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A BIOCHEMICAL ASSESSMENT OF THE POTENTIAL OF SPIRULINA PLATENSIS TO AMELIORATE THE ADVERSE EFFECTS OF HIGHLY ACTIVE ANTIRETROVIRAL THERAPY *IN VITRO*

By

Thabani Sibiya

B. Med. Sc. (Hons) (Medical Biochemistry), Master of Medical Science (Medical Biochemistry)

Submitted in fulfilment for the degree of Doctor of Philosophy (Medical Biochemistry), School of Laboratory Medicine and Medical Science, College of Health Sciences, University of KwaZulu-Natal

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DECLARATION

I, Thabani Sibiya, declare that:

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DEDICATION

To my late Grandmother (Florence F. Shezi), I love you and hope you are proud of me. I learned how to be a respectful and hard-working person from you, I am forever grateful for the role you played in my life.

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PUBLICATIONS

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2) Thabani Sibiya, Terisha Ghazi, Jivanka Mohan, Savania Nagiah and Anil A. Chuturgoon (2022). Spirulina platensis Ameliorates Oxidative Stress Associated with Antiretroviral Drugs in HepG2 Cells. ***Plants*** 2022, 11, 3143. doi.org/10.3390/plants11223143.

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3) Thabani Sibiya, Terisha Ghazi, Jivanka Mohan, Savania Nagiah and Anil A. Chuturgoon (2022). Spirulina Platensis mitigates the inhibition of selected miRNAs that promote inflammation in HAART treated HepG2 cells. ***Plants*** (*In Review*). Manuscript ID: plants-2084169

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ABBREVIATIONS

$\Delta\psi_m$	Mitochondrial membrane depolarisation
3TC	Lamivudine/ (-)-L-2',3'-dideoxy-3'-thiacytidine
AIDS	Acquired immuno-deficiency syndrome
ANOVA	One-way analysis of variance
Akt	Protein kinase B
ARE	Antioxidant response element
ARV	Antiretroviral
BSA	Bovine serum albumin
CAT	Catalase
cDNA	Complementary DNA
DCF	2',7'-dichlorofluorescein
CCM	Complete culture medium
Cox-1	Cyclooxygenase-1
Cox-2	Cyclooxygenase-2
dH_2O	Distilled water
DNA	Deoxyribose nucleic acid
EMEM	Eagle's Essential Minimal Media
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ETC	Electron transport chain
ETV	Entecavir
FAO	Food and agriculture organization
FTC	Emtricitabine/ 2',3'-dideoxy-5fluoro-3'-thiacytidine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPx	Glutathione peroxidase
GSH	Reduced glutathione
H2DCF-D	2',7'-dichlorodihydrofluorescein
H2DCF-DA	Dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
HAART	Highly active antiretroviral therapy

HepG2	Human hepatoma/ Human liver cells
HIV	Human Immuno-deficiency Virus
HO-1	Heme-oxygenase 1
HRP	Horse-radish peroxidase
IC_{50}	The half-maximal inhibitory concentration
IDV	Indinavir
iNOS	Inducible NO synthase
IκB-α	NF-κB inhibitor-alpha
IKKα	IkappaB Kinase alpha
IKKβ	IkappaB Kinase beta
IKK γ/NEMO	IkappaB Kinase gamma NEMO
IL-12	Interleukin-12
IL-1β	Interleukin-1 beta
JNK	C-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
LDH	Lactate dehydrogenase
LPV	Lopinavir
MetS	Metabolic syndrome
MDA	Malondialdehyde
mRNA	Messenger RNA
miR-146a	MicroRNA-146a
miR-155	MicroRNA-155
miRNA	MicroRNA
mt	Mitochondrial
mtDNA	Mitochondrial DNA
MTT	Methylthiazol tetrazolium
NADH	Nicotinamide adenine dinucleotide
NQO-1	NADH quinone oxidoreductase 1

NOX	NADPH oxidase
NF-κB	Nuclear factor-kappa-light chain-enhancer of activated B cell
NBD	Nucleotide binding domain
NFDM	Non-fat dry milk
NFV	Nelfinavir
NO	Nitric oxide
NMR	Nuclear magnetic resonance
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRF1	Nuclear respiratory factor 1
NRF-2	Nuclear erythroid 2 related factor 2
NRTI	Nucleoside/nucleotide reverse transcriptase inhibitor
O_2	Oxygen
O2•-	Superoxide anion
OH•	Hydroxyl radical
OH_2•	Hydroperoxyl radical
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEP	Potential exposure prophylaxis
PI	Protease inhibitor
pNRF-2	Phosphorylated NRF-2
pol	Polymerase
PrEP	Pre-exposure prophylaxis
Pri-miRNA	Primary transcript microRNA
qPCR	Quantitative polymerase chain reaction

RBD	Relative band density
RLU	Relative light units
RNA	Ribose nucleic acid
RT	Room temperature
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
SP	Spirulina platensis
TBARS	Thiobarbituric reactive substances
TCA	Tricarboxylic acid
TCEP	Tris-(2-carboxyethyl)-phosphine
TDF	Tenofovir disoproxil fumarate
TNF-α	Tumor necrosis factor alpha
TTBS	Tween 20-tris buffered saline
UNAIDS	Joint united nations programme on HIV/AIDS
WHO	World health organisation

ABSTRACT

The human immunodeficiency virus (HIV) has been one of the prevalent causes of diseases on a global scale over four decades of its emergence. It is estimated that about 37.7 million people are infected with HIV globally, and 8.2 million persons are in South Africa. The highly active antiretroviral therapy (HAART) involves combining various types of therapies that are dependent on the infected person's viral load. HAART helps to regulate the viral load and prevents its associated symptoms from progressing into acquired immune deficiency syndrome (AIDS). Despite its success in prolonging HIV-infected patients' lifespan, the long-term use of HAART promotes metabolic syndrome (MetS) through an inflammatory pathway, excess production of reactive oxygen species (ROS), and mitochondrial dysfunction. Interestingly, *Spirulina platensis* (SP), a blue-green microalga commonly used as a traditional food by Mexican and African people, has been demonstrated to mitigate MetS by regulating oxidative stress and inflammation. This study examined the protective role of SP against HAART-induced oxidative stress and inflammation in human hepatoma (HepG2) liver cells. The first published manuscript (appendix A) is a literature review on the potential of SP to ameliorate adverse effects of HAART: An update focusing on highlighting the potential positive synergistic effects of SP and HAART. This review provides introductory background of *spirulina* and its protective attributes. Thereafter, a study in an *in vitro* model was carried out by measuring oxidative stress, antioxidant, and inflammation markers.

The HepG2 cell line was used as an *in vitro* model. Changes were investigated in cellular redox status, inflammation, and antioxidant response. The data analysis followed prolonged [96 hours (hrs)] exposure to HAART and acute (24 hrs) exposure to SP. HAART (Lamivudine (3TC): 1.51 µg/ml, tenofovir disoproxil fumarate (TDF): 0.3 µg/ml and Emtricitabine (FTC): 1.8 µg/ml) in HepG2 cells was investigated for 96 hrs and thereafter, treated with 1.5 µg/ml SP for 24 hrs. The HepG2 cells that served as control contained complete culture medium (CCM) only. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to determine cell viability following SP treatment. Cellular redox status was assessed using the quantification of intracellular reactive oxygen species (ROS), lipid peroxidation, and lactate dehydrogenase (LDH) assay. The fluorometric, JC-1 assay was used to determine mitochondrial polarisation. Protein expression was determined using western blots. Quantitative Polymerase Chain Reaction (qPCR) was also employed for micro-RNA and gene expressions. The findings from these investigations led to further analyses as depicted and described in our second, third, and fourth manuscripts.

In the second published manuscript (chapter three), antioxidant markers and Nuclear erythroid 2 related factor 2 (NRF-2), a key regulator of antioxidants, was investigated. The results show that SP exposure induces an antioxidant response. The results further reveal that prolonged exposure with

HAART followed by SP treatment induced an antioxidant response through upregulating *NRF-2* ($p < 0.0001$), *CAT* ($p < 0.0001$), and *NQO-1* ($p < 0.0001$) mRNA expression. Furthermore, NRF-2 ($p = 0.0085$) and pNRF-2 ($p < 0.0001$) protein expression was upregulated in the HepG2 cells post-exposure to HAART-SP.

In the third manuscript (chapter four), microRNAs and genes involved in inflammatory response were analysed. SP prevented the inhibition of microRNAs involved in the regulation of inflammation. MiR-146a ($p < 0.0001$) and miR-155 ($p < 0.0001$) levels increased in SP treated cells. However, only miR-146a ($p < 0.0001$) in HAART-SP indicated an increase, while miR-155 ($p < 0.0001$) in HAART-SP treatment indicated a significant decrease expression. SP may mitigate the inhibition of selected miRNAs that regulate inflammation in HAART treated HepG2 cells. Further, analysis revealed that Cox-1 mRNA expression was significantly increased in HAART-SP treated cells ($p < 0.0001$). Moreover, HepG2 cells exposed to HAART-SP treatment showed a significant decreased Cox-2 ($p < 0.0001$) expression, therefore, SP potentially controls inflammation by regulating microRNA and gene expressions. Moreover, the positive synergistic effect is indicated by normalised intracellular ROS levels ($p < 0.0001$) in HAART-SP treated cells.

In the fourth manuscript (chapter five), it was shown how SP mitigates inflammation induced by HAART in HepG2 liver cells. SP inhibits the inflammatory pathway, by significantly decreasing iNOS ($p < 0.0001$), IκB-α ($p < 0.0001$), NF-κB ($p < 0.0001$), IL-1β ($p = 0.0002$) and TNF-α ($p = 0.0074$) mRNA levels. The HAART-SP post treatments reduced inflammation as evidenced by decreased mRNA levels of NF-κB ($p < 0.0001$), IL-1β ($p < 0.0001$), IL-12 ($p < 0.0001$), TNF-α ($p < 0.0001$). Furthermore, NF-κB ($p < 0.0001$) protein expression was downregulated. Thus, SP has the potential to inhibit inflammation induced by HAART (3TC, TDF and FTC) in HepG2 cells.

Finally, the overall results show that SP mitigates HAART-adverse drug toxicity in HepG2 cells, by activating the antioxidant response in HepG2 cells.

CHAPTER ONE

INTRODUCTION

Due to its rapid pace of widespread infection and concerning mortality rate, human immunodeficiency virus (HIV) has remained a global public health concern (WHO 2021). In its latest report published in November 2021, the Joint United Nations Programme on HIV/AIDS (UNAIDS) projected that around 37.7 million persons worldwide are HIV positive. Additionally, it was estimated that 680 000 deaths and 1.5 million new HIV infections were recorded in 2020 (Release 2021, UNAIDS 2021, UNAIDS 2021, World Health Organisation 2021, July 17). According to the South African population statistics 2021, approximately 8.2 million people are living with HIV in South Africa (Release 2021). Antiretrovirals (ARVs) are now widely accessible, thus contribute to the extension of HIV/AIDS patients' lives and significantly alter the death pattern. In 2020, of the 27.5 million HIV positive (HIV+) people worldwide who were receiving ARVs, around 5.6 million were South Africans (Africa 2020, Release 2021, WHO 2021, World Health Organisation 2021, July 17).

The use of various combinations of highly active antiretroviral therapy (HAART) treatment choices takes into account the HIV viral load in infected people, thus regulate the viral load and prevent development of AIDS-related symptoms. Since its discovery over three decades ago, HAART has greatly improved the identification and treatment of HIV-positive people (Mondal, Pradhan et al. 2004, Ngondi, Oben et al. 2006, Masiá, Padilla et al. 2007, Blas-Garcia, Apostolova et al. 2011, Manda, Banerjee et al. 2011, NIH 2012, Mohan, Ghazi et al. 2021). Despite its success in extending the life expectancy of HIV positive individuals, the use of HAART promotes metabolic syndromes (MetS) via an inflammatory pathway, excessive formation of reactive oxygen species (ROS), and mitochondrial dysfunction.

The most popular nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) under HAART are 2',3'-dideoxy-5-fluoro-3'-thiacytidine (FTC), (-)-L-2',3'-dideoxy-3'-thiacytidine (3TC), and tenofovir disoproxil fumarate (TDF), which form part of the first-line therapy (Gilks, Crowley et al. 2006, Sliwa, Hilfiker-Kleiner et al. 2010, Society 2011, Rakhmanina and la Porte 2012, Kahana, Rohan et al. 2013). Moreover, 3TC, FTC and TDF are recommended for pre-exposure prophylaxis (PrEP) by the World Health Organization (WHO) (Borroto-Esoda, Vela et al. 2006, Maserati, De Silvestri et al. 2010, Fonner, Dalglish et al. 2016, Spinner, Boesecke et al. 2016, Mugwanya, John-Stewart et al. 2017, Mujugira, Baeten et al. 2020). Additionally, 3TC can also be used to prevent HIV infection after potential exposure (Cheney, Barbaro et al. 2021). These NRTIs have however been associated with increased ROS, endothelial cell injury, cardiovascular complications (Cheney, Barbaro et al. 2021), mitochondrial alterations, oxidative stress (Milián, Peris et al. 2017), and development of lipodystrophy syndrome, as a result of low mitochondrial toxicity (Nelson and Schiavone 2004). It is therefore crucial to look into remedies to alleviate the side effects brought on by these ARV drugs.

Blue-green microalga known as *Spirulina platensis* (SP) is frequently consumed as a traditional cuisine by some African and Mexican populations (Estrada, Bescós et al. 2001, Bashandy, El Awdan et al. 2016). The United Nations' Food and Agriculture Organization (FAO) also recommends *spirulina* as a dietary supplement (Pelizer, Danesi et al. 2003). In addition to its well-known nutritional value, SP has been discovered to have numerous therapeutic benefits as its consumption can strengthen the immune system's cellular and humoral defences (Estrada, Bescós et al. 2001). Interestingly, SP has been associated with MetS-lowering qualities such as hypoglycemic (Iyer Uma, Sophia et al. 1999), hypolipidemic (Serban, Sahebkar et al. 2016), and antihypertensive (Finamore, Palmery et al. 2017). According to studies in rodent species, SP appears to be most helpful in the prevention of MetS (Finamore, Palmery et al. 2017). Oxidative stress inhibitors, phycocyanin, and phycocyanobilin are all found in SP (Miranda, Cintra et al. 1998, Hu and Liu 2001, Zheng, Inoguchi et al. 2013). Prior research has shown that SP prevents oxidative stress (Riss, Décordé et al. 2007, Zheng, Inoguchi et al. 2013, Abdelkhalek, Ghazy et al. 2015, Bashandy, El Awdan et al. 2016) and promotes mitochondrial health (Nawrocka, Kornicka et al. 2017, Jadaun, Yadav et al. 2018, Oriquat, Ali et al. 2019, Sun, Hou et al. 2019), thereby inhibiting inflammation (Zheng, Inoguchi et al. 2013, Izadi and Fazilati 2018). SP can also prevent the development of atherosclerosis (Riss, Décordé et al. 2007), and diabetes (Zheng, Inoguchi et al. 2013).

Specific microRNAs, such as miR-155 and miR-146a, were initially linked with the inflammatory response by virtue of their potent up-regulation in multiple immune cell lineages by Toll-like receptor ligands, inflammatory cytokines, and specific antigens. Moreover, the increased expression of miR-155 and miR-146a in MetS was found to contribute to inflammation-mediated glomerular endothelial injury (Huang, Liu et al. 2014). It is therefore imperative to explore microRNA involvement in HAART and SP synergy. Due to the alarmingly increasing number of HIV-infected people and their high dependence on HAART, supplementation with an antioxidant agent such as SP, which has been shown to protect against oxidative stress and inflammation (Miranda, Cintra et al. 1998, Hu and Liu 2001, Zheng, Inoguchi et al. 2013), can help to minimize the oxidative stress and inflammation during HAART treatment.

Although a number of studies have investigated the effect of SP as an antioxidant, there is insufficient research on the impact of SP on the oxidative and inflammatory pathway of HAART *in vitro*. This study focuses on the anti-inflammatory and antioxidant properties of SP and its amelioration of HAART-associated MetS.

It was hypothesized that SP inhibits oxidative stress and inflammation induced by HAART (3TC, TDF and FTC) in human hepatoma (HepG2) cells. In order to test the hypothesis, the cytoprotective effects

of SP against oxidative stress, inflammation, protein homeostasis and epigenetic regulation of cellular detoxification in HepG2 cells was determined.

The objectives of the study were to determine:

- the protective role of SP against HAART-induced oxidative stress in HepG2 cells.
- the effect of SP on HAART-induced alterations of micro-RNAs involved in inflammation in HepG2 cells.
- the ability of SP to ameliorate inflammation induced by HAART in HepG2 cells.

The thesis is submitted in a manuscript format (as per UKZN guidelines for doctoral thesis submission) comprising of the following manuscripts:

1. Review paper
2. Experimental paper 1
3. Experimental paper 2
4. Experimental paper 3

Experimental paper 1 (Chapter 3)

Spirulina platensis Ameliorates Oxidative Stress Associated with Antiretroviral Drugs in HepG2 Cells.

Sibiya T, Ghazi T, Mohan J, Nagiah S, Chuturgoon AA.

Plants (Basel). 2022 Nov 17;11(22):3143. doi: 10.3390/plants11223143.

PMID: 36432871 Free article.

Review paper (APPENDIX A)

The Potential of Spirulina platensis to Ameliorate the Adverse Effects of Highly Active Antiretroviral Therapy (HAART).

