress, and were too broad to fully capture the effects of thyme and thymol treatment. The CellTiter-Glo assay was used as an indirect indicator of mitochondrial integrity because ATP production depends on intact oxidative phosphorylation. Since ART-associated toxicity disrupts mitochondrial function, changes in ATP-linked viability provide a functional readout of oxidative stress-related cytotoxicity. However, mitochondrial dysfunction and oxidative damage can occur

prior to detectable reductions in cell viability. Cells therefore, may maintain ATP production through compensatory metabolic pathways even when mitochondrial function is impaired. As a result, oxidative stress may be present even when viability measurements appear relatively unchanged. To better assess the consistency of suspension preparation, the initial antioxidant assays (TPC, ORAC) should be run before pursuing the cell viability assays to determine whether antioxidant activity is consistent with measured viability. Unfortunately, the scope of this initial experiment was not feasible due to lack of resources and time availability, but is recommended for further investigation.

In an attempt to evaluate the shortcomings of the cell viability experiments, assays measuring intracellular antioxidant systems, such as glutathione, were later introduced in this study to provide additional insight into the earlier stages of drug-induced cellular stress. Intracellular glutathione levels were measured because reduced glutathione is a central regulator of cellular redox balance and is depleted during oxidative stress. Restoration of GSH levels following thyme treatment would provide mechanistic evidence of supporting antioxidant-mediated mitigation of ART-induced oxidative damage. Unfortunately, the results of the GSH assay did not provide consistent trends. Oxidative stress levels in cells undergoing dual treatment with thymol and TDF show conflicting data: GSH levels are higher in dual treatment groups at 30 µg/mL TDF than in TDF alone, but lower in dual-treatment groups at 60 and 120 µg/mL TDF than in TDF alone (Figure 9). As for the thyme and TDF-dual-treated cells, the results varied similarly to the cell viability data. Interestingly, GSH levels in TDF-only-treated cells ranged from higher (120 µg/mL) to lower (60 µg/mL), to similar (30 µg/mL) to those in control cells. This result was entirely at odds with the expected trend, in which TDF-only-treated cells should, as previously investigated, have demonstrated a significant decrease in GSH levels

due to oxidative stress (Ndlovou et al., 2023). Furthermore, a concentration-dependent decline in GSH levels would be expected as TDF concentrations increase, but this was not observed in Figures 9 or 10.

However, some significance was observed when comparing TDF-only-treated cells with cells dually treated with thyme and TDF. Intracellular GSH levels in cells treated with 120 μg/mL TDF and 2000 μg/mL thyme are significantly higher than in cells treated with TDF alone, suggesting potential improvement with this dual-treatment approach. Based on these results, it is reasonable to consider 2000 μg/mL the optimal tested concentration for combating oxidative stress in these dual-treated cells. It is likely that more significant findings could be observed at concentrations above this level; however, Figure 2 showed increasing cell death at thyme concentrations greater than 2500 μg/mL. It is inconclusive whether the cell death viewed above 2500 μg/mL is due to oxidative stress or to other cellular processes. Further investigation is needed to explore the effects of dual treatment conditions on these cells at higher thyme concentrations. As for GSH levels in thymol- and TDF-dual-treated cells, the data are too contradictory to draw any preliminary conclusions, further supporting the previous claim that thymol is not effective in this application.

Because antioxidant activity cannot be fully characterized using a single assay, multiple complementary approaches were used. TPC quantified total phenolic antioxidant potential; ABTS and ORAC assessed radical scavenging via distinct mechanisms; and cellular assays (CellTiter-Glo and GSH) evaluated whether these chemical antioxidant properties translated into biologically meaningful protection against oxidative stress in hepatocyte models. However, given the lack of consistent and significant trends in both the cell viability and GSH assays, it is important to consider other potential causes of adverse effects from ARTs beyond oxidative

stress. Firstly, although the models created *in vitro* provide insight into radical scavenging potential, they likely did not replicate the intracellular antioxidant dynamics or the conditions experienced by the human body during HIV infection and subsequent long-term ART treatment. The lack of reproducible cell viability from *in vitro* dual treatment, as well as the lack of improvement with increasing thyme concentrations, may have reflected the chosen study environment rather than indicating how the human body would react to this treatment. The cellular environment was established after 48 hours of TDF treatment, rather than the daily dosing required for PLWH taking this medication. Likely, the oxidative stress and inflammatory response by liver cells *in vivo* is much different than that created within Huh7.5.1 cells after such short exposure. It may be possible to obtain more consistent and significant results after much longer *in vitro* treatment of both TDF and thyme. Therefore, the lack of an observed effect in this *in vitro* model does not necessarily preclude potential benefits *in vivo*, where chronic exposure, immune interactions, and systemic metabolism play significant roles.

In addition, it is possible that the mechanism by which TDF induces toxicity or produces side-effects in clinical applications is not solely through induction of oxidative stress, and thus cannot be addressed solely by antioxidant supplementation. The prevailing model of toxicity involves depletion of mtDNA and inhibition of a mitochondrial polymerase, but TDF is also implicated in activation of apoptotic signaling pathways, leading to cell death via a mechanism distinct from oxidative stress or mitochondrial dysfunction. If this mechanism of cell death is responsible for a significant portion of toxicity, then antioxidant supplementation will be less effective in addressing TDF-induced toxicity, as it primarily mitigates toxicity related to oxidative stress and radical formation. In addition, even if ROS generated by mitochondrial dysfunction is neutralized by an antioxidant, the basal mitochondrial dysfunction, a currently

immutable property of TDF treatment, will not be resolved. Antioxidant supplementation serves as a means of neutralizing cellular radicals and preventing toxicity, but cannot currently address all mechanisms of toxicity.