Sibiya T, Ghazi T, Chuturgoon A.

Nutrients. 2022 Jul 27;14(15):3076. doi: 10.3390/nu14153076.

PMID: 35893930 Free article.

Experimental paper 2 (Chapter 4)

Spirulina Platensis mitigates the inhibition of selected miRNAs that promote inflammation in HAART treated HepG2 cells.

Thabani Sibiya, Terisha Ghazi, Jivanka Mohan, Savania Nagiah and Anil A. Chuturgoon (2022).

Plants (In Review). Manuscript ID: plants-2084169

At the time of the thesis submission this manuscript was corrected and submitted with revisions (Revision 1, Journal: Plants).

Experimental paper 3 (Chapter 5)

Spirulina platensis mitigates inflammation induced by highly active antiretroviral therapy (HAART) in HepG2 liver cells.

Thabani Sibiya, Terisha Ghazi, Jivanka Mohan, Savania Nagiah and Anil A. Chuturgoon (2022).

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CHAPTER TWO

LITERATURE REVIEW

2.1. Human Immuno-Deficiency Virus

HIV is a public health concern, deadly comorbidity, and a major cause of mortality worldwide. According to the Joint United Nations Programme on HIV/AIDS (UNAIDS), an estimated 37.7 million [30.2 million–45.1 million] people globally were living with HIV in 2020. Of this 1.5 million [1.0 million–2.0 million] people became newly infected with HIV in 2020, and 680 000 [480 000–1.0 million] people died from AIDS-related illnesses in 2020 (UNAIDS 2021). Ambitious targets that can reduce the impact of HIV in the future and end AIDS by the year 2030 were made by leaders globally (Mahy, Marsh et al. 2019); including initiatives such as early antiretroviral therapy (ART) to decline HIV prevalence (Dehne, Dallabetta et al. 2016). The primary responsibility of HAART is to treat HIV type 1 (HIV-1) infection. HAART has several classes, Nucleoside/Nucleotide reverse transcriptase inhibitors (NRTIs), Non-nucleoside reverse transcriptase inhibitors (NNRTIs) (Kakuda 2000, Martin, Nolan et al. 2004, Lyseng-Williamson, Reynolds et al. 2005, Yeni 2006, Lucas, Ross et al. 2014, Chang, Tsai et al. 2018, Saag, Benson et al. 2018, Chen, Stampley et al. 2019, Eggleton and Nagalli 2020), Protease inhibitors (PIs) (Eastone and Decker 1997, Yeni 2006, Soliman, Lundgren et al. 2011, Lennox, Landovitz et al. 2014), Integrase strand transfer inhibitors (INSTIs) (Hazuda, Felock et al. 2000, Schafer and Squires 2010, Gutierrez, Fulladosa et al. 2014, Fettiplace, Stainsby et al. 2017, Hoffmann, Welz et al. 2017, Zash, Makhema et al. 2018), Fusion inhibitors (FIs) (Hardy and Skolnik 2004), and Chemokine receptor antagonists (CCR5 antagonists) (Dorr, Westby et al. 2005, De Luca, Pezzotti et al. 2019, Miao, De Clercq et al. 2020). HAART is a specifically selected combination of NRTIs and NNRTI, PI or INSTIs drugs responsible for inhibition of viral replication by multiple virus targets (Kitahata, Koepsell et al. 1996, Cunningham, Tisnado et al. 1999, Shafer and Vuitton 1999, Sax, Tierney et al. 2009, Post, Moyle et al. 2010, Rackal, Tynan et al. 2011, Thompson, Aberg et al. 2012, Group 2015, Günthard, Saag et al. 2016, Ford, Migone et al. 2018). However, HAART can cause adverse drug reactions. 3TC (lamivudine), FTC (emtricitabine), TDF (Tenofovir Disoproxil Fumarate), ZDV (Zidovudine) and d4T (Stavudine) are associated with mitochondrial toxicity and oxidative stress (Venhoff, Setzer et al. 2007, Brown, Jin et al. 2014, Canale, de Bragança et al. 2014, Glover, Hebert et al. 2014), while NNRTIs are linked to toxic hepatitis and PIs are implicated in insulin resistance and hyperlipidemia (Yeni 2006). Chronic side effects linked to HAART include ROS-induced insulin resistance (dos Santos, Paes et al. 2013, Bonomini, Rodella et al. 2015), lipodystrophy, gastrointestinal disorders (Sharma 2011), and cardiovascular disease (Masiá, Padilla et al. 2007, Csányi 2014). It has been shown that HAART drugs induce oxidative stress (Mondal, Pradhan et al. 2004, Ngondi, Oben et al. 2006, Masiá, Padilla et al. 2007) and mitochondrial dysfunction (Blas-Garcia, Apostolova et al. 2011, Manda, Banerjee et al. 2011).

2.1.1 HIV morphology

HIV morphology has been the centre of intervention in therapy, especially biochemical and structural composition. The virus is a member of the lentivirus genus, a branch of the Retroviridae family. HIV is spherical in shape, and has a diameter of 100–120 nm (Sierra, Kupfer et al. 2005). HIV-1 and HIV-2 are the two known strains of the virus. Globally, HIV-1 is the more contagious and frequently seen strain. HIV-2 is mostly found in some regions of West Africa and has a substantially reduced virulence/infectivity. There are two identical 9.2 kb single RNA strands that make up the HIV genome (Figure 2.1) (Sierra, Kupfer et al. 2005, Rossi, Meuser et al. 2021). Three structural protein open reading frames (ORFs) are present in the genome. Matrix (MA), capsid (CA), and nucleocapsid (NC) structural proteins are all encoded by group specific antigen (Gag). Protease (PR), reverse transcriptase (RT), integrase (IN), and ribonuclease are all encoded by the polymerase (pol) ORF. Finally, the envelope (Env) ORF encodes the glycoprotein (gp)120 and gp41 viral envelope proteins (Figure 2.2) (Wilk and Fuller 1999, Rossi, Meuser et al. 2021). Additional accessory genes involved in the control of viral replication are encoded by the HIV genome, including the trans-activator of transcription (Tat), the regulator of virion protein expression (Rev), the negative factor (Nef), the viral infectivity factor (Vif), the viral protein R (Vpr), and the viral protein U. (Vpu).

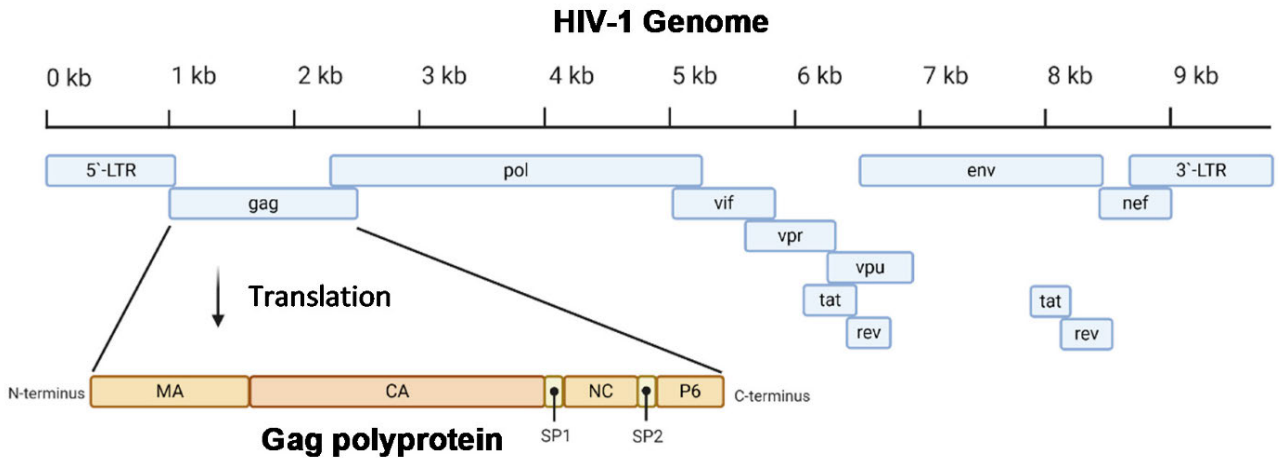

Figure 2.1. A diagram of the 9.8 kb HIV-1 genome. The Gag polyprotein, which is made up of the matrix (MA), capsid (CA), nucleocapsid (NC), P6, and two spacer peptides (SP1 and SP2), is produced from the Gag part of the genome. The polyprotein separates into its component parts during maturity (Rossi, Meuser et al. 2021).

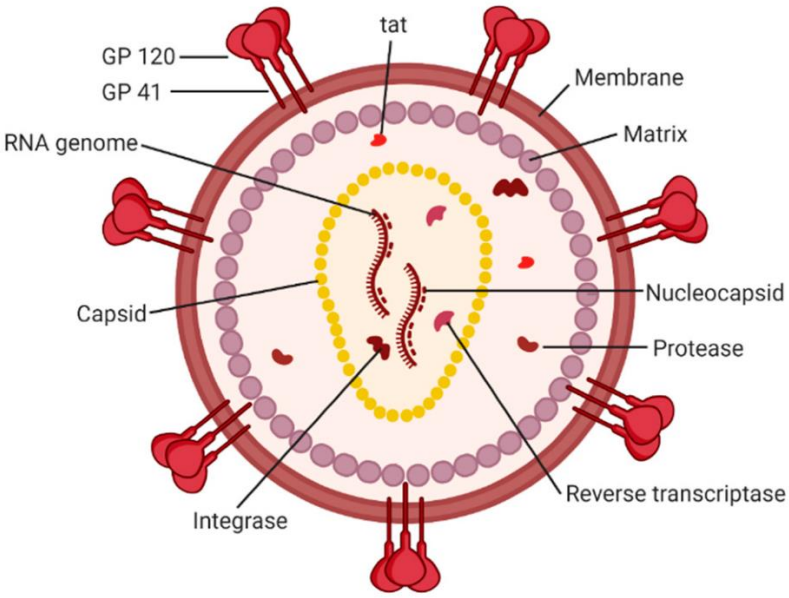

Figure 2.2. Diagram of HIV virion. The host cell serves as the source of the lipid bilayer. The host-derived membrane surface is surrounded by envelop proteins (GP41 and GP120), which is lined internally with a layer of matrix protein (p17). The structural proteins forming the core are matrix protein (p17), capsid protein (p24), and nucleocapsid (p7). The viral capsid contains reverse transcriptase and integrase, as well as the two viral RNA strands. (Karlsson Hedestam, Fouchier et al. 2008, Rossi, Meuser et al. 2021)

A bilayer of cellularly derived phospholipids surrounds the virion, forming an envelope. Protein protrusions that promote binding to the host cell are present on the viral envelope. The viral protein gp120 extends from the viral envelope to aid host cell attachment and eventual fusion. Viral protein p17 makes up the viral MA, which is enclosed in the viral envelope and keeps the virion's structural integrity. CA form part of the viral core and is conical in shape, which is made up of viral protein p24. Two strands of viral RNA are strongly linked to p7, the viral NC protein, within the CA. The viral replication enzymes RT, IN, and PR are also found in NC (Turner and Summers 1999, Sierra, Kupfer et al. 2005, Ganser-Pornillos, Yeager et al. 2008, Rossi, Meuser et al. 2021).

2.1.2. Viral life cycle

Immune cells including CD4+ helper T cells, macrophages, dendritic cells, and microglial cells are the main targets of HIV. Gp120 interacts with the CD4 receptor on the immune cell. Gp120 also engages in further interaction with chemokine co-receptors, mainly C-X-C chemokine receptor type 4 (CXCR4) and C-C chemokine receptor type 5 (CCR5), once the attached to the host cell (Doranz, Rucker et al. 1996, Feng, Broder et al. 1996, Clapham and Weiss 1997). The hydrophobic domain of gp41 is exposed as a result of this contact, enabling fusion with the host cell. The CA, which contains viral genetic material and enzymes, undergoes an uncoating process that releases its contents into the host cell's

cytoplasm (Figure 2.3). The viral pre-integration complex is created when RT reverse transcribes viral RNA after it enters the cytosol, a process regulated by the accessory protein Vif (Turner and Summers 1999). The active process of nuclear localization of the viral DNA is controlled by MA, IN, RT, and Vpr (Bukrinsky, Haggerty et al. 1993, von Schwedler, Kornbluth et al. 1994). In the nucleus, virus and host DNA are integrated following three successive integration stages mediated by the viral enzyme IN. During integration, the viral DNA is first assembled, then its 3' end must undergo endonucleolytic processing, and ultimately the strand is joined to cellular DNA (Arts and Hazuda 2012). Both spliced and unspliced mRNA transcripts are produced as a result of this process. Tat, Rev, and Nef are encoded by the short-spliced RNA species. Regulatory protein Tat is essential for transcriptional activation. The introns encoding for Gag and Pol are present in both the full-length and singly spliced viral RNA. For synthesis to occur, these must be exported to the cytosol. Rev, which binds to the Rev-response element, regulates the viral RNA's export. When this complex forms, protein exportin 1 is attracted, allowing the viral DNA to pass via a nuclear pore (Ohno, Fornerod et al. 1998).

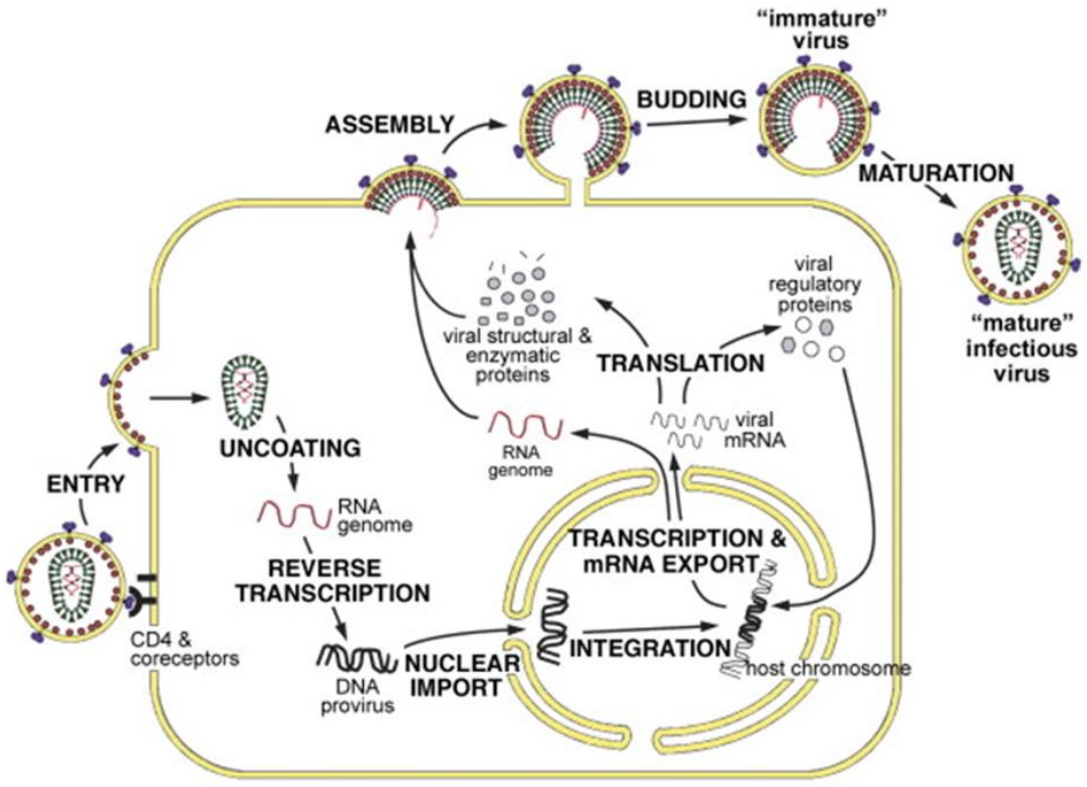

Figure 2.3. Viral replication of HIV-1 (Ganser-Pornillos, Yeager et al. 2008)

The endoplasmic reticulum (ER) produces the viral spike gp120, which derives from gp160, a genetic derivative of Env. The polyprotein gp120 is transported from the ER through the Golgi apparatus and

split into gp120 and gp41 by furin. The assembly phase is made possible by the processed proteins, Gag, Gag-Pol, and viral RNA aggregating at the plasma membrane. The virion then starts to bud off the host cell (Figure 2.3). Gag polyproteins will still require additional cleavage by PR. A complete, infectious virion is created once all the structural elements have come together. A mature virus has the ability to penetrate CD4+ cells and use cellular resources for viral reproduction. As long as viral replication is maintained, the CD4+ T cell pool will inevitably become much smaller. Apoptotic cell death accounts for the majority of CD4+ cell loss (Gougeon, Lecoeur et al. 1996). The gradual loss of immune cells impairs both innate and adaptive immunity, ultimately making people more vulnerable to opportunistic infections. Kaposi sarcoma, lymphoma, and infections with tuberculosis, bacterial pneumonia, Cryptococcus, herpes simplex, and cytomegalovirus (CMV) are among the health effects that are frequently seen (Benson, Brooks et al. 2009). Viral replication is a critical target in lowering the infectivity of HIV because it is necessary to keep CD4+ T cells at a stable level in order to provide protection. To reduce an HIV-positive person's viral load, HAART includes a variety of ARV drugs, ensuring multiple targets are covered (NIH 2012, Brouwer, Napravnik et al. 2014, Mohan, Ghazi et al. 2021). However, studies have demonstrated the ability of natural medicine such SP in the inhibition of HIV-1 replication (Ayehunie, Belay et al. 1998, Ali and Saleh 2012). Unlike HAART, SP is an antioxidant with the ability to inhibit MetS, which make it a suitable supplement for people on life-long HAART.

2.2. Spirulina Species (Please see appendix A)

Three *spirulina* species are frequently researched because of their great nutritional value and potential for medicinal use. These *Spirulina* species include *Spirulina platensis* (SP) (*Arthrospira platensis*), *Spirulina maxima* (*Arthrospira maxima*), and *Spirulina fusiformis* (*Arthrospira fusiformis*). Additionally, Cyanobacteria and Prochlorales subclassify these *Spirulina* species as oxygenic photosynthetic bacteria (Whitton 1992, Turner 1997, Vonshak 1997, Khan, Bhadouria et al. 2005, Gershwin and Belay 2007, Karkos, Leong et al. 2011). SP is abundantly present in alkaline water with high concentration of bicarbonate and saline (Ciferri 1983, Estrada, Bescós et al. 2001). The majority of *spirulina* species are microstructures with a three-dimensional helix (Kamata, Piao et al. 2014) covered by a cell wall made of intricate proteins and carbohydrates (Estrada, Bescós et al. 2001). SP is regarded as an anti-inflammatory and antioxidant (Henrikson 1989). Due to the abundance of carotenoid, phycocyanin, and chlorophyll in it, it is also regarded as a dietary resource. Chlorophyll is anti-mutagenic and an antioxidant (Ferruzzi and Blakeslee 2007, Hosikian, Lim et al. 2010), Carotenoids are essential antioxidants that can prevent cancer (Khan, Bhadouria et al. 2005), The Bili protein phycocyanin has antioxidant and radical-scavenging abilities (Hoseini, Khosravi-Darani et al. 2013). Additionally, SP has been praised for its capacity to prevent cancer and viral infections (Asghari, Fazilati et al. 2016). According to studies, SP contains a variety of bioactive compounds, such as carotenoid, phenol, chlorophyll, phycocyanin, polyunsaturated fatty acids (PUFAs), glycosides, flavonoids, and alkaloids

(Jaime, Mendiola et al. 2005, Herrero, Vicente et al. 2007, El-Baky, Hanaa et al. 2009, Kannan, Pushparaj et al. 2014, Prabakaran, Sampathkumar et al. 2020).

2.2.1. Nutritional Composition

Spirulina was chemically determined to be an excellent source of proteins, vitamins and minerals (Ali and Saleh 2012), constituted by 60% to 70% content of proteins in its dry form (Ciferri 1983). Rich in β-carotene (Belay 1997), vitamin B12 and useful in anemia treatment, hence, it is used as iron supplement during gestation in anemic woman (Belay 1997, Ali and Saleh 2012, Becker 2017). 4% to 7% is lipid content (Misbahuddin, Maidul Islam et al. 2006). Composition of carbohydrates is approximately 13.6% (Shekharam, Venkataraman et al. 1987). RNA composition is 2.2% to 3.5% and DNA is between 0.6% to 1% (Ciferri 1983). It is also rich in carotenes, chlorophyll and phycocyanin (Ciferri 1983, Saxena, Ahmad et al. 1983).

2.2.2. Health Benefits

Spirulina is a potent inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, resulting in reduction of oxidative stress (McCarty 2007). It shares structure and physiological activities with bilirubin, an important modulator of chronic inflammation and oxidative stress in MetS (Miranda, Cintra et al. 1998, Hu and Liu 2001, Jiang, Roberts et al. 2006, Riss, Décordé et al. 2007, Zheng, Inoguchi et al. 2013). *Spirulina* has therapeutic effects against vascular diseases, cancer, diabetes, neurodegenerative diseases, and inflammatory disorders (McCarty 2007). It has central neuroprotective effects in rodents (Chamorro, Pérez-Albiter et al. 2006). Food and Agriculture Organization (FAO) of the United Nations recommends *Spirulina* as an excellent dietary supplement (Pelizer, Danesi et al. 2003). The compound has been shown to have the ability to inhibit oxidative stress (Riss, Décordé et al. 2007, Zheng, Inoguchi et al. 2013, Abdelkhalek, Ghazy et al. 2015, Bashandy, El Awdan et al. 2016), and enhance mitochondrial health (Nawrocka, Kornicka et al. 2017, Jadaun, Yadav et al. 2018, Oriquat, Ali et al. 2019, Sun, Hou et al. 2019).

2.2.3. Antiviral activity of Spirulina

Spirulina possesses inhibitory effects against numerous viruses such as HIV-1, herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), human cytomegalovirus (HCMV), influenza type A, measles and other enveloped viruses (Hayashi, Hayashi et al. 1996, Ayehunie, Belay et al. 1998, Hernández-Corona, Nieves et al. 2002, Luescher-Mattli 2003, Khan, Bhadouria et al. 2005). Moreover, it has antimutagenic and anticancer effects (Ali and Saleh 2012). Studies have demonstrated the ability of SP to inhibit HIV-1 replication in human T-cell lines, peripheral blood mononuclear cells (PBMC), and Langerhans cells (LC). Inhibition of viral production by *spirulina* extract (between 0.3 and 1.2μg/ml) has been proven to be approximately 50% in PBMCs (Ayehunie, Belay et al. 1998, Ali and Saleh 2012).

2.2.4. Anti-Cancer Effects

Studies have demonstrated that *spirulina* anticancer effects are potent in both humans and animals, this may be achieved through enhancement of cell nucleus enzyme function and DNA repair synthesis (Ali and Saleh 2012, Konícková, Vanková et al. 2014, Liu, Huang et al. 2016, Marková, Koníčková et al. 2020).

2.2.5. Immunological Benefits

Spirulina can build up both the humoral and cellular mechanisms of the immune system when consumed (Estrada, Bescós et al. 2001). *Spirulina* consumption can enhance components within both the mucosal and systemic immune systems (Balachandran, Pugh et al. 2006). It has antiallergic effects (Karkos, Leong et al. 2011). *Spirulina* is an effective treatment against chronic arsenic poisoning with melanosis and keratosis (Choudhary, Jetley et al. 2007). *Spirulina* has been shown to have protective effects against allergic rhinitis in humans (Mao, Water et al. 2005). It prevents cellular ageing, infectious diseases and promotes strong immune system (Ali and Saleh 2012). Furthermore, it prevents free radicals (FR) induced apoptotic cell death (Chu, Lim et al. 2010).

2.3. Common Highly Active Antiretroviral Therapy (HAART) (Please see appendix A)

The purpose of HAART is to prevent viral replication by targeting several virus targets with a carefully chosen mix of NRTI and NNRTI, PI, or INSTI drugs (Kitahata, Koepsell et al. 1996, Cunningham, Tisnado et al. 1999, Shafer and Vuitton 1999, Sax, Tierney et al. 2009, Post, Moyle et al. 2010, Rackal, Tynan et al. 2011, Thompson, Aberg et al. 2012, Group 2015, Günthard, Saag et al. 2016, Ford, Migone et al. 2018). HAART is primarily in charge of treating people with HIV-1 infection and preventing viral replication. These core tasks of HAART are accomplished by addressing various viral lifecycle stages, ensuring suppression even if the virus is resistant to one of the drugs (Kitahata, Koepsell et al. 1996, Cunningham, Tisnado et al. 1999, Shafer and Vuitton 1999, Sax, Tierney et al. 2009, Post, Moyle et al. 2010, Rackal, Tynan et al. 2011, Thompson, Aberg et al. 2012, Group 2015, Günthard, Saag et al. 2016, Ford, Migone et al. 2018). In comparison to other drug combinations, the combination of two NRTIs (most commonly FTC and TDF) and one NNRTI (for example, EFV; Efavirenz) is preferable since it is easier to dose and has less toxic side effects (Yeni 2006, McColl, Margot et al. 2011, Hong, Jonas et al. 2015). Three NRTIs together are less efficient than two NRTIs plus an NNRTI (Gulick, Ribaudo et al. 2004). Due to its high toxicity, the combination of d4T with ddI is not frequently recommended (Robbins, De Gruttola et al. 2003). Cytidine analogs (XTC), FTC, 3TC, and TDF are the most widely used NRTIs and are used as first-line treatments (Gilks, Crowley et al. 2006, Sliwa, Hilfiker-Kleiner et al. 2010, Society 2011, Rakhmanina and la Porte 2012, Kahana, Rohan et al. 2013). The molecular structures of FTC and 3TC are comparable, but they have different pharmacokinetic and

pharmacodynamic characteristics, and they have required deoxynucleosides for HIV DNA synthesis. They are phosphorylated by intracellular kinases to produce the triphosphate metabolites FTC-TP and 3TC-TP, with FTC-TP being more effectively integrated into HIV DNA synthesis than 3TC-TP (Schinazi, McMillan et al. 1992, Schinazi, Lloyd et al. 1993, Margot, Enejosa et al. 2009, Maserati, De Silvestri et al. 2010, McColl, Margot et al. 2011).

2.3.1 Approved PrEP and PEP HAART drugs

Clinical trials showed that pre-exposure prophylaxis (PrEP) with daily oral emtricitabine/tenofovir, disoproxil, and fumarate reduced the risk of HIV infection by more than 90%, and the US Food and Drug Administration (FDA) authorized it for this use in 2012 (Touger and Wood 2019). Additionally, 3TC, FTC, and TDF are suggested by WHO for PrEP (Borroto-Esoda, Vela et al. 2006, Maserati, De Silvestri et al. 2010, Fonner, Dalglish et al. 2016, Spinner, Boesecke et al. 2016, Mugwanya, John-Stewart et al. 2017, Mujugira, Baeten et al. 2020). Furthermore, 3TC is utilized to stop HIV infection after potential exposure (Cheney, Barbaro et al. 2021). PrEP is a strategy for preventing HIV infections in HIV-negative people who exhibit major risk factors by using ARV drugs as a prophylactic measure (Kelesidis and Landovitz 2011). Animal studies have shown that the NRTI TDF, especially when combined with emtricitabine (FTC; TDF/FTC), is effective in preventing sexual HIV transmission (García-Lerma, Otten et al. 2008, García-Lerma, Cong et al. 2010). Literature has shown more effective risk-reduction for anal sexual intercourse by PrEP compared with vaginal sexual intercourse. Rectal tissue has been found to have higher PrEP tissue concentrations than vaginal tissue (Patterson, Prince et al. 2011). TDF and FTC, two oral ART medications used as PrEP for those at risk for HIV, are very effective at lowering HIV acquisition when used as directed (Grant, Lama et al. 2010, Molina, Capitant et al. 2015, McCormack, Dunn et al. 2016). Following HIV exposure, the WHO advises one month of triple ART as post-exposure prophylaxis (PEP) (Organization 2014). UNAIDS targets to lower the number of new HIV infections globally by guaranteeing that 90% of those at risk of contracting HIV have access to PEP and PrEP (Prevention 2020).

2.4 Inflammation

The immune system's reaction to a toxic stimulus, infections, and damaged cells is inflammation. Therefore, inflammation serves as defence mechanism to clear out harmful stimuli and begin the healing process. However, chronic inflammation is harmful as it contributes as a side consequence of many disorders (Chen, Deng et al. 2018). Given that it promotes cell regeneration in the event of cellular damage, regulated inflammation has positive effects. Furthermore, it sets off an immune response that scavenge for and eliminates abnormal cells. Chronic inflammation is however detrimental because it accelerates the development of fibrosis, cirrhosis, and ultimately hepatocellular carcinoma as a result of ongoing liver injury and the associated regenerative damage healing mechanisms (Luedde and Schwabe

2011, Hoesel and Schmid 2013, Bishayee 2014). One of the main mediators of inflammation is nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Beinke and Ley 2004). There may be three distinct mediators of inflammation, which include NF-κB, interleukin-6 and signal transducer and activator of transcription 3 (STAT3). The focus on NF-κB is due to its key regulatory role in the inflammation pathway. NF-κB is also involved in the regulation of micro-RNAs linked to inflammation. For example, the inflammation linked to arsenite carcinogenesis is promoted by exosomal miR-155, which is regulated by NF-κB (Chen, Luo et al. 2017). Alteration in inflammation-related miR-146a expression of NF-κB signaling pathway in Diabetic Rat Hippocampus (Habibi, Soufi et al. 2016), indicates a clear link and the involvement of NF-κB in MetS.

2.4.1 Nuclear factor kappa -light-chain-enhancer of activated B cells (NF-κB)

An immunological response and inflammation are induced by the activation of NF-κB in response to environmental stimuli. NF-κB is a dimeric complex made up of subunits from the Rel family of DNA binding proteins: p105/p50, p100/p52, RelB, c-Rel and p65 (RelA) which controls the transcription of genes involved in immunological responses, inflammation, cell growth and differentiation, and cell survival (Beinke and Ley 2004, Sharma, Tate et al. 2018). The Rel homology domain (RHD) in NF-κB proteins allows for DNA binding, dimerization, and nuclear localization. Tumor necrosis factor alpha (TNF-α), Interleukin-1 beta (IL-1β), lipopolysaccharide (LPS), and ultraviolet (UV) radiation are examples of activator molecules that can induce NF-κB phosphorylation and NF-κB inhibitor-alpha (IκB-α) degradation (Figure 2.4). The IκB proteins, which are composed of the catalytic subunits IkappaB Kinase alpha (IKKα), IkappaB Kinase beta (IKKβ), and the regulatory component IkappaB Kinase gamma NEMO (IKK γ/NEMO), bind to NF-κB to keep it confined to the cytoplasm under normal physiological conditions (Ben-Neriah and Karin 2011). IκB is phosphorylated in response to a particular stimulus, such as chemotherapy, stressors, or cytokines, which leads to its ubiquitination and breakdown by the 26S proteasome. The nucleus receives the activated NF-κB, which changes the expression of the target genes (Ahn, Moon et al. 2003, Nishikori 2005). The consensus DNA sequence 5'-GGGRNNYYCC-3', often known as the kB site, is where NF-κB binds to start transcription of genes in the nucleus (where Y denotes cytosine or thymine, N denotes any base, and R denotes either adenine or guanine) (Antonaki, Demetriades et al. 2011). Protein phosphorylation as well as post-translational changes, such as protein acetylation, control the NF-κB dimerised complex further. It is also responsible for the activation of genes that prevent cell death, encourage the invasion and migration of altered cells, and promote the development of mesenchymal stem cells linked to cancer (Ben-Neriah and Karin 2011). There is also a clear link between NF-κB activation and cancer, the majority of cancer cells maintain active NF-κB through mutations in signaling molecules upstream of it or in response to external stimuli in the tumor microenvironment (Ben-Neriah and Karin 2011). The activation of the inflammatory response and NF-κB -mediated pro-oncogenic activity depends on the RelA and p50 NF-κB heterodimer

(Yu, Pardoll et al. 2009). Inflammatory and immunological responses are mediated by Th2 cytokines, chemokines, COX-2, matrix metallopeptidases (MMPs), and apoptotic factors, depending on whether the NF-κB transcription genes are repressed or activated (Messadi, Doung et al. 2004). It has been discovered that iron and copper chelators inhibit NF-κB from being activated. (Schmilz 1995).

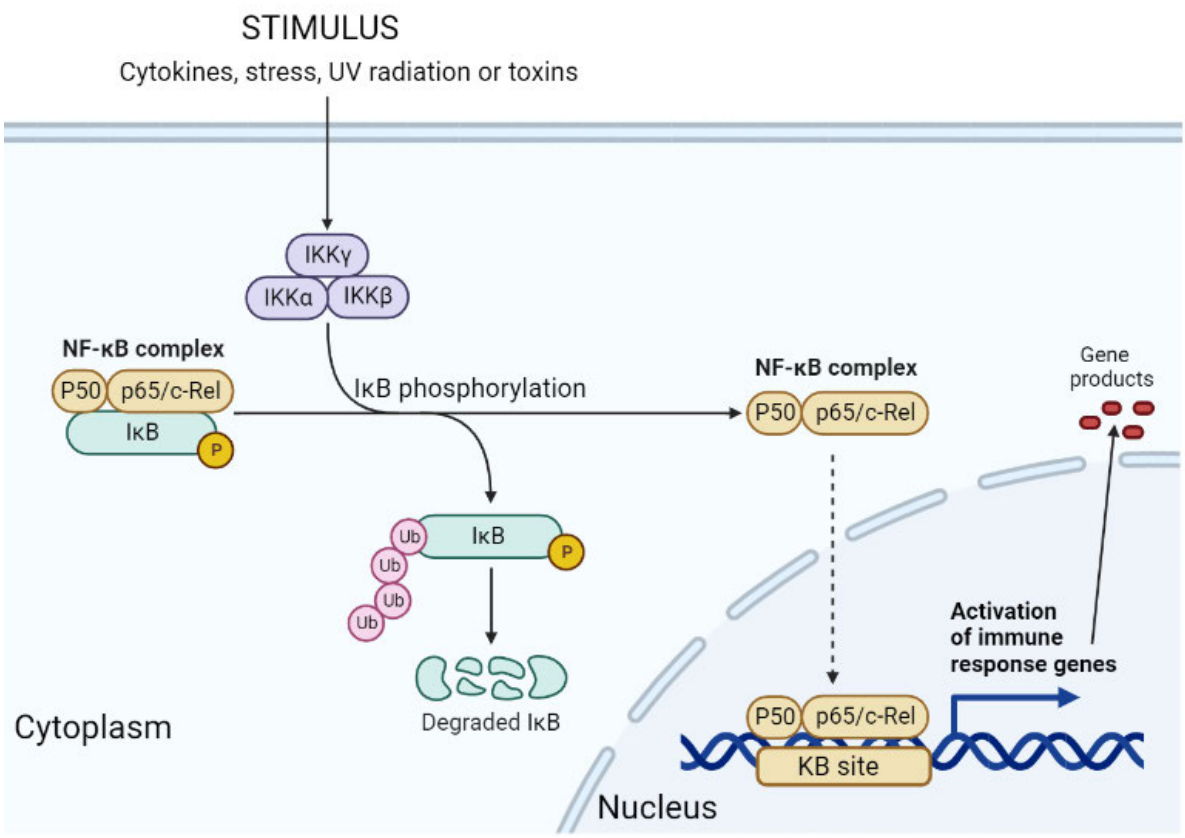

Figure 2.4. The pathway of NF-κB activation (Prepared by author with BioRender.com, accessed on 07 October 2022).