Another consideration is the complex relationship between ART treatment and HIV infection. This study explored non-infected cells to independently analyze the effects of TDF as an ART on cell viability from oxidative stress. However, it is known that ARTs work by reducing HIV replication, subsequently decreasing chronic inflammation and immune activity from HIV. Overall, this lowers the initially high levels of oxidative species caused by the HIV virus itself. Due to the mechanism of action of this medication, it may be possible that adverse effects and oxidative stress is only observed within cells after HIV infection and long-term ART usage. Although ARTs prove capable of viral suppression, redox imbalances persist due to the lack of the ART's ability to repair oxidative damage fully, and are only observed after long-term treatment with an ART for HIV infection. Our study, therefore, may have been limited in scope in analyzing non-HIV-infected cells and TDF treatment without HIV infection.

Although herbs such as thyme contain antioxidant compounds that may alleviate oxidative stress in cells, the clinical relevance of our findings remains unclear. Previous studies have found that antioxidant supplementation has had no significant effect on HIV outcomes, possibly due to variation in individual baseline serum micronutrient levels that limit the efficacy of supplementation (Wilkinson et al., 2018). Further, it is widely accepted among nutrition researchers that overall dietary patterns have a greater impact on health outcomes than supplementation with specific nutrients. Balanced diets high in fruits and vegetables, which contain high amounts of bioactive compounds (including antioxidants), significantly improve overall health and reduce risk of disease (Woodside et al., 2005). This suggestion is an important

consideration, especially given that our research specifically aims to find an accessible supplementation option for demographics often affected by low socioeconomic status (Pellowski et al., 2013). Income level is strongly associated with diet quality, and individuals with lower socioeconomic status tend to have reduced intake of fruits and vegetables due to factors such as limited physical access to grocery stores in food deserts (McCullough et al., 2022). While antioxidant supplementation may show promise in reducing ART-induced oxidative stress, lack of access to a complete, nutritious diet may continue to pose a barrier for individuals with HIV and limit the efficacy of antioxidant supplementation using a single source.

There are several directions for further research in this field. For example, in a 2023 study, *Moringa oleifera* extract was shown to reduce cellular ROS and upregulate expression of proteins involved in oxidative stress protection, including nuclear factor erythroid 2 p45–related factor 2 and superoxide catalase, as well as mitochondrial protective proteins such as Sirt3 in TDF-treated cells (Ndlovu et al., 2023). Another known antioxidant and dietary supplement, *Spirulina platensis*, relieves oxidative stress by inhibiting NADPH oxidase, a source of cellular ROS, and by radical scavenging through antioxidant compounds such as chlorophyll and phycocyanin (Sibiya et al., 2022). Comparative studies could be conducted on the effectiveness of various antioxidant herbs used in combination as treatments. In addition, while this study largely focused on the effects of thyme and TDF treatment on liver cells and, preliminarily, kidney cells, clinical studies of TDF have also been associated with high levels of bone toxicity. Further research could be done comparing the impact of thyme treatment on bone cells.

Future studies should also investigate whether the antioxidant capacity of *Thymus vulgaris* translates into direct reductions in intracellular ROS levels using fluorescent ROS-detection assays. Because ART-associated toxicity is closely linked to mitochondrial

dysfunction, additional measurements of mitochondrial membrane potential, oxygen consumption rate, and mtDNA copy number would further clarify the mechanism of protection. Expanding the analysis to include endogenous antioxidant enzymes, such as superoxide dismutase and catalase, would help determine whether thyme enhances intrinsic redox defenses. Furthermore, chromatographic characterization of thyme extracts could identify specific bioactive compounds responsible for the observed antioxidant effects.

Another extrapolation of this data concerns antioxidant supplementation for cancer patients undergoing chemotherapy to improve prognosis (Wieland et al., 2021). A 2016 study concluded that although antioxidant supplementation reduces chemotoxic adverse effects of chemotherapy, inconsistencies were found and warrant further exploration (Yasueda et al., 2016). Testing alternative treatment schedules, additional cell types affected by TDF toxicity, and eventual *in vivo* models will also be essential for evaluating the effectiveness and relevance of thyme supplementation. Finally, future work should assess whether antioxidant treatment alters the antiviral efficacy of TDF to ensure compatibility with ART regimens.

This research can serve as a starting point for future studies investigating thyme's ability to mitigate the toxicity of oxidative stress-inducing medications, including ART, chemotherapy, antipsychotics, and analgesics (Deavall et al., 2012). Bridging the gap between modern medicine and herbal treatment may make HIV treatment more comfortable and reduce the burden associated with maintaining strict treatment adherence. The possible use of antioxidant supplementation to treat the harmful side effects of ART highlights the importance of and connection between nutrition and health. Overall, these findings suggest that although *Thymus vulgaris* demonstrates antioxidant potential, its ability to mitigate TDF-induced cytotoxicity in this model remains inconclusive and likely depends on factors not captured in this study.

Chapter 6: Conclusion

This study evaluated the antioxidant capacity of *Thymus vulgaris* and its potential to mitigate oxidative stress associated with treatment with TDF, a widely used antiretroviral therapy. Results from ORAC, ABTS, and TPC assays confirmed that thyme extracts possess measurable antioxidant activity, supporting their suitability for further investigation as a plant-derived antioxidant source. While antioxidant activity was supported by phenolic content and assay results, variability in antioxidant measurements suggested this activity was not consistently quantified across all assays. *In vitro* experiments demonstrated concentration-dependent effects of thyme on hepatocyte viability. They suggested that co-treatment with thyme may modulate cellular responses to TDF-induced oxidative stress, as reflected in changes in cell viability and GSH levels. These effects suggest a potential role for thyme in modulating cellular redox balance, possibly through reactive oxygen species scavenging or maintenance of intracellular antioxidant systems such as glutathione. However, these mechanisms were not directly confirmed. Overall, our results suggest that further investigation is needed to conclude if thyme has a protective effect against TDF-induced toxicity under the conditions tested.

While these findings do not establish therapeutic efficacy, they provide preliminary evidence that *Thymus vulgaris* may help reduce the oxidative burden associated with ART exposure. This study is limited by the variability of antioxidant assay results, a limited number of experimental replicates, reliance on cell viability as an indirect measure of oxidative stress, and the use of a single hepatocyte line. Because thyme is inexpensive and widely accessible, it represents a promising area of exploration for future investigation as a supportive antioxidant intervention, particularly for populations disproportionately affected by HIV and barriers to

treatment access. This work contributes to ongoing research by identifying *Thymus vulgaris* as a potentially accessible antioxidant candidate to mitigate ART-associated toxicity. Further studies are needed to clarify mechanisms of action, including direct measurements of ROS and mitochondrial function, optimization of dosing conditions, and evaluation of potential effects in more complex biological models before clinical relevance can be concluded.

References

Aboelwafa, H. R., & Yousef, H. N. (2015). The ameliorative effect of thymol against hydrocortisone-induced hepatic oxidative stress injury in adult male rats. *Biochemistry and Cell Biology*, *93*(4), 282–289. https://doi.org/10.1139/bcb-2014-0154

Abraham, P., Ramamoorthy, H., & Isaac, B. (2013). Depletion of the cellular antioxidant system contributes to tenofovir disoproxil fumarate—Induced mitochondrial damage and increased oxido-nitrosative stress in the kidney. *Journal of Biomedical Science*, *20*(1), 61. https://doi.org/10.1186/1423-0127-20-61

Allard, J. P., Aghdassi, E., Chau, J., Tam, C., Kovacs, C. M., Salit, I. E., & Walmsley, S. L. (1998). Effects of vitamin E and C supplementation on oxidative stress and viral load in HIV-infected subjects. *AIDS (London, England)*, *12*(13), 1653–1659. https://doi.org/10.1097/00002030-199813000-00013

Ammerman, N. C., Beier‐Sexton, M., & Azad, A. F. (2008). Growth and Maintenance of Vero Cell Lines. *Current Protocols in Microbiology*, *11*(1). https://doi.org/10.1002/9780471729259.mca04es11

Apostolova, N., Blas-García, A., & Esplugues, J. V. (2011). Mitochondrial interference by anti-HIV drugs: Mechanisms beyond Pol-γ inhibition. *Trends in Pharmacological Sciences*, *32*(12), 715–725. https://doi.org/10.1016/j.tips.2011.07.007

Arts, E. J., & Hazuda, D. J. (2012). HIV-1 Antiretroviral Drug Therapy. *Cold Spring Harbor Perspectives in Medicine*, *2*(4), a007161. https://doi.org/10.1101/cshperspect.a007161

Assiri, A. M. A., Elbanna, K., Abulreesh, H. H., & Ramadan, M. F. (2016). Bioactive Compounds of Cold-pressed Thyme (Thymus vulgaris) Oil with Antioxidant and

Antimicrobial Properties. *Journal of Oleo Science*, *65*(8), 629–640. https://doi.org/10.5650/jos.ess16042

Ayesh, B. M., Abed, A. A., & Faris, D. M. (2014). In vitro inhibition of human leukemia THP-1 cells by Origanum syriacum L. and Thymus vulgaris L. extracts. *BMC Research Notes*, *7*(1), 612. https://doi.org/10.1186/1756-0500-7-612

Battistini Garcia, S., Zubair, M., & Guzman, N. (2025). *CD4 Cell Count and HIV*. https://www.ncbi.nlm.nih.gov/books/NBK513289/

Bepe, N., Madanhi, N., Mudzviti, T., Gavi, S., Maponga, C. C., & Morse, G. D. (2011). The impact of herbal remedies on adverse effects and quality of life in HIV-infected individuals on antiretroviral therapy. *Journal of Infection in Developing Countries*, *5*(1), 48–53.

Bienstock, R. J., & Copeland, W. C. (2004). Molecular insights into NRTI inhibition and mitochondrial toxicity revealed from a structural model of the human mitochondrial DNA polymerase. *Mitochondrion, Mitochondria, HIV and Antiretroviral Therapy*, *4*(2), 203–213. https://doi.org/10.1016/j.mito.2004.05.018

Bingham, A., Shrestha, R. K., Khurana, N., Jacobson, E. U., & Farnham, P. G. (2021). Estimated Lifetime HIV–Related Medical Costs in the United States. *Sexually Transmitted Diseases*, *48*(4), 299. https://doi.org/10.1097/OLQ.0000000000001366

Bouayed, J., & Bohn, T. (2010). Exogenous antioxidants—Double-edged swords in cellular redox state. *Oxidative Medicine and Cellular Longevity*, *3*(4), 228–237. https://doi.org/10.4161/oxim.3.4.12858

Brewer, M. S. (2011). Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications. *Comprehensive Reviews in Food Science and Food Safety*, *10*(4), 221–247. https://doi.org/10.1111/j.1541-4337.2011.00156.x

Burton, G. J., & Jauniaux, E. (2011). Oxidative stress. *Best Practice & Research Clinical Obstetrics & Gynaecology, Placental Bed & Maternal - Fetal Disorders*, *25*(3), 287–299. https://doi.org/10.1016/j.bpobgyn.2010.10.016

CDC. (2022, August 18). *HIV and Women: HIV Diagnoses*. Centers for Disease Control and Prevention. https://www.cdc.gov/hiv/group/gender/women/diagnoses.html

CDC. (2024a, May 21). *Fast Facts: HIV in the US by Race and Ethnicity*. CDC. https://www.cdc.gov/hiv/data-research/facts-stats/race-ethnicity.html

CDC. (2024b, October 21). *Fast Facts: HIV and Hispanic/Latino People*. CDC. https://www.cdc.gov/hiv/data-research/facts-stats/hispanic-latino-people.html#:~:text=or%20not%20identified.-,Source:%20CDC

Chen, W.-T., Shiu, C.-S., Yang, J. P., Simoni, J. M., Fredriksen-Goldsen, karen I., Lee, T. S.-H., & Zhao, H. (2013). Antiretroviral Therapy (ART) Side Effect Impacted on Quality of Life, and Depressive Symptomatology: A Mixed-Method Study. *Journal of AIDS & Clinical Research*, *4*, 218. https://doi.org/10.4172/2155-6113.1000218