2.4.2 Inflammation and reactive oxygen species

Diabetes-related consequences such as nephropathy, atherosclerosis, cardiomyopathy, neuropathy, and retinopathy are caused by metabolic alterations that include hyperglycemia, hyperlipidaemia, and insulin resistance along with an increase in oxidative stress and inflammation (Sharma, Tate et al. 2018). Oxidative stress and inflammation are linked in a cyclical process. Reactive oxygen species (ROS) levels are stimulated by elevated pro-inflammatory cytokine expression. High levels of ROS also cause an elevation in the expression of pro-inflammatory cytokines (Sharma, Tate et al. 2018). A very pro-inflammatory and pro-oxidant condition that has been shown to be harmful to type 2 diabetes mellitus (T2DM) and exacerbate problems is driven by this positive feedback loop. The function of NF-κB is crucial to this positive feedback loop (Morgan and Liu 2011). In MetS, including T2DM, an essential inflammasome activating signal is ROS. ROS indirectly promotes inflammation through its effects on

NF-κB (Figure 2.5) (Sharma, Tate et al. 2018). Kim, Na et al. (2008) revealed that ROS stimulates NF-κB indirectly by activating Akt. IκBα inhibitory proteins are oxidized and broken up as a result of Akt activation and IKK phosphorylation, which then permits the phosphorylation of ser-276 and NF-κB translocation (Schreck, Rieber et al. 1991, Kim, Na et al. 2008). In addition to being triggered by ROS for inflammatory purposes, NF-κB also stimulates the expression of many proteins, which increases ROS generation. In order to promote oxidative stress, NF-κB directly upregulates the enzymes NADPH oxidase 2 (NOX2), a dedicated ROS generator, and cyclo-oxygenase 2 (COX2) , which generates superoxide by-products (Figure 2.5) (Morgan and Liu 2011).

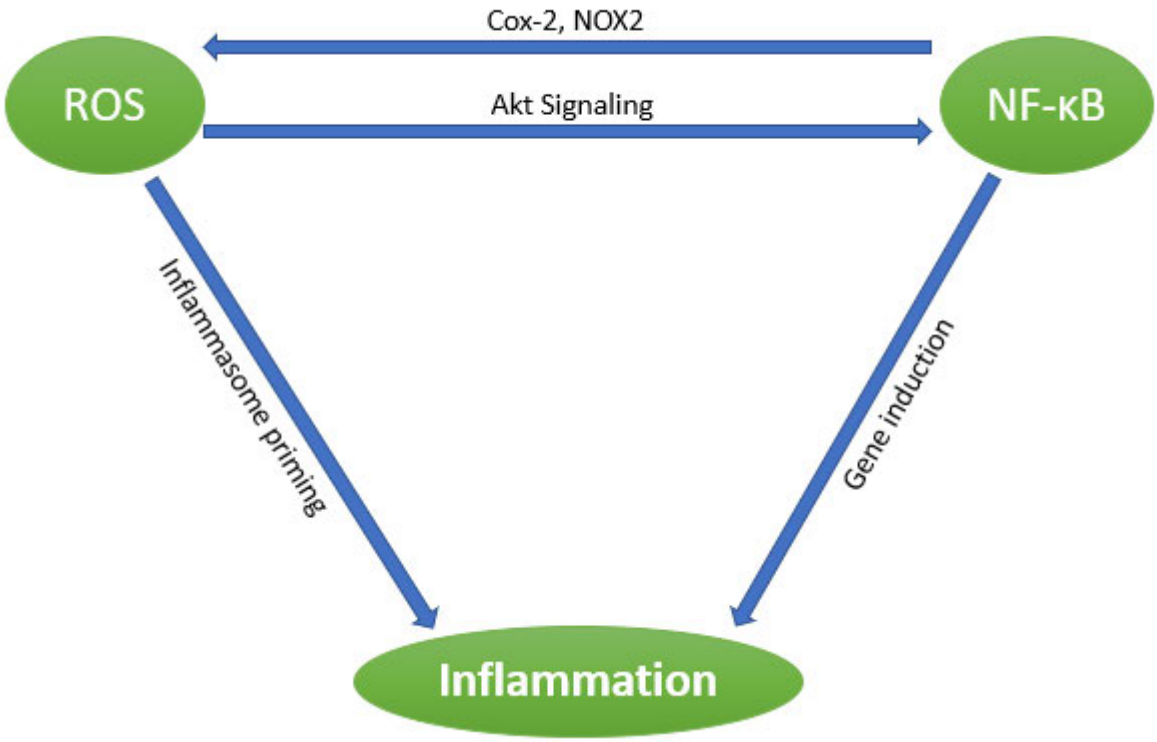

Figure 2.5. Diagram of the link between NF-κB, ROS and inflammation (Prepared by author)

2.5 Oxidative Stress

Oxidative stress is defined as a disturbance in the balance between ROS production and antioxidant defence, shifted towards excess ROS (Betteridge 2000). ROS consists of free radicals and reactive forms of oxygen. FR are molecules with one or more unpaired electrons in the valence shells, the family of FR also includes reactive nitrogen species (RNS) and reactive sulphur species (RSS) (Finaud, Lac et al. 2006). Cells deal with abnormal levels of ROS by employing antioxidants to scavenge ROS and ameliorate ROS induced damage (Kabel 2014).

Vertebrates require dioxygen (O_2) during oxidation of energetic substances since its acts as an electron acceptor. O_2 forms a reactive anion by gaining an e^-, the superoxide ($O_2^{\bullet-}$) formed can be reduced to hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^\bullet$) (Apel and Hirt 2004). Electron transport chain (ETC) is the major producer of intracellular ROS via complex I and III of the ETC in the mitochondria, which produces $O_2^{\bullet-}$ anions during the Q cycle. The increased respiration is directly proportional to the increased ROS production, since the Q cycle is non-enzymatic. There are numerous other components that are involved in the mitochondrial production of ROS such as monoamine oxidase, p66sch and α-ketoglutarate dehydrogenase (Phaniendra, Jestadi et al. 2015).

The second major source of ROS is the respiratory burst that results from NADPH oxidase when there is phagocytosis of microbes (Borza, Muntean et al. 2013). Endogenous ROS comes from various contributors such as the endoplasmic reticulum, lysosomes and a number of cytochromes (Borza, Muntean et al. 2013). Negative environmental changes induced or naturally occurring can introduce ROS insults to the body, e.g., cigarette smoke contains many free radicals. $O_2^{\bullet-}$ and nitric oxide are the main contributors from cigarette smoke, oxidative injury results from activated endogenous ROS producing mechanisms by smoke inhalation (Birben, Sahiner et al. 2012). Photosensitizing agents from ultra violet and ionizing radiation induces ROS synthesis through the conversion of $O_2^{\bullet-}$ and organic radicals to H_2O_2 and organic hydroperoxides, which results in oxidative stress (Borza, Muntean et al. 2013). ROS promotes the unfolding of proteins, shortening of telomeres, destruction of lipids, damage to nucleic acids and other small organic molecules (Phaniendra, Jestadi et al. 2015). ROS has positive attributes in muscular contraction (Andrade, Reid et al. 1998, Coombes, Powers et al. 2001, Reid 2001, Linnane, Zhang et al. 2002), Biogenesis of cells (Sen and Packer 1996, Rimbach, Höhler et al. 1999, Reid 2001, SEN 2001, Linnane, Zhang et al. 2002), fighting against inflammation during phagocytosis of antigens (Jenkins 1988, Rimbach, Höhler et al. 1999, Fehrenbach and Northoff 2001), and facilitation of enzyme activation, drug detoxification and repletion of glycogen (Jenkins 1988). However, ROS are implicated in degenerative pathologies such as cancer, cataract formation, Alzheimer's and other cell aging related diseases (Golden, Hinerfeld et al. 2002). More specifically, ROS is capable of damaging all types of cells (Tavazzi, Di Pierro et al. 2000).

Cytochrome P450-dependant enzymes activates O_2 and formation of ROS during detoxification of drugs consumed (Borza, Muntean et al. 2013). ROS formation and lipid peroxidation can be induced by exposure to O_3 and enhanced atmospheric O_2 levels (Birben, Sahiner et al. 2012). Alcohol consumption initiates ROS synthesis via xanthine oxidase and aldehyde oxidase activation. Oxidation of aldehyde results in the formation of $O_2^{\bullet-}$ (Borza, Muntean et al. 2013). Increased central temperature, catecholamine and lactic acid can initiate ROS production, since these have the ability to convert $O_2^{\bullet-}$ into $OH^\bullet$ (Clarkson and Thompson 2000). HAART drugs can also impact oxidant profile since they

inhibit cytochrome P450 3A-mediated synthesis of cis-9-retinoic acid; one of the key activator of PPAR-γ (Carr, Samaras et al. 1998), resulting in impaired fat storage and lipid release (Carr, Samaras et al. 1998, dos Santos, Paes et al. 2013, Bonomini, Rodella et al. 2015).

2.5.1 Effects of oxidative stress

Reactive species oxidize lipids to produce aldehydes and peroxides during the process known as lipid peroxidation. Lipid peroxidation happens when ROS and RNS are produced at a rate that is significantly higher than it is during a normal physiological state. Lipid peroxidation produces compounds that are more stable than the parent reactive species, which leave the site of synthesis and cause tissue damage and dysfunction. Due to their high reactivity, the lipid peroxidation products can have an impact on DNA, proteins, and cell signalling (Ramana, Srivastava et al. 2013). Lipid peroxidation is caused by the addition of oxygen radicals or the removal of hydrogen atoms from lipids with two or more carbon atoms, which causes excessive oxidative damage to PUFAs (Halliwell 2006, Repetto, Semprine et al. 2012, Ayala, Muñoz et al. 2014). As a result, the carbon atom receives a single unpaired electron, creating a "carbon-centered radical" (Repetto, Semprine et al. 2012). In order to stabilize the double carbon bonds, the lipid radical first goes through a chemical rearrangement. After that, it interacts with oxygen to produce peroxyl radicals. By itself, the peroxyl radical is capable of detaching a hydrogen atom from another PUFA, which triggers a cascade of harmful events (Repetto, Semprine et al. 2012). The oxidation of proteins within the cell membrane is mediated by peroxyl radicals, which readily target nearby side chains. eventually causing the lipid membrane to be destroyed, along with the production of other breakdown products like ethers, aldehydes, ketones, and hydroperoxides that have genotoxic and cytotoxic consequences (Esterbauer 1993, Niki, Yoshida et al. 2005, Halliwell 2006, Repetto, Semprine et al. 2012). Thus, the primary molecular mechanism for cellular oxidative damage is lipid peroxidation, which can also cause cell death via the toxicity process (Repetto, Semprine et al. 2012, Ayala, Muñoz et al. 2014). Only when two lipid radicals combine to generate a non-radical product or when they come into contact with an antioxidant electron donor does lipid peroxidation come to an end (Kaufman, Kolesar et al. 2003, Ayala, Muñoz et al. 2014). To keep the equilibrium between oxidants and antioxidants, antioxidants scavenge free radicals and decrease their reactivity (Casas-Grajales and Muriel 2015). The by-products of lipid peroxidation are harmful and contribute to a number of pathogenic illnesses, such as metabolic problems, pre-eclampsia, kidney damage, asthma, and neurological disorders (Pham-Huy, He et al. 2008). Numerous studies demonstrate that the creation of ROS promotes the occurrence and progression of cardiovascular illnesses (Figure 2.6) and is directly related to the process of cell death (Xu, Ding et al. 2019). Malondialdehyde (MDA) and 4-hydroxynonenal are the reactive aldehydes that are most frequently examined by researchers and are used as indicators of oxidative stress (Requena, Fu et al. 1996).

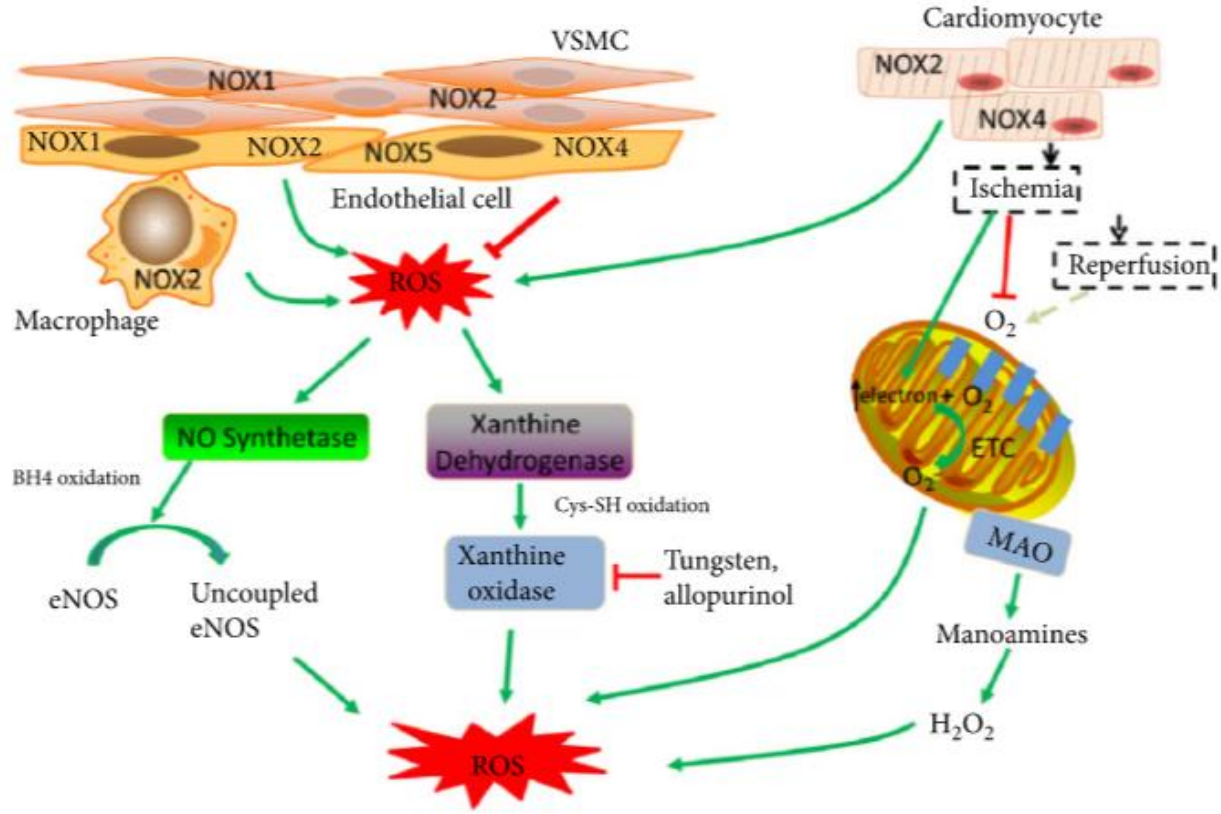

Figure 2.6. ROS resources during cardiovascular diseases (Xu, Ding et al. 2019)

The primary cause of numerous vascular dysfunctions is the excessive formation of ROS, which is brought on by pathogenic stimuli or the malfunction of the ROS clearance mechanism. Through the activation of transcription factors, elevation of adhesion molecules, stimulation of chemokine synthesis, and recruitment of inflammatory cells, oxidative stress may cause vascular inflammation and damage (Förstermann 2008, Thomas, Witting et al. 2008). Although there are several sources of intracellular ROS, the only primary ROS sources are the actions of NADPH oxidases (NOXs) (Figure 2.6) (Drummond, Selemidis et al. 2011, Lassègue, San Martín et al. 2012). In the vascular smooth muscle cell (VSMC), endothelial cells, and fibroblasts, NOXs may produce a significant burst of O_2^-, with NADPH acting as an electron donor (Griendling, Sorescu et al. 2000). Through the oxidative breakdown of the NO synthase cofactor, BH4, NOX-derived ROS can also decouple the NO synthase and promote O_2^- generation. Five distinct NOX isoforms have been found thus far (Griendling, Sorescu et al. 2000, Sumimoto, Miyano et al. 2005). The NOX1, NOX2, NOX4, and NOX5 are all expressed in endothelial cells (Montezano and Touyz 2012), and are differently regulated under various pathological conditions. However, the immune cells and VSMCs in the vascular wall express NOXs as well, which under certain circumstances also contributes to the formation of ROS (Figure 2.6) (Lassegue and Clempus 2003, Arruda and Barja-Fidalgo 2009, Dharmarajah, Arthur et al. 2010, Montezano and Touyz 2012). Under pathological circumstances, NOX2 is probably the most significant ROS producer, whereas NOX4

serves a protective role by encouraging NO bioavailability and reducing cell death (Rouhanizadeh, Hwang et al. 2005, Judkins, Diep et al. 2010, Rivera, Sobey et al. 2010).