Cihlar, T., Birkus, G., Greenwalt, D. E., & Hitchcock, M. J. M. (2002). Tenofovir exhibits low cytotoxicity in various human cell types: Comparison with other nucleoside reverse transcriptase inhibitors. *Antiviral Research*, *54*(1), 37–45. https://doi.org/10.1016/S0166-3542(01)00210-8

Contract Pharma. (n.d.). *Gilead Sciences: Top 20 Pharma & BioPharma in 2022*. Retrieved December 12, 2023, from https://www.contractpharma.com/heaps/view/10305/4/435469/

De Almeida, A. J. P. O., De Oliveira, J. C. P. L., Da Silva Pontes, L. V., De Souza Júnior, J. F., Gonçalves, T. A. F., Dantas, S. H., De Almeida Feitosa, M. S., Silva, A. O., & De Medeiros, I. A. (2022). ROS: Basic Concepts, Sources, Cellular Signaling, and its Implications in Aging Pathways. *Oxidative Medicine and Cellular Longevity*, *2022*(1), 1225578. https://doi.org/10.1155/2022/1225578

De Cock, K. M., Jaffe, H. W., & Curran, J. W. (2011). Reflections on 30 Years of AIDS. *Emerging Infectious Diseases*, *17*(6), 1044–1048. https://doi.org/10.3201/eid1706.100184

Deavall, D. G., Martin, E. A., Horner, J. M., & Roberts, R. (2012). Drug-Induced Oxidative Stress and Toxicity. *Journal of Toxicology*, *2012*, 645460. https://doi.org/10.1155/2012/645460

Deeks, S. G., Overbaugh, J., Phillips, A., & Buchbinder, S. (2015). HIV infection. *Nature Reviews. Disease Primers*, *1*, 15035. https://doi.org/10.1038/nrdp.2015.35

Eggleton, J. S., & Nagalli, S. (2023). Highly Active Antiretroviral Therapy (HAART). In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK554533/

Feriotto, G., Marchetti, N., Costa, V., Beninati, S., Tagliati, F., & Mischiati, C. (2018). Chemical Composition of Essential Oils from *Thymus vulgaris* , *Cymbopogon citratus* , and *Rosmarinus officinalis* , and Their Effects on the HIV‐1 Tat Protein Function. *Chemistry & Biodiversity*, *15*(2), e1700436. https://doi.org/10.1002/cbdv.201700436

Fioroti, C. E. A., Distenhreft, J. I. Q., Paulino, B. B., Lacchine, K., Ramos, D. R., Seguro, A. C., & Luchi, W. M. (2022). Tenofovir-induced renal and bone toxicity: Report of two cases and literature review. *Revista Do Instituto de Medicina Tropical de São Paulo*, *64*, e10. https://doi.org/10.1590/S1678-9946202264010

Firat, Y. Y., Cicek, B., Kara, A., Ozturk, N. K., & Ilgun, S. (2025). Effects of Thyme, Cumin, and Sumac Extracts on Apoptosis and Paraptosis in Hepatocellular Carcinoma: Synergistic, Antagonistic, or Additive Properties. *Food Science & Nutrition*, *13*(4), e70106. https://doi.org/10.1002/fsn3.70106

Fleming, A. M., Muller, J. G., Dlouhy, A. C., & Burrows, C. J. (2012). Structural Context Effects in the Oxidation of 8-Oxo-7,8-dihydro-2′-deoxyguanosine to Hydantoin Products: Electrostatics, Base Stacking, and Base Pairing. *J. Am. Chem. Soc.* https://doi-org.proxy-um.researchport.umd.edu/10.1021/ja306077b

Gambo, A., Moodley, I., Babashani, M., Babalola, T. K., & Gqaleni, N. (2021). A double-blind, randomized controlled trial to examine the effect of Moringa oleifera leaf powder supplementation on the immune status and anthropometric parameters of adult HIV patients on antiretroviral therapy in a resource-limited setting. *PloS One*, *16*(12), e0261935. https://doi.org/10.1371/journal.pone.0261935

Ganguly, P. (2025). Retrovirus. *National Human Genome Research Institute*. https://www.genome.gov/genetics-glossary/Retrovirus

Gonsalves, G., & Staley, P. (2014). Panic, Paranoia, and Public Health—The AIDS Epidemic's Lessons for Ebola. *New England Journal of Medicine*, *371*(25), 2348–2349. https://doi.org/10.1056/NEJMp1413425

Guicciardi, M. E., Malhi, H., Mott, J. L., & Gores, G. J. (2013). Apoptosis and Necrosis in the Liver. *Compr Physiol.* https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3867948/#:~:text=Macrophages%2C%20upon%20activation%2C%20in%20turn,in%20liver%20inflammation%20and%20injury

Gwads, M., Cleland, C. M., Freeman, R., Wilton, L., Collins, L. M., Hawkins, R. L., Ritchie, A. S., Leonard, N. R., Jonas, D. F., Korman, A., Cluesman, S., He, N., & Sherpa, D. (2021). Stopping, starting, and sustaining HIV antiretroviral therapy: A mixed-methods exploration among African American/Black and Latino long-term survivors of HIV in an urban context. *BMC Public Health*, *21*, 419. https://doi.org/10.1186/s12889-021-10464-x

Hammoudi Halat, D., Krayem, M., Khaled, S., & Younes, S. (2022). A Focused Insight into Thyme: Biological, Chemical, and Therapeutic Properties of an Indigenous Mediterranean Herb. *Nutrients*, *14*(10), Article 10. https://doi.org/10.3390/nu14102104

Hashwin Singh, T. S., Jashwin Singh, T. S., & Chin, K.-Y. (2024). Effects of Tenofovir Disoproxil Fumarate on Bone Quality beyond Bone Density—A Scoping Review of the Literature. *Pharmaceuticals*, *17*(2), Article 2. https://doi.org/10.3390/ph17020146