2.5.2. ROS and cell death

The induction of cell death, which is the primary cause of numerous cardiovascular illnesses under diverse clinical situations, is a direct result of excessive ROS production. As it is controlled by gene products, programmed cell death is a crucial therapeutic target for the treatment of diseases (Xu, Ding et al. 2019). Necrotic cell death occurs before the activation of catabolic enzymes, but in apoptotic cell death, the activation of proteases and endonucleases occurs prior to the lysis of the cell (Kroemer, Dallaporta et al. 1998). ROS encourage the development of lipid raft-derived signalling platforms, initiating apoptosis that is mediated by the death receptor (Xu, Ding et al. 2019). Numerous studies demonstrate that apoptosis plays a role in the chronic loss of cardiomyocytes in chronic heart failure as well as the acute loss of cardiomyocytes in cardiac ischemia/reperfusion damage (Xu, Ding et al. 2019). The enlargement and rupture of intracellular organelles, which results in the disintegration of the plasma membrane, is a hallmark of necrotic cell death. Cytoplasmic enzymes and other cell components are expelled into the extracellular matrix (Chan, Moriwaki et al. 2013). Inflammatory reactions are mediated by the plasma membrane leaking. It has been discovered that cytokines from the TNF family cause necrosis. The leakage of lactate dehydrogenase (LDH), which is located in the cytoplasm of the cell, is a significant indicator of necrotic death and membrane damage (Chan, Moriwaki et al. 2013).

2.5.3. Link between highly active antiretroviral therapy (HAART) and mitochondrial toxicity

Elevated generation of mitochondrial FR is one of the most frequent effects of mitochondrial dysfunction. The NRTIs mitochondrial toxicity is expected to cause an imbalance in the cellular redox state. Previous research has demonstrated a connection between the usage of HAART and increased FR generation as well as oxidative damage indicators (Mondal, Pradhan et al. 2004, Ngondi, Oben et al. 2006, Masiá, Padilla et al. 2007). Protein synthesis, mitochondrial biogenesis, mitochondrial membrane integrity, mitochondrial matrix metabolism, ATP production, and mitochondrial DNA replication and transcription have all been identified as potential targets for NRTI-related mitochondrial toxicity (Apostolova, Blas-García et al. 2011).

2.6 Antioxidants

Antioxidants are substances that are responsible for reducing harmfulness of oxidative stress in several ways including forming a less active radical or inhibiting the damaging FR chain reaction on substrates such as proteins, carbohydrates, lipids or DNA (Dekkers, van Doornen et al. 1996). To keep the equilibrium between oxidants and antioxidants, antioxidants scavenge free radicals and reduce their reactivity (Casas-Grajales and Muriel 2015, Basak, Sadhukhan et al. 2017). There are enzymatic

(endogenous) and non-enzymatic (mostly from food) antioxidants active in the human body, either found intracellularly or extracellularly (Powers and Lennon 1999). Enzymatic antioxidants include Superoxide dismutase (SOD), Catalase (CAT) and glutathione peroxidase (GPx). While non-enzymatic antioxidants consist of several FR inhibitors such as vitamin A (retinol), vitamin C (ascorbic acid), vitamin E (tocopherol), flavonoids, thiols (including glutathione GSH, ubidecarenone (ubiquinone Q_{10}), uric acid, bilirubin, ferritin) and micronutrients (iron, copper, Zinc, selenium, manganese), which act as enzymatic cofactors. Endogenous antioxidant enzyme production and vitamin-micronutrients base nutritional intakes form a stable antioxidant system (Dekkers, van Doornen et al. 1996). An adaptive reaction to facilitate the excretion of pharmacological compounds is the chemical alteration of xenobiotics. Phase I and II enzymes play a huge role in the endogenous antioxidant defence system. CYP450 mono-oxygenases make up the majority of the phase I enzymes in biotransformation and frequently create electrophilic metabolites (Payne, Fancey et al. 1987). 3TC is an example of CYP450 mono-oxygenases' substrates (Veal and Back 1995). Phase II enzymes are stimulated by the production of CYP450 metabolites, which makes it easier for electrophiles to bind to reduced glutathione (GSH) and glucuronic acid. Due to the high expression of phase I and II enzymes, the liver is essential for xenobiotic metabolism. As a result, xenobiotic metabolism's electrophilic attack mostly affects the liver (Park, Pirmohamed et al. 1995). The cytoprotective arsenal would not be complete without the endogenous antioxidant defence mechanism. The master initiator of the transcription of antioxidant genes is Nuclear-Factor-erythroid 2 Related Factor 2 (NRF-2) (Motohashi and Yamamoto 2004).

2.6.1. NRF-2 pathway

The transcription factor NRF-2 is a key regulator of the transcriptional response to oxidative stress (Solst, Rodman et al. 2017). The expression of NRF-2 is increased in organs exposed to the outside environment (lungs, skin, gastrointestinal tract) and in tissues engaged in detoxification (liver, kidney) (Copple, Goldring et al. 2008). NRF-2 masterfully regulates cellular redox homeostasis. It is tightly linked with the coordination of the basal and inducible expression of antioxidants and Phase II detoxification enzymes that are essential for adaption to different stress conditions. Kelch-like ECH-associated protein 1 (Keap1) regulates the stability of NRF-2 and its cellular distribution. NRF-2 activities are also controlled by other numerous mechanisms such as posttranslational, transcriptional, translational and epigenetic, as well as by other protein partners (Nguyen, Sherratt et al. 2003, Nguyen, Nioi et al. 2009, Huang, Li et al. 2015).The classical pathway of NRF-2-regulated antioxidant response is via the NRF-2-Keap1 system (Ishii, Itoh et al. 2000). The separation of KEAP1 from NRF-2 is necessary for this mechanism. NRF-2 has a high affinity for KEAP1 dimers. Keap1 directly binds NRF-2 in the cytoplasm, repressing NRF-2 transactivation activity (Itoh, Wakabayashi et al. 1999). Under basal conditions, Keap1 ubiquitinates NRF-2 and localizes NRF-2 near the proteasome, thereby enhancing its degradation in the cytoplasm of quiescent cells. During elevated ROS concentrations, the cysteine residues in the NRF-2/Keap1 complex oxidize, allowing NRF-2 to dissociate from Keap1 and translocate to the nucleus

(Ishii, Itoh et al. 2000). NRF-2 is a member of the Cap 'n' Collar (CNC) family of basic region leucine zipper (bZIP) transcription factors (Moi, Chan et al. 1994, Huang, Li et al. 2015). In the nucleus, NRF-2 forms heterodimers with a group of nuclear bZIP proteins called small Maf proteins (Itoh, Igarashi et al. 1995). NRF-2 then induces expression of antioxidant genes via its interaction with regulatory DNA sequences in the antioxidant response element (ARE) (Solst, Rodman et al. 2017). A cis-acting element called the ARE can be found either on the promoter or upstream of the promoters of important phase II and antioxidant genes (Kobayashi and Yamamoto 2006). The ARE or electrophile responsive element (EpRE) regulates the coordination in regulatory sites of target genes during the activation of antioxidants such as *GPx*, *SOD*, *HO-1*, *NQO1* (Figure 2.7) (Friling, Bensimon et al. 1990, Rushmore, Morton et al. 1991, Eggler, Gay et al. 2008). However, ARE and the mitochondrial biogenesis regulator NRF1 share motifs. It has been demonstrated that NRF-2 and NRF1 interact, hence mediating mitochondrial biogenesis (Piantadosi, Carraway et al. 2008). Furthermore, NRF-2 has been linked to metabolic reprogramming (Mitsuishi, Taguchi et al. 2012). More research is still needed to determine how NRF-2 contributes to drug transporter induction during xenobiotic detoxification (Maher, Cheng et al. 2005, Aleksunes, Slitt et al. 2008).

It is well established that NRF-2 is essential for preventing drug-induced cytotoxicity (Copple, Goldring et al. 2008). In order for NRF-2 to be activated it has to be phosphorylated (Figure 2.7) (Huang, Nguyen et al. 2002). Protein Kinase C (PKC) catalyses the phosphorylation of NRF-2 at Ser40; this is a critical signalling event that results in cellular antioxidant response mediated by ARE (Huang, Nguyen et al. 2002). Ser40 is a critical residue of the domain in NRF-2-Keap1 interaction believed to be part of Neh2 (comprised of ≈100 N-terminal amino acids), which Keap1 binds through this domain of NRF-2 and PKC phosphorylates this domain at Ser40 upon oxidative stress, leading to a dissociation of NRF-2 from Keap1 (Huang, Nguyen et al. 2002). Detoxification of ROS is mediated by a network of antioxidant enzymes. SOD, CAT, and GPx are the major antioxidant enzymes involved in radical scavenging (Birben, Sahiner et al. 2012).

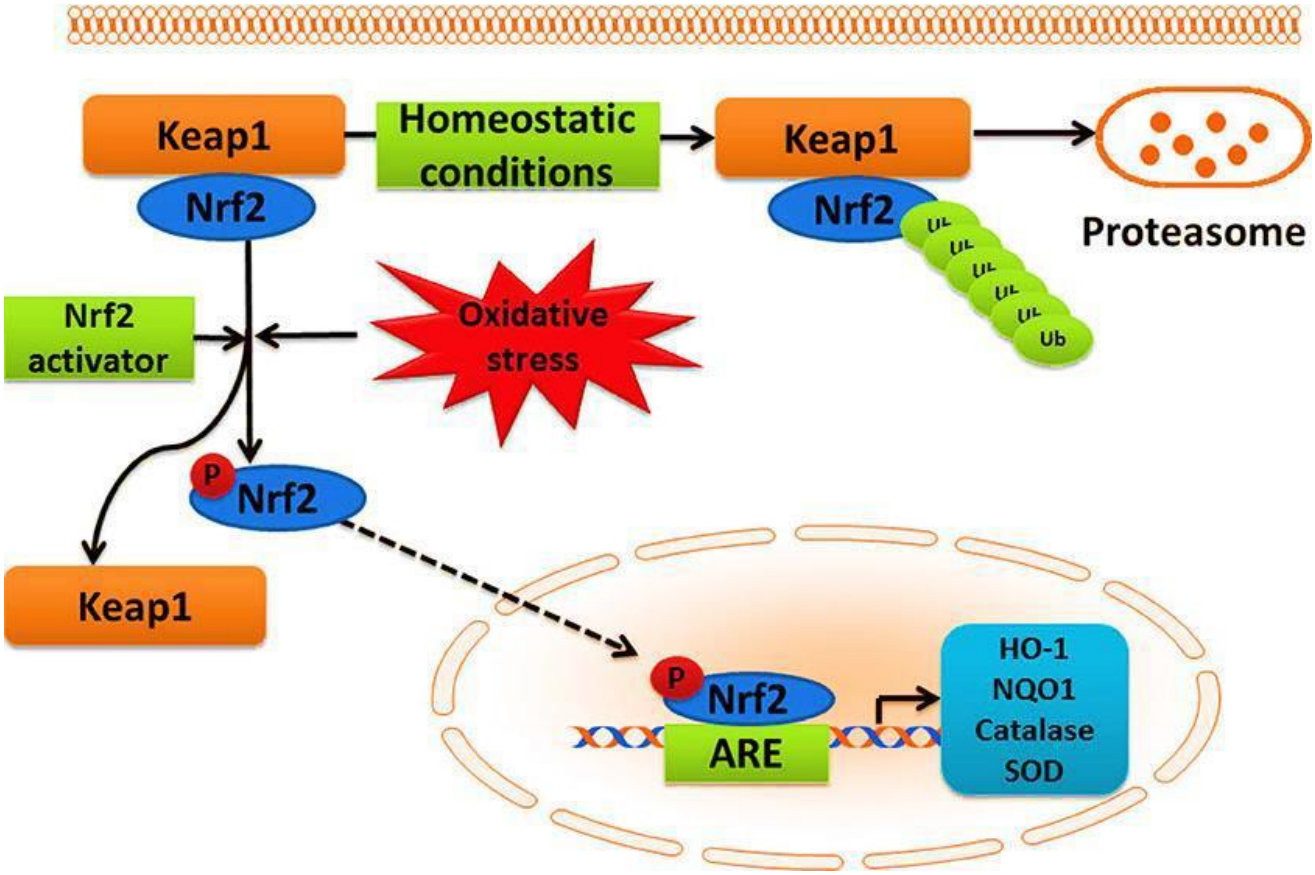

Figure 2.7. NRF-2-KEAP1 pathway showing cellular control of antioxidants (Chen, Zhang et al. 2014).

2.6.2. Superoxide detoxification

One of the most frequently produced ROS from the ETC is the $O_2^{\bullet-}$ anion radical. $O_2^{\bullet-}$ reactions frequently produce additional reactive species, which feeds the oxidative insult cycle (Sies 1991). SOD serves as the initial line of defence against $O_2^{\bullet-}$. Depending on where in the cell the SOD enzyme is located, it has several isoforms: SOD1 (cytosol; mitochondrion), SOD2 (mitochondrion), and SOD3 (extracellular) (Tainer, Getzoff et al. 1983, McCord and Fridovich 1988). Superoxide dismutase converts $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2). Due to the Fenton reaction's potential for quickly converting hydrogen peroxide to $OH^{\bullet}$, extra CAT processing is necessary to convert $OH^{\bullet}$ into H_2O (Figure 2.8).

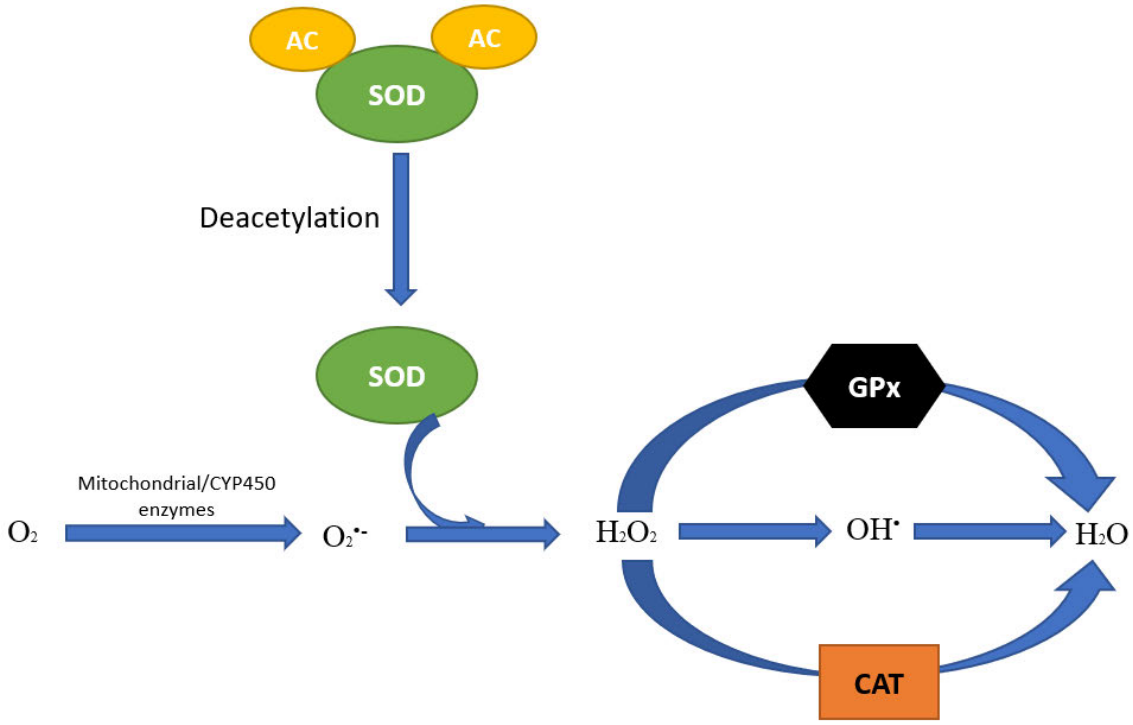

Figure 2.8. Schematic representation of the first line of defence against mitochondrial ROS production. SOD catalyses the reaction of $O_2^{\bullet-}$ to H_2O_2. Hydrogen peroxide may be further processed by CAT or GPx and converted to H_2O (Figure by author).

2.7. Intramitochondrial Antioxidant defence systems

Mitochondria have a built-in antioxidant system since they are highly vulnerable to redox damage. Antioxidants are phenol-containing substances that prevent substrate oxidation and counteract the negative effects of ROS (Noori 2012). Antioxidants act as reducing agents since they undergo self-oxidation to eliminate other radical intermediates, which prevents FR-producing oxidation processes (Lü, Lin et al. 2010). These antioxidants can be either enzymatic (SOD, GPx1) or non-enzymatic (GSH) (Noori 2012).

2.8. Metabolic Syndrome (MetS)

The co-existence of numerous established cardiovascular risk factors, such as insulin resistance, obesity, atherogenic dyslipidemia, elevated glucose, elevated blood pressure, pro-thrombotic state, pro-inflammatory state and hypertension, is referred to as the MetS (Huang 2009, Grundy 2016). Understanding the pathophysiology of MetS can help improve pharmacological, and preventive treatment approaches (Figure 2.9). Type 2 diabetes (T2D) and atherosclerotic cardiovascular disease

(CVD) are both risk factors for MetS (Grundy 2016). Oxidative stress is one of the key factors in MetS, as overproduction of ROS participate in the development of insulin resistance and CVD (Ando and Fujita 2009).