Hebert, S. P., & Schlegel, H. B. (2019). Computational Study of the Oxidation of Guanine To Form 5-Carboxyamido-5-formamido-2-iminohydantoin (2Ih). *Chemical Research in Toxicology*, *32*(11), 2295–2304. https://doi.org/10.1021/acs.chemrestox.9b00304

Horne, R., Cooper, V., Gellaitry, G., Date, H. L., & Fisher, M. (2007). Patients' Perceptions of Highly Active Antiretroviral Therapy in Relation to Treatment Uptake and Adherence: The Utility of the Necessity-Concerns Framework. *JAIDS Journal of Acquired Immune Deficiency Syndromes*, *45*(3), 334. https://doi.org/10.1097/QAI.0b013e31806910e3

Jin, X., Zhao, L.-M., Wang, S., Huang, W.-J., Zhang, Y.-X., Pannecouque, C., De Clercq, E., & Chen, F.-E. (2022). Structure-Based Discovery of Novel NH_2 -Biphenyl-Diarylpyrimidines as Potent Non-Nucleoside Reverse Transcriptase Inhibitors with Significantly Improved Safety: From NH_2 -Naphthyl-Diarylpyrimidine to NH_2

-Biphenyl-Diarylpyrimidine. *Journal of Medicinal Chemistry*, *65*(12), 8478–8492. https://doi.org/10.1021/acs.jmedchem.2c00468

Kamitani, E., Mizuno, Y., & Koenig, L. J. (2024). Strategies to Eliminate Inequity in PrEP Services in the US South and Rural Communities. *Journal of the Association of Nurses in AIDS Care*, *35*(2), 153–160. https://doi.org/10.1097/JNC.0000000000000437

Kanzler, S., & Galle, P. R. (2000). Apoptosis and the liver. *Seminars in Cancer Biology*, *10*(3), 173–184. https://doi.org/10.1006/scbi.2000.0318

Katerji, M., Filippova, M., & Duerksen-Hughes, P. (2019). Approaches and Methods to Measure Oxidative Stress in Clinical Samples: Research Applications in the Cancer Field. *Oxidative Medicine and Cellular Longevity*, *2019*, 1–29. https://doi.org/10.1155/2019/1279250

Kaur, R., Sharma, P., Gupta, G. K., Ntie-Kang, F., & Kumar, D. (2020). Structure-Activity-Relationship and Mechanistic Insights for Anti-HIV Natural Products. *Molecules*, *25*(9), 2070. https://doi.org/10.3390/molecules25092070

Kehm, R., Baldensperger, T., Raupbach, J., & Höhn, A. (2021). Protein oxidation—Formation mechanisms, detection and relevance as biomarkers in human diseases. *Redox Biology*, *42*, 101901. https://doi.org/10.1016/j.redox.2021.101901

Kemnic, T. R., & Gulick, P. G. (2023). HIV Antiretroviral Therapy. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK513308/

Khnissi, S., Ben Salem, I., Bejaoui, B., Fattouch, S., Mustapha, S. B., Haj‐Kacem, R., M'Hamdi, N., Martin, P., Dattena, M., & Lassoued, N. (2025). Antioxidant Capacity of Thyme (*Thymus vulgaris*) Essential Oil and Its Effect on In Vivo Fertility of Rams Subjected to

Testicle Heat Stress. *Journal of Animal Physiology and Animal Nutrition*, *109*(2), 437–448. https://doi.org/10.1111/jpn.14063

Khoddami, A., Wilkes, M. A., & Roberts, T. H. (2013). Techniques for Analysis of Plant Phenolic Compounds. *Molecules*, *18*(2), 2328–2375. https://doi.org/10.3390/molecules18022328

Khurana, R. K., Jain, A., Jain, A., Sharma, T., Singh, B., & Kesharwani, P. (2018). Administration of antioxidants in cancer: Debate of the decade. *Drug Discovery Today*, *23*(4), 763–770. https://doi.org/10.1016/j.drudis.2018.01.021

Kim, Y.-S., Hwang, J.-W., Kang, S.-H., Kim, E.-H., Jeon, Y.-J., Jeong, J.-H., Kim, H.-R., Moon, S.-H., Jeon, B.-T., & Park, P.-J. (2014). Thymol from Thymus quinquecostatus Celak. Protects against tert-butyl hydroperoxide-induced oxidative stress in Chang cells. *Journal of Natural Medicines*, *68*(1), 154–162. https://doi.org/10.1007/s11418-013-0786-8

Kudin, A. P., Bimpong-Buta, N. Y.-B., Vielhaber, S., Elger, C. E., & Kunz, W. S. (2004). Characterization of Superoxide-producing Sites in Isolated Brain Mitochondria. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M310341200

Kumar, S., Chashoo, G., Saxena, A. K., & Pandey, A. K. (2013). *Parthenium hysterophorus:* A Probable Source of Anticancer, Antioxidant and Anti-HIV Agents. *BioMed Research International*, *2013*, e810734. https://doi.org/10.1155/2013/810734

Landovitz, R. J., Desmond, K. A., & Leibowitz, A. A. (2017). Antiretroviral therapy: Racial disparities among publicly insured Californians with HIV. *Journal of Health Care for the Poor and Underserved*, *28*(1), 406–429. https://doi.org/10.1353/hpu.2017.0031

Losada-Barreiro, S., Sezgin-Bayindir, Z., Paiva-Martins, F., & Bravo-Díaz, C. (2022). Biochemistry of Antioxidants: Mechanisms and Pharmaceutical Applications. *Biomedicines*, *10*(12), 3051. https://doi.org/10.3390/biomedicines10123051

Lott, B. E., Loveluck, J., Benton, A., Golson, L., Kahle, E., Lam, J., Bauermeister, J. A., & Veinot, T. C. (2022). The impact of stigma on HIV testing decisions for gay, bisexual, queer and other men who have sex with men: A qualitative study. *BMC Public Health*, *22*(1), 471. https://doi.org/10.1186/s12889-022-12761-5

Łupina, K., Nowak, K., Lorek, D., Nowak, A., Romac, A., Głowacka, E., & Janczura, J. (2025). Pharmacological advances in HIV treatment: From ART to long-acting injectable therapies. *Archives of Virology*, *170*(9), 195. https://doi.org/10.1007/s00705-025-06381-8

Margolis, A. M., Heverling, H., Pham, P. A., & Stolbach, A. (2014). A Review of the Toxicity of HIV Medications. *Journal of Medical Toxicology*, *10*(1), 26–39. https://doi.org/10.1007/s13181-013-0325-8

Marianna Usai, Mauro Marchetti, Marzia Foddai, Alessandra Del Caro, Roberta Desogus, Iser Sanna, Antonio Piga, Influence of different stabilizing operations and storage time on the composition of essential oil of thyme (Thymus officinalis L.) and rosemary (Rosmarinus officinalis L.), *LWT - Food Science and Technology*, Volume 44, Issue 1, 2011, Pages 244-249, ISSN 0023-6438, https://doi.org/10.1016/j.lwt.2010.05.024.

McCullough, M. L., Chantaprasopsuk, S., Islami, F., Rees-Punia, E., Um, C. Y., Wang, Y., Leach, C. R., Sullivan, K. R., & Patel, A. V. (2022). Association of Socioeconomic and Geographic Factors With Diet Quality in US Adults. *JAMA Network Open*, *5*(6), e2216406. https://doi.org/10.1001/jamanetworkopen.2022.16406

Miller, N. J., Sampson, J., Candeias, L. P., Bramley, P. M., & Rice-Evans, C. A. (1996). Antioxidant activities of carotenes and xanthophylls. *FEBS Letters*, *384*(3), 240–242. https://doi.org/10.1016/0014-5793(96)00323-7

Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., & Malik, A. B. (2014). Reactive oxygen species in inflammation and tissue injury. *Antioxidants & Redox Signaling*, *20*(7), 1126–1167. https://doi.org/10.1089/ars.2012.5149

Mokhtari, R., Kazemi Fard, M., Rezaei, M., Moftakharzadeh, S. A., & Mohseni, A. (2023). Antioxidant, Antimicrobial Activities, and Characterization of Phenolic Compounds of Thyme (Thymus vulgaris L.), Sage (Salvia officinalis L.), and Thyme–Sage Mixture Extracts. *Journal of Food Quality*, *2023*(1), 2602454. https://doi.org/10.1155/2023/2602454

Moore, J., Hao, Z., Zhou, K., Luther, M., Costa, J., & Yu, L. (Lucy). (2005). Carotenoid, Tocopherol, Phenolic Acid, and Antioxidant Properties of Maryland-Grown Soft Wheat. *Journal of Agricultural and Food Chemistry*, *53*(17), 6649–6657. https://doi.org/10.1021/jf050481b

Muñoz-Muela, E., Mejías-Trueba, M., Serna-Gallego, A., Saborido-Alconchel, A., Fernández-Pérez, S., Herrero, M., Sotomayor, C., Gutiérrez-Valencia, A., Trujillo-Rodríguez, M., & López-Cortés, L. F. (2025). Mitochondrial Disorders After 12 Months of Human Immunodeficiency Virus Type 1 Preexposure Prophylaxis Based on Tenofovir Disoproxil Fumarate Plus Emtricitabine in Healthy Adults. *The Journal of Infectious Diseases*, *231*(6), 1430–1439. https://doi.org/10.1093/infdis/jiaf156

Munteanu, I. G., & Apetrei, C. (2021). Analytical Methods Used in Determining Antioxidant Activity: A Review. *International Journal of Molecular Sciences*, *22*(7), 3380. https://doi.org/10.3390/ijms22073380

Nagoor Meeran, M. F., Jagadeesh, G. S., & Selvaraj, P. (2015). Thymol attenuates altered lipid metabolism in β-adrenergic agonist induced myocardial infarcted rats by inhibiting tachycardia, altered electrocardiogram, apoptosis and cardiac hypertrophy. *Journal of Functional Foods*, *14*, 51–62. https://doi.org/10.1016/j.jff.2015.01.013

Nagoor Meeran, M. F., Javed, H., Al Taee, H., Azimullah, S., & Ojha, S. K. (2017). Pharmacological Properties and Molecular Mechanisms of Thymol: Prospects for Its Therapeutic Potential and Pharmaceutical Development. *Frontiers in Pharmacology*, *8*, 380. https://doi.org/10.3389/fphar.2017.00380

Nagoor Meeran, M. F., & Stanely Mainzen Prince, P. (2012). Protective effects of thymol on altered plasma lipid peroxidation and nonenzymic antioxidants in isoproterenol‐induced myocardial infarcted rats. *Journal of Biochemical and Molecular Toxicology*, *26*(9), 368–373. https://doi.org/10.1002/jbt.21431

Ndlovu, S. S., Chuturgoon, A. A., & Ghazi, T. (2023). Moringa oleifera Lam Leaf Extract Stimulates NRF2 and Attenuates ARV-Induced Toxicity in Human Liver Cells (HepG2). *Plants*, *12*(7), 1541. https://doi.org/10.3390/plants12071541

Nieddu, M., Rassu, G., Boatto, G., Bosi, P., Trevisi, P., Giunchedi, P., Carta, A., & Gavini, E. (2014). Improvement of thymol properties by complexation with cyclodextrins: In vitro and in vivo studies. *Carbohydrate Polymers*, *102*, 393–399. https://doi.org/10.1016/j.carbpol.2013.10.084

NIH. (2020). *LiverTox: Clinical and Research Information on Drug-Induced Liver Injury*. https://www.ncbi.nlm.nih.gov/books/NBK548917/