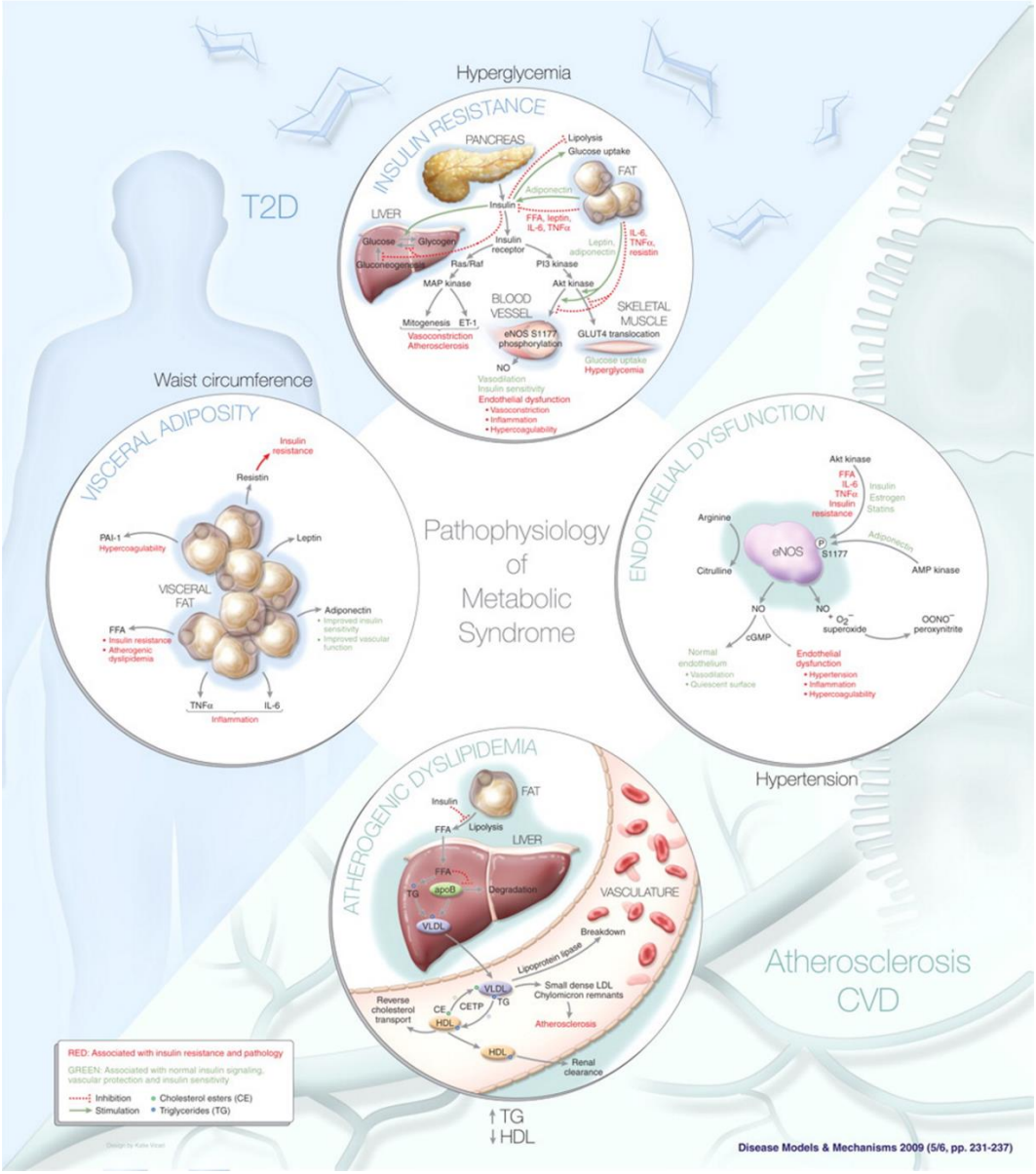

Figure 2.9. Pathophysiology of Metabolic Syndrome (Huang 2009).

2.8.1. Diabetes and antioxidant implications

In the diseased environment, diminished antioxidant capability is equally important to elevated ROS. In the context of diabetes, this goes beyond the idea that high levels of ROS passively deplete the antioxidant supply and leans toward having a more direct impact on antioxidants. Numerous investigations supported the hypothesis that the diabetes condition causes the decline in antioxidant levels of CAT, SOD, and GPx (Loven, Schedl et al. 1986, Wohaieb and Godin 1987, Sukalski, Pinto et

al. 1993). Two theoretically sound reasons for the negative effects of diabetes on antioxidant processes. The first theory centres on glycation, the non-enzymatic binding of glucose to proteins that alters their structure and functional properties. Szaleczky, Prechl et al. (1999) hypothesized that increased glycation in the high glucose state would lead to a decrease in antioxidant enzyme activity (Szaleczky, Prechl et al. 1999). The second theory proposes that the micronutrient environment is modified by diabetes. To function, antioxidant enzymes need specific cofactors and micronutrients. For example, CAT needs haem, GPx needs selenium, and superoxide dismutase uses copper, zinc, or manganese. It was proposed that changes in the micronutrient environment in the diabetic state impacted the cofactor/enzyme ratio, affecting the activation statuses of antioxidant enzymes (Szaleczky, Prechl et al. 1999). Therefore, diabetes causes an abnormal antioxidant profile and elevated ROS levels.

2.8.2. JNK pathway

The tripeptide's sequence serves as the basis for classifying kinases. Due to their functions in inflammatory and environmental stress responses, JNK and p38 are known as stress-activated protein kinases (SAPK) (Plattner and Bibb 2012). P38 has been associated with the regulation of proinflammatory cytokines such as IL-1, IL-6, IL-8, TNF-α, and cyclooxygenase-2 (COX-2) (Cuenda and Rousseau 2007). JNK is involved in a variety of processes, including gene expression, cell death, and survival pathways in addition to neuronal and immune functions (Zeke, Misheva et al. 2016). JNK is phosphorylated twice at Tyr and Thr by the protein kinases mitogen-activated protein kinase 4 (MKK4) and mitogen-activated protein kinase 7 (MKK7) (Weston and Davis 2002). JNK is in charge of making c-Jun active, which boosts the expression of activator protein-1 (AP-1). The transcription of cytokines, cell proliferation, and differentiation are all controlled by AP-1 (c-Jun & c-Fos) (Fisher and Voorhees 1998, Xie, Zhang et al. 2013).

2.9. MicroRNAs (miRNAs)

Biological processes such as cell proliferation and apoptosis, require small non-coding RNAs called miRNA for gene regulation (Ambros 2004). MiRNA are approximately 22 nucleotides in length and are generated from long primary miRNA transcripts. The main function of miRNA is to control gene expression at the post-transcriptional level through degrading or repressing target mRNAs (Chen 2005). It is estimated that 30% of all human genes expression are regulated by miRNAs (Lewis, Burge et al. 2005). MiRNAs play important roles in coordinating many cellular processes such as regulating apoptosis, proliferation, differentiation, development, and metabolism (Maziere and Enright 2007, Gusev 2008, Chuturgoon, Phulukdaree et al. 2014). MiRNAs play important regulatory roles in a variety of biological processes including metabolic processes (metabolic integration, insulin resistance and appetite regulation) (Heneghan, Miller et al. 2010). MiRNAs fall under the field of epigenetics, which refers to genomic modifications independent of changes to the actual gene sequence. Examples of epigenetic modifications include DNA methylation, chromatin and histone modification and miRNAs.

The full description of miRNA biogenesis and mode of action are described in Figure 2.10. Translational repression or mRNA degradation occur as a result of the establishment of the mRNA-miRNA complex. These molecules' ability to modulate gene expression by inhibiting protein translation has been described by research. This has given rise to notions that contend miRNA can serve as targets for therapeutic interventions as well as biomarkers for disease. Moreover, numerous clinical illnesses and biological processes, such as cancer (Jansson and Lund 2012), diabetes (McClelland and Kantharidis 2014), cardiovascular disease (Small, Frost et al. 2010), and most recently medication transport and metabolism (Peng and Zhong 2015), have been linked to microRNA.

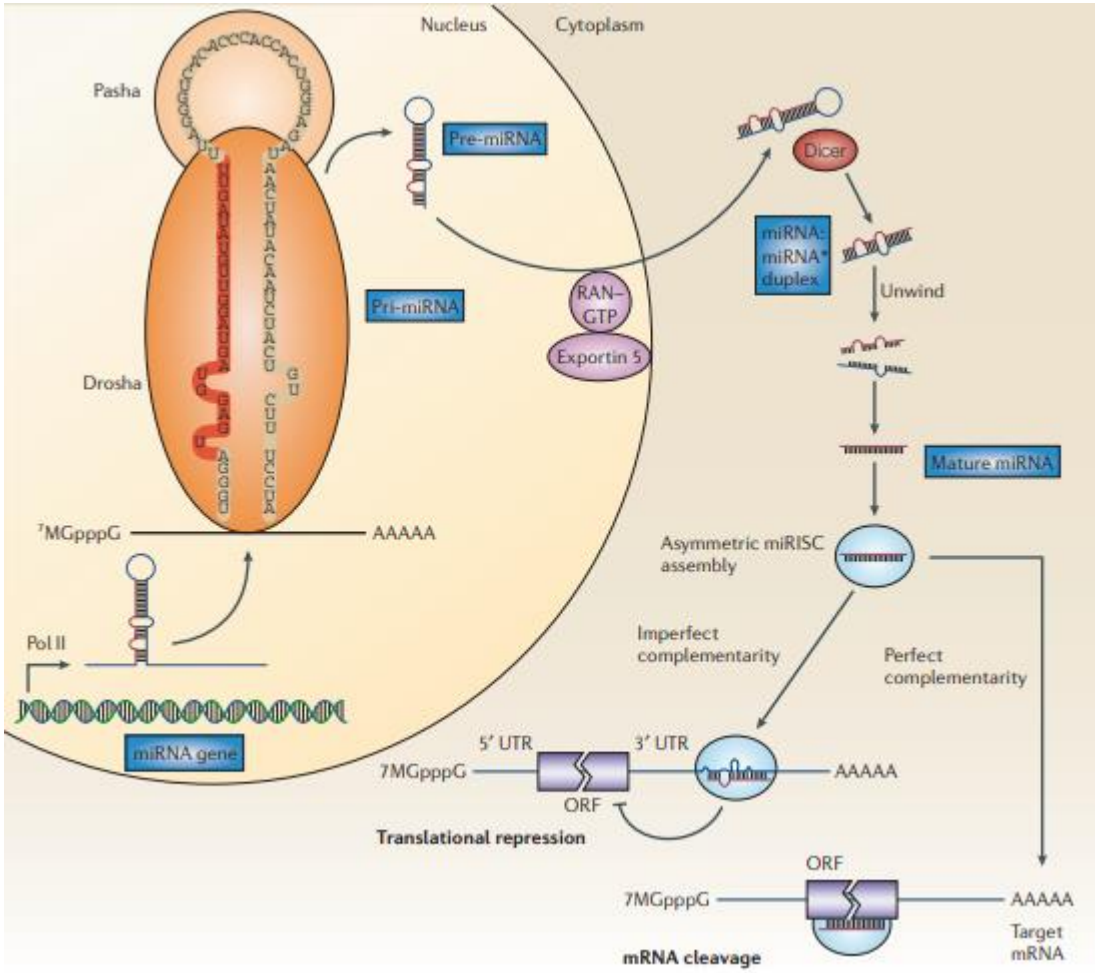

Figure 2.10. Mechanism of miRNA biogenesis and mode of RNA silencing. MiRNAs can be generated from either their own genes or the introns of protein-coding genes and are transcribed as independent units. Transcription of miRNA is carried out by RNA polymerase II (Pol II), producing large capped primary transcripts (Pri-miRNA). Primary transcripts are characterized by stem loops. The RNA III enzyme, Drosha, together with its co-factor (Pasha) cleave pri-miRNA at the stem of the loop, releasing 70-nucleotide long pre-miRNA products. The nuclear transporter, Exportin 5, and GTPase Ran facilitate the export of the pre-miRNA from the nucleus to the cytoplasm. In the cytoplasm, pre-miRNA is subject to further processing by Dicer, producing a

miRNA duplex. The duplex is loaded into an RNA-inducing silencing complex (RISC), consisting of multiple proteins, including Argonaute proteins. Mature miRNAs are unwound and remain tethered to Argonaute proteins within the complex. MicroRNA have more than one target gene. The mature miRNA binds the 3' untranslated region (UTR) of the target gene, under the guidance of Argonaute protein, by complementary base pairing. Perfect complementarity with a target gene results in degradation of the target mRNA. Imperfect complementarity causes translational repression (Esquela-Kerscher and Slack 2006)

2.9.1. MiRNA-146a and MiRNA-155

Due to the powerful up-regulation of particular microRNAs, such as miR-155 and miR-146a, by Toll-like receptor ligands, inflammatory cytokines, and particular antigens in numerous immune cell lineages, these microRNAs were initially associated with the inflammatory response. Additionally, it was discovered that the MetS's elevated expression of miR-155 and miR-146a contributes to inflammation-mediated glomerular endothelial damage (Huang, Liu et al. 2014). Due to the widespread oncogene features and stimulation of proliferation in human cancer, studies have shown that miRNA-155 may be a target for cancer treatment. The fact that hepatocellular carcinoma (HCC) proliferated more quickly when miRNA-155 expression was elevated, indicates the significant involvement of miR-155 in the disease pathogenesis (Gao, Ning et al. 2015).

There is evidence that miRNAs play a significant role in the inflammation process by controlling both innate and adaptive immunity (Ge, Brichard et al. 2014). MicroRNAs, which are metabolic disease biomarkers, and other signalling pathways connected to metabolism and epigenetics are dysregulated as a result of oxidative stress. Numerous miRNAs involved in the control of lipid and glucose metabolism as well as the endothelium are expressed differently in various organs under different conditions of oxidative stress, according to studies. Dysregulated microRNAs affect the expression and activity of molecules in antioxidative signalling pathways, as well as genes for numerous signalling pathways related to inflammation, insulin sensitivity, and lipid metabolism, either directly or indirectly, which accelerates the development of metabolic imbalance (Włodarski, Strycharz et al. 2020).

2.10. SP as a potential supplement

Remarkably, SP has anti-inflammatory (Izadi and Fazilati 2018), and powerful antioxidant properties (Miranda, Cintra et al. 1998, Hu and Liu 2001), making it a viable supplement in the reduction of oxidative stress brought on by HAART adverse reactions. According to studies, antioxidants may have therapeutic benefits that can help people with HIV/AIDS avoid developing comorbidities that are brought on by oxidative stress and HAART medication (Mondal, Pradhan et al. 2004). Increased oxidative stress and lipid peroxidation have been linked to HIV and HAART, which promotes ROS by activating NADPH oxidase (Wang, Liao et al. 2009, Yeligar, Guidot et al. 2015). One of the main

sources of ROS and FR in HIV-infected people taking HAART is NADPH oxidase, which SP can suppress (Wang, Liao et al. 2009, Yeligar, Guidot et al. 2015, Izadi and Fazilati 2018), resulting in reduced oxidative stress (Riss, Décordé et al. 2007). TDF and lopinavir produce both acute and long-term renal impairment (Boffito 2004, Patel, Patel et al. 2010, Fernandez-Fernandez, Montoya-Ferrer et al. 2011, Shafi, Choi et al. 2011, Cao, Han et al. 2013). Normalization of urinary and renal oxidative stress markers and inhibition of NADPH-dependent superoxide generation in renal mesangial cells are both achieved by phycocyanin from SP (Zheng, Inoguchi et al. 2013), ameliorating renal dysfunction. By inhibiting oxidative stress, SP has proven to be a viable treatment method for preventing diabetic nephropathy (Zheng, Inoguchi et al. 2013). These characteristics point to SP as a possible treatment for HAART-induced renal impairment. NRTIs inhibit mitochondrial DNA polymerase (Grigsby, Pham et al. 2010, Moss, Neary et al. 2014). Studies *in vitro* revealed that SP can repair DNA synthesis and improve cell nucleus enzyme performance (Ali and Saleh 2012), and enhance mitochondrial health (Kamble, Gaikar et al. 2013, Nawrocka, Kornicka et al. 2017, Jadaun, Yadav et al. 2018, Oriquat, Ali et al. 2019, Sun, Hou et al. 2019). SP can treat mitochondrial toxicity, which manifests as lactic acidosis and peripheral neuropathy, by supplying trace minerals for the production of antioxidant enzymes (Nasirian, Dadkhah et al. 2018) and also reduce chronic inflammation (Jensen, Attridge et al. 2015).

SP's phycocyanin has the ability to prevent liver microsomal lipid peroxidation. (Romay, Armesto et al. 1998, Romay, Ledon et al. 1998, Gonzalez, Rodriguez et al. 1999, Romay, Delgado et al. 2001, Remirez, Fernández et al. 2002, Remirez, Ledon et al. 2002, Romay, Gonzalez et al. 2003, Khan, Varadharaj et al. 2006, Patel, Mishra et al. 2006, Cherng, Cheng et al. 2007, Riss, Décordé et al. 2007, Manconia, Pendás et al. 2009, Shih, Cheng et al. 2009), hence, reducing toxic hepatitis. Diabetes mellitus and hyperlipidemia are brought on by PI treatment (Jivanka Mohan 2021, Mohan, Ghazi et al. 2021). HAART is also connected to myocardial infarction (Yeni 2006). Diabetes, cancer, vascular disease, and neurological disorders can all be treated with SP (McCarty 2007). SP may exert its neuroprotective activities through antioxidant and anti-inflammatory effects (Lima, Joventino et al. 2017). The list of advantages makes SP a recommended antioxidant for usage as a dietary supplement. SP also strengthens the immune system and guards against infectious diseases and cellular aging (Ali and Saleh 2012). SP's support of a robust immune system can aid in raising CD4 cell counts, reducing HIV viral loads, and delaying the development of AIDS. In terms of chemistry, SP is a good source of proteins, vitamins, and minerals (Ali and Saleh 2012), important nutrients for individuals on the HAART program. Finally, as SP has been demonstrated to reduce viral generation in PBMCs, it can support HAART in its goal of preventing HIV-1 replication.