NIH. (2021, September 17). *Cost Considerations and Antiretroviral Therapy | NIH*. https://clinicalinfo.hiv.gov/en/guidelines/hiv-clinical-guidelines-adult-and-adolescent-arv/antiretroviral-therapy-cost-considerations

NIH. (2024a, September 4). *HIV and Women*. NIH. https://hivinfo.nih.gov/understanding-hiv/fact-sheets/hiv-and-women

NIH. (2024b, October 24). *HIV Medicines and Side Effects*. NIH. https://hivinfo.nih.gov/understanding-hiv/fact-sheets/hiv-medicines-and-side-effects

NIH. (2025a). HIV and AIDS: The Basics. *NIH*. https://hivinfo.nih.gov/understanding-hiv/fact-sheets/hiv-and-aids-basics#:~:text=HIV%20attacks%20and%20destroys%20the,and%20the%20onset%20of%20AIDS

NIH. (2025b, March 31). *HIV and Kidney Disease | NIH*. https://hivinfo.nih.gov/understanding-hiv/fact-sheets/hiv-and-kidney-disease

NIH. (2026, March 20). *HIV and Gay and Bisexual Men*. NIH. https://hivinfo.nih.gov/understanding-hiv/fact-sheets/hiv-and-gay-and-bisexual-men

Nworu, S, C., Okoye, L, E., Ezeifeka, O, G., Esimone, & O, C. (2013). Extracts of Moringa oleifera Lam. Showing inhibitory activity against early steps in the infectivity of HIV-1 lentiviral particles in a viral vector-based screening. *African Journal of Biotechnology*, *12*(30), 4866–4873. https://doi.org/10.5897/AJB2013.12343

Owen, J. B., & Butterfield, D. A. (2010). Measurement of Oxidized/Reduced Glutathione Ratio. In P. Bross & N. Gregersen (Eds.), *Protein Misfolding and Cellular Stress in Disease and*

Aging (Vol. 648, pp. 269–277). Humana Press. https://doi.org/10.1007/978-1-60761-756-3_18

Parham, S., Kharazi, A. Z., Bakhsheshi-Rad, H. R., Nur, H., Ismail, A. F., Sharif, S., RamaKrishna, S., & Berto, F. (2020). Antioxidant, Antimicrobial and Antiviral Properties of Herbal Materials. *Antioxidants (Basel, Switzerland)*, *9*(12), E1309. https://doi.org/10.3390/antiox9121309

Patel, P. H., & Zulfiqar, H. (2023). Reverse Transcriptase Inhibitors. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK551504/

Pedro, M. N., Rocha, G. Z., Guadagnini, D., Santos, A., Magro, D. O., Assalin, H. B., Oliveira, A. G., Pedro, R. D. J., & Saad, M. J. A. (2018). Insulin Resistance in HIV-Patients: Causes and Consequences. *Frontiers in Endocrinology*, *9*, 514. https://doi.org/10.3389/fendo.2018.00514

Pellowski, J. A., Kalichman, S. C., Matthews, K. A., & Adler, N. (2013). A pandemic of the poor: Social disadvantage and the U.S. HIV epidemic. *The American Psychologist*, *68*(4), 197–209. https://doi.org/10.1037/a0032694

Pham-Huy, L. A., He, H., & Pham-Huy, C. (2008). Free Radicals, Antioxidants in Disease and Health. *International Journal of Biomedical Science : IJBS*, *4*(2), 89–96.

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D., & Bitto, A. (2017). Oxidative Stress: Harms and Benefits for Human Health. *Oxidative Medicine and Cellular Longevity*, *2017*, 8416763. https://doi.org/10.1155/2017/8416763

Proskurnina, E. V., Izmailov, D. Yu., Sozarukova, M. M., Zhuravleva, T. A., Leneva, I. A., & Poromov, A. A. (2020). Antioxidant Potential of Antiviral Drug Umifenovir. *Molecules*, *25*(7), 1577. https://doi.org/10.3390/molecules25071577

Richman, D. D., Margolis, D. M., Delaney, M., Greene, W. C., Hazuda, D., & Pomerantz, R. J. (2009). The Challenge of Finding a Cure for HIV Infection. *Science*, *323*(5919), 1304–1307. https://doi.org/10.1126/science.1165706

Roby, M. H. H., Sarhan, M. A., Selim, K. A.-H., & Khalel, K. I. (2013). Evaluation of antioxidant activity, total phenols and phenolic compounds in thyme (*Thymus vulgaris* L.), sage (*Salvia officinalis* L.), and marjoram (*Origanum majorana* L.) extracts. *Industrial Crops and Products*, *43*, 827–831. https://doi.org/10.1016/j.indcrop.2012.08.029

Sakai, A., Nakanishi, M., Yoshiyama, K., & Maki, H. (2006). *Impact of reactive oxygen species on spontaneous mutagenesis in Escherichia coli*. https://doi.org/10.1111/j.1365-2443.2006.00982.x

Saki, M., De Villiers, H., Ntsapi, C., & Tiloke, C. (2023). The Hepatoprotective Effects of Moringa oleifera against Antiretroviral-Induced Cytotoxicity in HepG2 Cells: A Review. *Plants*, *12*(18), Article 18. https://doi.org/10.3390/plants12183235

Shadel, G. S., & Horvath, T. L. (2015). Mitochondrial ROS signaling in organismal homeostasis. *Cell*, *163*(3), 560–569. https://doi.org/10.1016/j.cell.2015.10.001

Sharma, B. (2014). Oxidative stress in HIV patients receiving antiretroviral therapy. *Current HIV Research*, *12*(1), 13–21. https://doi.org/10.2174/1570162x12666140402100959

Sibiya, T., Ghazi, T., & Chuturgoon, A. (2022). The Potential of Spirulina platensis to Ameliorate the Adverse Effects of Highly Active Antiretroviral Therapy (HAART). *Nutrients*, *14*(15), 3076. https://doi.org/10.3390/nu14153076