The review paper was published, and the original paper is included in appendix (APPENDIX A). The Potential of Spirulina platensis to Ameliorate the Adverse Effects of Highly Active Antiretroviral Therapy (HAART).

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CHAPTER THREE

Spirulina platensis ameliorates oxidative stress associated with antiretroviral drugs in HepG2 cells

Thabani Sibiya[1], Terisha Ghazi[1], Jivanka Mohan[1], Savania Nagiah[1,2], Anil A. Chuturgoon[1], *

[1]Discipline of Medical Biochemistry and Chemical Pathology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Howard College Campus, Durban 4013, South Africa

[2]Department of Human Biology, Medical Programme, Faculty of Health Sciences, Nelson Mandela University Missionvale, Bethelsdorp, Port Elizabeth, South Africa, 6059

*Corresponding author: Professor Anil A Chuturgoon, Discipline of Medical Biochemistry and Chemical Pathology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Howard College Campus, Durban 4013, South Africa. Telephone: +27312604404. Email: chutur@ukzn.ac.za

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Spirulina platensis Ameliorates Oxidative Stress Associated with Antiretroviral Drugs in HepG2 Cells

Thabani Sibiya [1], Terisha Ghazi [1], Jivanka Mohan [1], Savania Nagiah [1,2] and Anil A. Chuturgoon [1,*]

[1] Discipline of Medical Biochemistry and Chemical Pathology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, Howard College Campus, University of KwaZulu-Natal, Durban 4013, South Africa

[2] Medical Programme, Department of Human Biology, Faculty of Health Sciences, Nelson Mandela University Missionvale, Port Elizabeth 6059, South Africa

* Correspondence: chutur@ukzn.ac.za

Abstract: Lately, *Spirulina platensis* (SP), as an antioxidant, has exhibited high potency in the treatment of oxidative stress, diabetes, immune disorder, inflammatory stress, and bacterial and viral-related diseases. This study investigated the possible protective role of *Spirulina platensis* against ARV-induced oxidative stress in HepG2 cells. Human liver (HepG2) cells were treated with ARVs ((Lamivudine (3TC): 1.51 µg/mL, tenofovir disoproxil fumarate (TDF): 0.3 µg/mL and Emtricitabine (FTC): 1.8 µg/mL)) for 96 h and thereafter treated with 1.5 µg/mL *Spirulina platensis* for 24 h. After the treatments, the gene and protein expressions of the antioxidant response pathway were determined using a quantitative polymerase chain reaction (qPCR) and Western blots. The results show that *Spirulina platensis* decreased the gene expressions of *Akt* ($p < 0.0001$) and *eNOS* ($\downarrow p < 0.0001$) while, on the contrary, it increased the transcript levels of *NRF-2* ($\uparrow p = 0.0021$), *Keap1* ($\uparrow p = 0.0002$), *CAT* ($\uparrow p < 0.0001$), and *NQO-1* ($\uparrow p = 0.1432$) in the HepG2 cells. Furthermore, the results show that *Spirulina platensis* also decreased the protein expressions of NRF-2 ($\downarrow p = 0.1226$) and pNRF-2 ($\downarrow p = 0.0203$). Interestingly, HAART-SP induced an NRF-2 pathway response through upregulating NRF-2 (except for FTC-SP) ($\uparrow p < 0.0001$), *CAT* ($\uparrow p < 0.0001$), and *NQO-1* (except for FTC-SP) ($\uparrow p < 0.0001$) mRNA expression. In addition, NRF-2 ($\uparrow p = 0.0085$) and pNRF-2 ($\uparrow p < 0.0001$) protein expression was upregulated in the HepG2 cells post-exposure to HAART-SP. The results, therefore, allude to the fact that *Spirulina platensis* has the potential to mitigate HAART-adverse drug reactions (HAART toxicity) through the activation of antioxidant response in HepG2 cells. We hereby recommend further studies on *Spirulina platensis* and HAART synergy.

Keywords: highly active antiretroviral therapy (HAART); *Spirulina platensis*; oxidative stress; reactive oxygen species; antioxidant; HAART toxicity

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1. Introduction

Due to high infection and fatality rates, the human immunodeficiency virus (HIV) remains a global health problem [1]. In 2021, around 8.2 million people in South Africa were living with HIV [2]. Prior to the year 2021, an estimated 1.5 million additional people became newly infected with HIV globally, with 680,000 AIDS-related deaths [1–4]. The availability of antiretrovirals (ARVs) has helped those living with HIV/AIDS live longer. In 2020, around 27.5 million HIV-infected people worldwide had access to ARVs, while roughly 5.6 million HIV-infected South Africans had access [1,2,5].

Highly active antiretroviral therapy (HAART) combines many types of antiretroviral drugs to lower the viral load of an HIV-positive person [6–8]. HAART has several negative side effects that lead to metabolic syndrome, which includes oxidative stress and inflammation, despite its success in extending the lives of HIV-infected patients. The most popular nucleoside/nucleotide reverse-transcriptase inhibitors (NRTIs) are 2′,3′-dideoxy-5fluoro-3′-thiacytidine (FTC), (-)-L-2′,3′-dideoxy-3′-thiacytidine (3TC), and tenofovir disoproxil

fumarate (TDF), which form part of the first-line therapy [9–13]. Moreover, 3TC, FTC, and TDF are recommended for pre-exposure prophylaxis (PrEP) by the World Health Organization (WHO) [14–19]. Additionally, 3TC is part of post- exposure prophylaxis (PEP) [20]. It is imperative to explore solutions to the negative side effects associated with these ARV drugs. 3TC, an important component of HAART, which can also be used to prevent HIV infection after potential exposure, has been linked to increased ROS, endothelial cell injury, and cardiovascular complications in HIV-infected individuals [20]. Another HAART backbone component, TDF, is a prodrug of tenofovir that is used to treat HIV infection and is associated with mitochondrial alterations and oxidative stress [21]. FTC, an analogue of 3TC, is used to inhibit the replication of HIV-1 and HIV-2. However, FTC is linked to the development of lipodystrophy syndrome, which is a result of low mitochondrial toxicity [22]. Supplementation with an antioxidant agent such as *Spirulina platensis*, which has been shown to protect against oxidative stress [23–25], can help to minimize the oxidative stress during HAART treatment.

Spirulina platensis is a blue-green microalga that Mexican and African people eat as a traditional cuisine [26,27]. *Spirulina platensis* can be found in volcanic lakes' alkaline water [23–25]. *Spirulina platensis* has also been shown to reduce oxidative stress [23,26,28,29] and increase mitochondrial health [30–33]. *Spirulina platensis* has been discovered to have a variety of therapeutic characteristics in addition to its well-known nutritional value. The health benefits found in *Spirulina platensis* include the inhibition of viruses, such as HIV-1 [34,35], and the inhibition of metabolic diseases [23,28]. *Spirulina* has also been credited with cancer- and viral infection-suppressing abilities [36]. When ingested, it can strengthen the immune system's humoral and cellular systems [27]. *Spirulina platensis* has been associated with hypoglycaemia [37], hypolipidemic [38], and antihypertensive [39] effects. Furthermore, antioxidant complexes such as phycocyanin and phycocyanobilin are found in *Spirulina platensis* [23–25]. Moreover, it also contains high amounts of carotenoids and phenolic compounds [40]. Studies have identified that the bioactive compounds found in *Spirulina platensis* include carotenoids, phenols, chlorophylls, phycocyanin, polyunsaturated fatty acids (PUFAs), glycosides, flavonoids, and alkaloids [41–45]. *Spirulina platensis* can also help to prevent the onset of atherosclerosis [28] and diabetes [23]. In view of the above information, the aim of this study was to investigate the possible protective role of *Spirulina platensis* against HAART-induced oxidative stress in HepG2 cells.

2. Results

2.1. Akt

The previous studies showed Akt expression increased rapidly in both neurons and vascular cells following cellular stress or damage [46]. *Spirulina platensis* (SP) (24 h) and HAART (excluding 3TC) (96 h)-treated HepG2 cells expressed lower *Akt* mRNA expression B: ($p < 0.0001$), B: ($p < 0.0001$), significantly for FTC. However, as compared to the control, the HAART-SP treated cells showed a substantial reduction in *Akt* mRNA expression B: ($p < 0.0001$) (Figure 1).

2.2. Endothelial Nitric Oxide Synthase (eNOS)

ENOS plays a role in primary hepatocytes to induce activation of the stress-responsive transcription factor, nuclear factor-erythroid 2-related factor 2 (NRF-2), under metabolic syndrome-promoting conditions [47]. The upregulation of eNOS is mostly promoted under stress conditions, such as oxidative stress and metabolic syndrome. It is noteworthy that favourable cellular conditions may not necessarily upregulate eNOS for survival or stress-related struggles. In SP- and HAART-SP-treated cells, the expression of *eNOS* mRNA was considerably reduced: A: ($p < 0.0001$), B: ($p < 0.0001$) (Figure 2). The HAART-treated cells, on the other hand, showed a significant increase in *eNOS* mRNA expression B ($p < 0.0001$) (Figure 2).

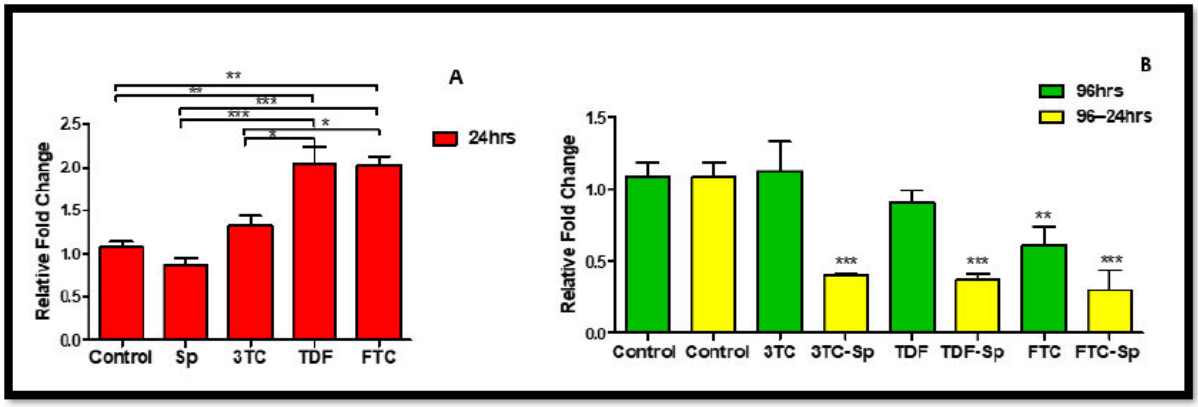

Figure 1. Effects of SP and HAART (3TC, TDF, and FTC) on *Akt* mRNA levels. *Akt* mRNA levels after exposure of HepG2 cells to (**A**): SP and HAART for 24 h, (**B**): HAART (96 h), and HAART (96 h) followed by SP (24 h); * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

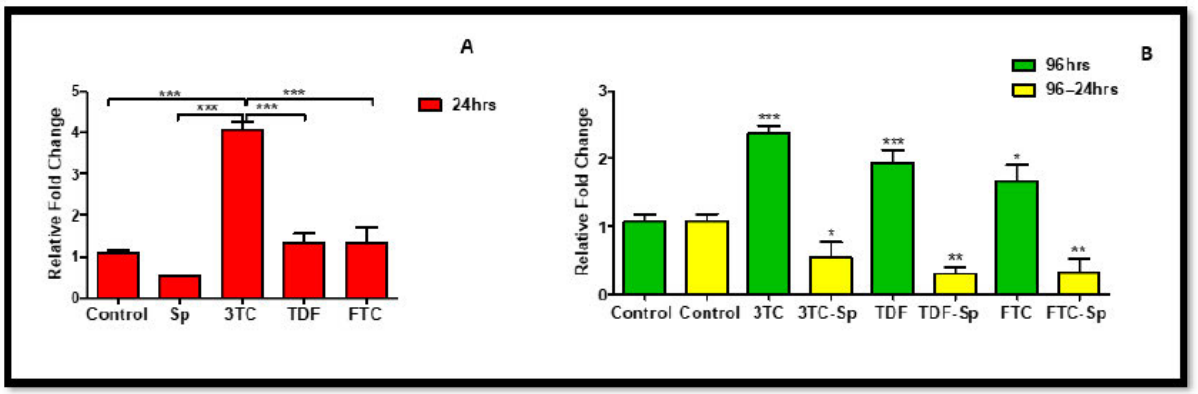

Figure 2. Effects of SP and HAART (3TC, TDF, and FTC) on *eNOS* mRNA levels. *eNOS* mRNA levels after exposure of HepG2 cells to (**A**): SP and HAART for 24 h, (**B**): HAART (96 h), and HAART (96 h) followed by SP (24 h); * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

2.3. The Role of *Spirulina platensis* in the Regulation of eNOS

The endothelial NO synthase (eNOS) is activated by hydrogen peroxide calcium- and Akt-dependent pathways. It has been demonstrated that eNOS activation by hydrogen peroxide is accompanied by Akt activation and increased eNOS phosphorylation [48]. Moreover, under enhanced oxidative stress conditions, endothelial cells employ this mechanism to maintain NO bioactivity [48]. The NADPH oxidase (NOX) family of enzymes is the major source of reactive oxygen species (ROS) involved in both metabolic syndrome and cardiovascular pathophysiology [49,50]. Oxidative stress, which is defined as a disturbance in the balance between the ROS production and the antioxidant defence, shifted towards excess ROS, a state characterized by elevated ROS levels. ROS involves two main categories: (a) free radicals such as superoxide (O_2^-), hydroxyl (OH·) and nitric oxide (NO·); and (b) non-radical derivatives of O_2, such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) [51]. Oxidative stress is involved in metabolic syndrome and numerous cardiovascular diseases [51]. Normally, oxidative stress causes damage to proteins, lipids, and DNA, resulting in cellular dysfunction [52]. Akt expression is known to be upregulated rapidly in both neurons and vascular cells following cellular dysfunction [46]. Therefore, Akt does not need to be upregulated in a cellular non-threatening environment. Interest-

ingly, the phycocyanin from *Spirulina platensis* is responsible for reducing oxidative stress and NADPH oxidase [28]. The inhibition of NADPH oxidase can also inhibit the increase of NO production through the suppression of inducible NO synthase (iNOS) [53]. However, it has been demonstrated that *Spirulina platensis* promotes the activation and expression of endothelial nitric oxide synthase (eNOS) protein under cellular dysfunction, such as atherosclerotic vascular disease [54]. Herein, *Spirulina platensis* downregulated the mRNA expression of eNOS; this might be due to the fact that *Spirulina platensis* promotes a healthy environment by reducing oxidative stress, which may result in the downregulation of genes that are mostly initiated by cellular dysfunction, such as *Akt* and *eNOS* (Figure 3).

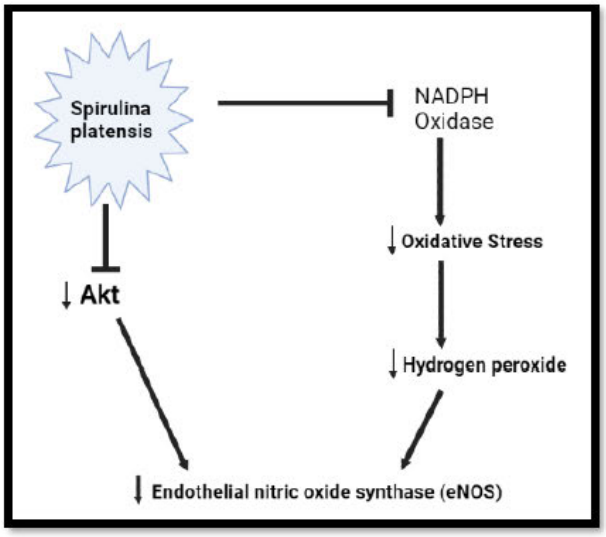

Figure 3. Diagrammatic representation of *Spirulina platensis* reduction of oxidative stress via inhibition of NADPH oxidase, leading up to *eNOS* mRNA downregulation. *Spirulina platensis* inhibits NADPH oxidase, reduces ROS, and blocks free radical species including H_2O_2, leading to the reduction of oxidative stress and reduced demand for eNOS (created with BioRender.com).

2.4. Antioxidant Response

NRF-2 mRNA levels rose in SP- and HAART-SP-treated cells (A: $p = 0.0021$ and B: $p < 0.0001$, respectively) (Figure 4). *NRF-2* mRNA expression, on the other hand, was reduced in HAART-treated cells after prolonged exposure B: ($p < 0.0001$) (Figure 4).

2.4.1. Keap1 Response

Keap1 controls the stability and cellular distribution of NRF-2. SP-and HAART-SP (excluding TDF-SP)-treated cells had higher levels of *Keap1* mRNA expression A: ($p = 0.0002$), B: ($p < 0.0001$). The HAART-treated cells, on the other hand, showed a substantial reduction in Keap1 mRNA expression after prolonged exposure B: ($p < 0.0001$) (Figure 5).