Sieniawska, E., Baj, T., Sawicki, R., Wanat, A., Wojtanowski, K. K., Ginalska, G., Zgorka, G., & Szymanska, J. (2017). LC‐QTOF‐MS Analysis and Activity Profiles of Popular Antioxidant Dietary Supplements in Terms of Quality Control. *Oxidative Medicine and Cellular Longevity*, *2017*(1), 8692516. https://doi.org/10.1155/2017/8692516

Sierra, S., Kupfer, B., & Kaiser, R. (2005). Basics of the virology of HIV-1 and its replication. *Journal of Clinical Virology, Focus on HIV*, *34*(4), 233–244. https://doi.org/10.1016/j.jcv.2005.09.004

Simoni, J. M., Huh, D., Wilson, I. B., Shen, J., Goggin, K., Reynolds, N. R., Remien, R. H., Rosen, M. I., Bangsberg, D. R., & Liu, H. (2012). Racial/Ethnic Disparities in ART Adherence in the United States: Findings From the MACH14 Study. *Journal of Acquired Immune Deficiency Syndromes (1999)*, *60*(5), 466–472. https://doi.org/10.1097/QAI.0b013e31825db0bd

Sluis-Cremer, N., & Tachedjian, G. (2008). Mechanisms of inhibition of HIV replication by nonnucleoside reverse transcriptase inhibitors. *Virus Research*, *134*(1–2), 147–156. https://doi.org/10.1016/j.virusres.2008.01.002

Stankovic, J. S. K., Selakovic, D., Mihailovic, V., & Rosic, G. (2020). Antioxidant Supplementation in the Treatment of Neurotoxicity Induced by Platinum-Based Chemotherapeutics—A Review. *International Journal of Molecular Sciences*, *21*(20), 7753. https://doi.org/10.3390/ijms21207753

Stringfellow, H. M., Jones, M. R., Green, M. C., Wilson, A. K., & Francisco, J. S. (2014). Selectivity in ROS-induced peptide backbone bond cleavage. *The Journal of Physical Chemistry. A*, *118*(48), 11399–11404. https://doi.org/10.1021/jp508877m

Su, L.-J., Zhang, J.-H., Gomez, H., Murugan, R., Hong, X., Xu, D., Jiang, F., & Peng, Z.-Y. (2019). Reactive Oxygen Species-Induced Lipid Peroxidation in Apoptosis, Autophagy, and Ferroptosis. *Oxidative Medicine and Cellular Longevity*, *2019*, e5080843. https://doi.org/10.1155/2019/5080843

Taanman, J. W. (1999). The mitochondrial genome: Structure, transcription, translation and replication. *Biochimica Et Biophysica Acta*, *1410*(2), 103–123. https://doi.org/10.1016/s0005-2728(98)00161-3

Volberding, P. A., & Deeks, S. G. (2010). Antiretroviral therapy and management of HIV infection. *The Lancet*, *376*(9734), 49–62. https://doi.org/10.1016/S0140-6736(10)60676-9

Wei, S., Evans, P. C., Strijdom, H., & Xu, S. (2025). HIV infection, antiretroviral therapy and vascular dysfunction: Effects, mechanisms and treatments. *Pharmacological Research*, *217*, 107812. https://doi.org/10.1016/j.phrs.2025.107812

White, A. J. (2001). Mitochondrial toxicity and HIV therapy. *Sexually Transmitted Infections*, *77*(3), 158–173. https://doi.org/10.1136/sti.77.3.158

WHO. (2025). *HIV*. World Health Organization. https://www.who.int/data/gho/data/themes/hiv-aids

Wieland, L. S., Moffet, I., Shade, S., Emadi, A., Knott, C., Gorman, E. F., & D"Adamo, C. (2021). Risks and benefits of antioxidant dietary supplement use during cancer treatment:

Protocol for a scoping review. *BMJ Open*, *11*(4), e047200. https://doi.org/10.1136/bmjopen-2020-047200

Wilkinson, A. L., Huey, S. L., & Mehta, S. (2018). Antioxidants and HIV/AIDS: Zinc, Selenium, and Vitamins C and E. In S. Mehta & J. L. Finkelstein (Eds.), *Nutrition and HIV: Epidemiological Evidence to Public Health*. CRC Press. http://www.ncbi.nlm.nih.gov/books/NBK572228/

Wood, E., Kerr, T., Hogg, R. D., Palepu, A., Zhang, R., Strathdee, S. A., & Montaner, J. S. G. (2006). Impact of HIV testing on uptake of HIV therapy among antiretroviral naive HIV-infected injection drug users. *Drug and Alcohol Review*, *25*, 451–454. https://doi.org/10.1080/09595230600883313

Woodside, J. V., McCall, D., McGartland, C., & Young, I. S. (2005). Micronutrients: Dietary intake v. supplement use. *Proceedings of the Nutrition Society*, *64*(4), 543–553. https://doi.org/10.1079/PNS2005464

Yao, Y., Whent, M., Li, Y., Liu, Z., Pehrsson, P., Sun, J., Chen, P., Huang, D., Wang, T. T. Y., Wu, X., & Yu, L. (2023). Chemical Composition of Thyme (*Thymus vulgaris*) Extracts, Potential Inhibition of SARS-CoV-2 Spike Protein–ACE2 Binding and ACE2 Activity, and Radical Scavenging Capacity. *Journal of Agricultural and Food Chemistry*, *71*(49), 19523–19530. https://doi.org/10.1021/acs.jafc.3c05432

Yasueda, A., Urushima, H., & Ito, T. (2016). Efficacy and Interaction of Antioxidant Supplements as Adjuvant Therapy in Cancer Treatment: A Systematic Review. *Integrative Cancer Therapies*, *15*(1), 17–39. https://doi.org/10.1177/1534735415610427

Zorov, D. B., Juhaszova, M., & Sollott, S. J. (2014). Mitochondrial Reactive Oxygen Species (ROS) and ROS-Induced ROS Release. *Physiological Reviews*, *94*(3), 909–950. https://doi.org/10.1152/physrev.00026.2013