2.4.2. NQO-1 Response

The oxidative stress response caused NQO-1 to be elevated. *NQO-1* mRNA expression increased in SP-and HAART-SP (excluding FTC-SP)-treated cells A: ($p = 0.1432$), B: ($p < 0.0001$) (Figure 5), but not in HAART-treated cells (Figure 6).

2.5. Detoxification of Peroxides

The redox equilibrium is maintained by catalase (CAT). *CAT* mRNA expression rose considerably in SP-and HAART-SP-treated cells, although more significantly in HAART-treated cells A: ($p < 0.0001$), B: ($p < 0.0001$) (See Figure 7).

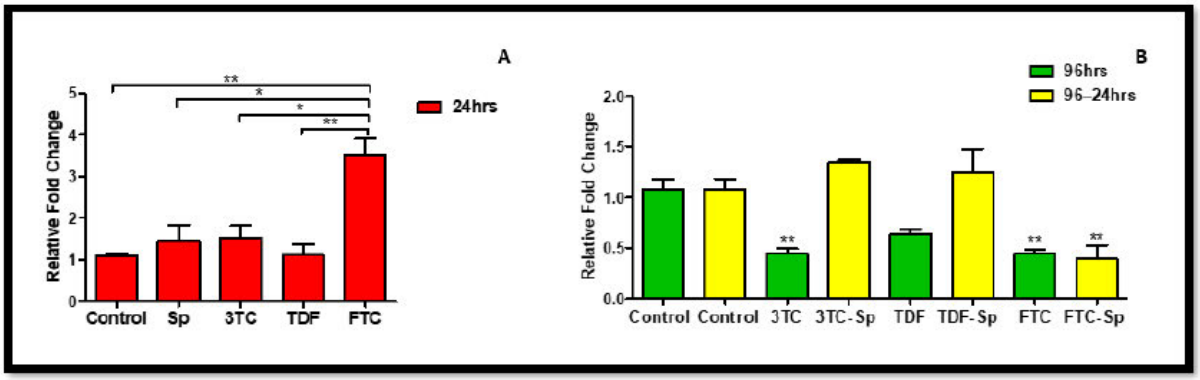

Figure 4. Effects of SP and HAART (3TC, TDF, and FTC) on *NRF-2* mRNA levels. *NRF-2* mRNA levels after exposure of HepG2 cells to (**A**): SP and HAART for 24 h, (**B**): HAART (96 h), and HAART (96 h) followed by SP (24 h); * $p < 0.05$, ** $p < 0.005$.

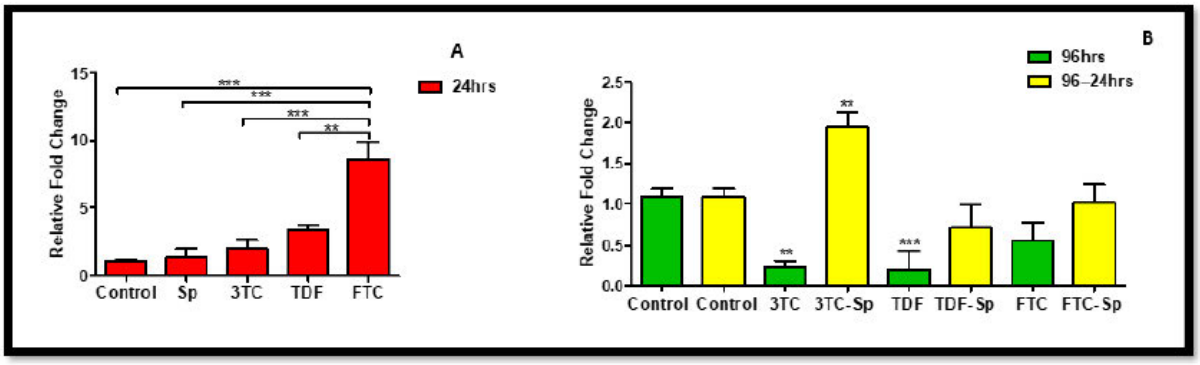

Figure 5. Effects of SP and HAART (3TC, TDF, and FTC) on *Keap1* mRNA levels. *Keap1* mRNA levels after exposure of HepG2 cells to (**A**): SP and HAART for 24 h, (**B**): HAART (96 h), and HAART (96 h) followed by SP (24 h); ** $p < 0.005$, *** $p < 0.0001$.

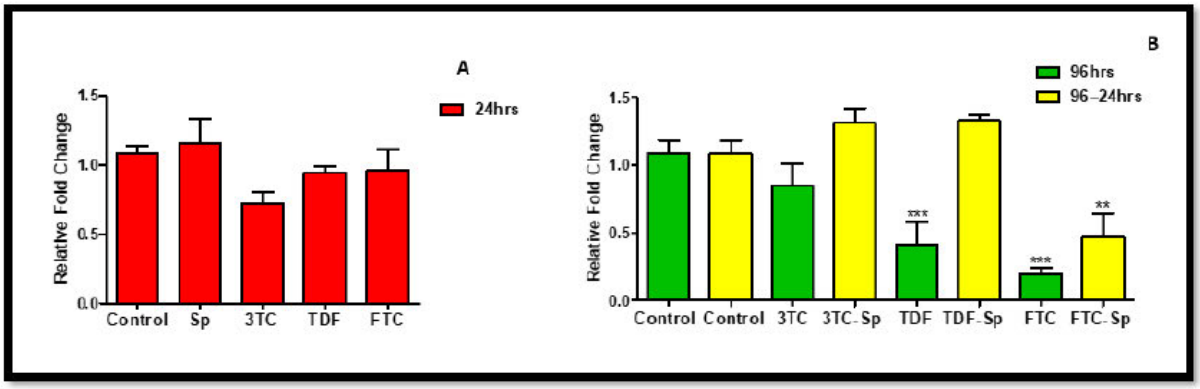

Figure 6. Effects of SP and HAART (3TC, TDF, and FTC) on *NQO-1* mRNA levels. *NQO-1* mRNA levels after exposure of HepG2 cells to (**A**): SP and HAART for 24 h, (**B**): HAART (96 h), and HAART (96 h) followed by SP (24 h); ** $p < 0.005$, *** $p < 0.0001$.

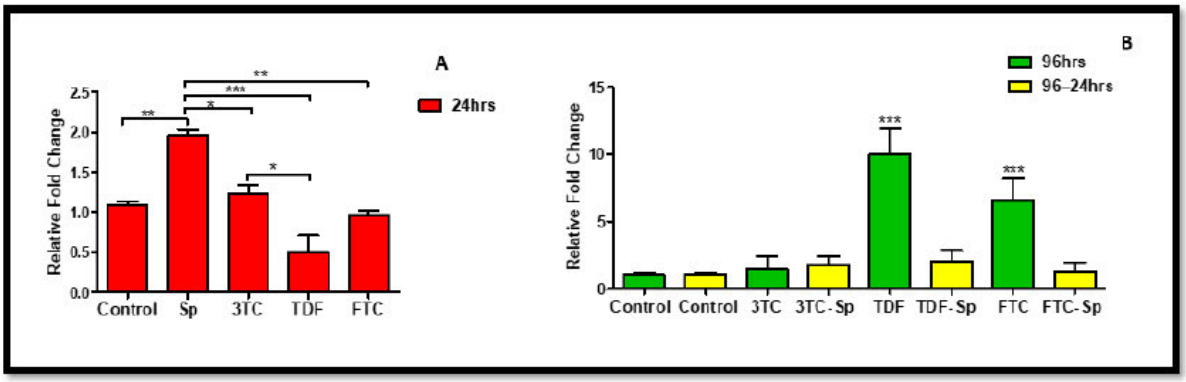

Figure 7. Effects of SP and HAART (3TC, TDF, and FTC) on *CAT* mRNA levels. *CAT* mRNA levels after exposure of HepG2 cells to (**A**): SP and HAART for 24 h, (**B**): HAART (96 h), and HAART (96 h) followed by SP (24 h); * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

NRF-2 Protein Response

NRF-2 is a master regulator of the oxidative stress transcriptional response. The expression of the pNRF-2 protein was reduced in the SP-treated cells A: ($p = 0.0203$) (Figure 8). However, pNRF-2 protein expression was increased in the HAART- and HAART-SP-treated cells B: ($p < 0.0001$) (Figure 8). The NRF-2 protein expression was shown to be lower in SP-treated cells C: ($p = 0.1226$) after acute exposure, higher in HAART-treated cells D: ($p = 0.0085$), and considerably higher in HAART-SP-treated cells, following prolonged exposure D: ($p = 0.0085$) (Figure 8).

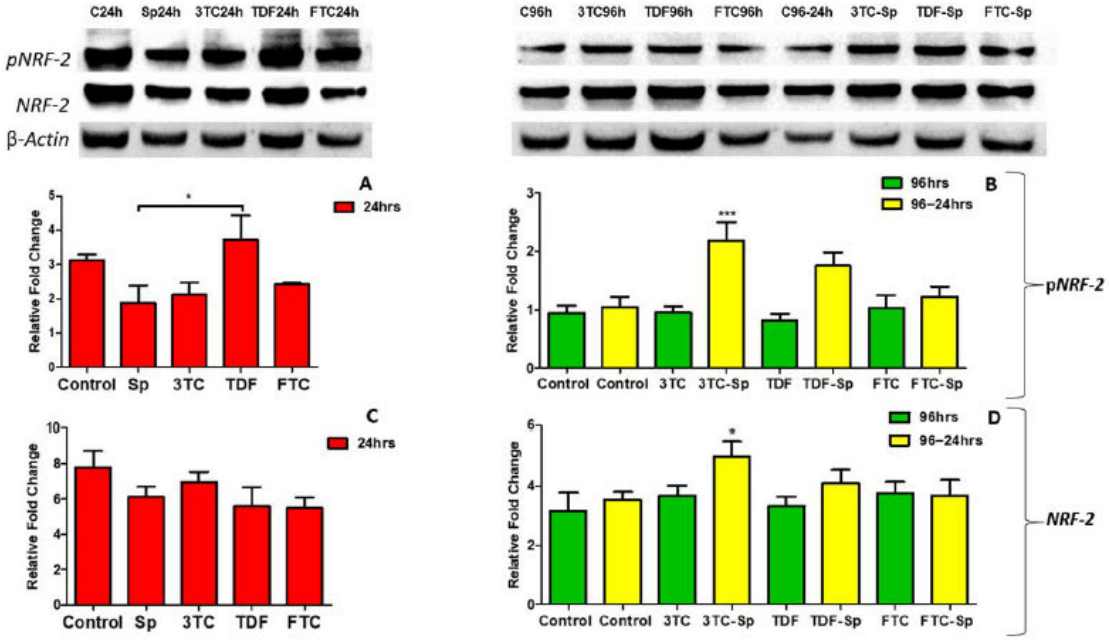

Figure 8. Effects of SP and HAART (3TC, TDF, and FTC) on pNRF-2 and NRF-2 protein expression. pNRF-2 protein expression after exposure of HepG2 cells to (**A**): SP and HAART for 24 h, (**B**): HAART (96 h), and HAART (96 h) followed by SP (24 h); NRF-2 protein expression after exposure of HepG2 cells to (**C**): SP and HAART for 24 h, (**D**): HAART (96 h), and HAART (96 h) followed by SP (24 h); * $p < 0.05$, *** $p < 0.0001$.

3. Discussion

Several types of HAART drugs are commonly used in combination against HIV and these backbone drugs (3TC, TDF, and FTC) have been implicated in oxidative stress and mitochondrial toxicity [55–58]. Oxidative stress and mitochondrial toxicity are characteristic pathologies of metabolic syndrome. Metabolic syndrome effects may be ameliorated by antioxidants such as *Spirulina platensis*. *Spirulina platensis* has been reported to inhibit oxidative stress [23,26,28,29], and is highly recommended to promote mitochondrial health [30–33]. We investigated the effect of *Spirulina platensis* on the oxidative pathway after exposure of HepG2 cells to the HAART backbone (3TC, TDF, and FTC). *Spirulina platensis* exerts direct antioxidant activity and modulation of the antioxidant response element (ARE)/NRF-2 pathway in HepG2 cells [59].

In the early in vitro toxicity evaluation for pharmaceutical development, HepG2 cells are good models for analysis [60]. The liver is at significant risk of oxidative insults since the hepatocytes are rich in mitochondria and are the oxidative centre for several metabolic reactions [61]. Akt and eNOS can regulate NRF-2 activation independently and sequentially, breaking the Keap1/NRF-2 complex [62]. Akt is responsible for the activation of eNOS which takes part in the dissociation of the keap1/NRF-2 complex. The phosphorylation of NRF-2 triggers its disassociation from Keap1 ubiquitination, allowing it to translocate to the nucleus and promote the transcription of antioxidants [63]. The pNRF-2 translocates to the nucleus. In the nucleus, the pNRF-2 forms a complex with an antioxidant response element (ARE) and activates the antioxidant genes such as catalase (CAT) and NQO-1 [64].

Spirulina platensis is rich in antioxidant properties [24,27], and also contains phycocyanin, responsible for reducing oxidative stress and NADPH oxidase [28]. The antioxidant response properties of *Spirulina platensis* are evident by the significantly increased expression of *CAT* mRNA (Figure 7A) and the elevated expression of NRF-2 (Figure 4A) and NQO-1 (Figure 6A) mRNAs. In the presence of *Spirulina platensis*, the expression of *Akt* and *eNOS* (Figures 1A and 2A) was reduced. The reduction of Akt may be an indication that *Spirulina platensis* does not induce cellular damage, since increased expression of Akt commonly occurs following cellular stress or damage [46]. The reduction in *eNOS* expression agrees with the reduction in *Akt* expression, as Akt is responsible for its activation. These results suggest that *Spirulina platensis* does not induce cellular stress that might require eNOS to activate the stress-responsive transcription factor NRF-2 in the hepatocytes [47]. Hence, the increased expression of *Keap1* (Figure 5A) may have prevented NRF-2 phosphorylation, as seen by the reduction in the pNRF-2 expression (Figure 8A) in the presence of only *Spirulina platensis*. Herein, the Keap1/NRF-2 complex was not dissociated by Akt or eNOS (Figure 9A). However, Strasky et al. (2013) demonstrated that *Spirulina platensis* promotes the activation and expression of eNOS protein under cellular dysfunction such as atherosclerotic vascular disease [54]. Moreover, Strasky et al. (2013) investigated protein expression, while in this present study we investigated mRNA expression.

Interestingly, *Spirulina platensis* also contains vitamin D3, vitamin C, and magnesium, whose components have been suspected to stimulate NO production [65]. Moreover, this makes sense, since *Spirulina platensis* also contains total essential amino acids, with an abundance of arginine [66–68]. Nitric Oxide can modify the cysteine residues on Keap1 to augment NRF-2 activity [62]. Total *NRF-2* was upregulated at the gene level (Figure 4A); hence, detoxification antioxidant genes such as CAT and NQO-1 were upregulated. Enhanced NRF-2/ARE activation results in decreased ROS production and an enhanced concentration of antioxidant glutathione (GSH) [62]. The decreased NRF-2 protein expression (Figure 8C) makes sense, since there was an increased *Keap1* mRNA expression (Figure 5A). NRF-2 transcriptional activity is low when Keap1 is upregulated [62]. *Spirulina platensis* managed to significantly increase *CAT* mRNA expression (Figure 7A). CAT plays a major role in the detoxification of ROS products [69,70]. Previous studies have shown that NRF-2 activation by *Spirulina platensis* results in the production and increased expression of antioxidant enzymes such as catalase (CAT) [71]. *Spirulina platensis* also increased the expression of the antioxidant gene, *NQO-1* (Figure 6A).

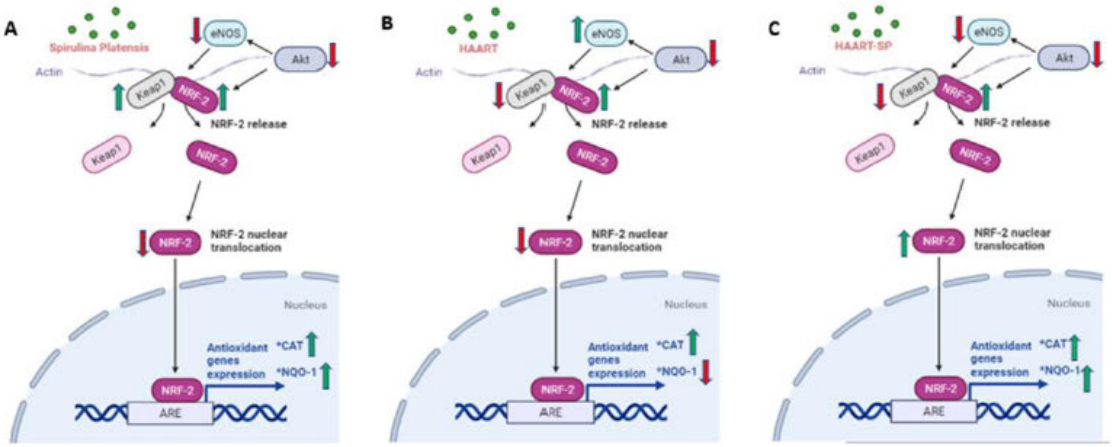

Figure 9. Effects of *Spirulina platensis* and HAART (3TC, TDF, and FTC) on antioxidant induction pathway in HepG2 cells; (**A**): the effect of *Spirulina platensis*; (**B**): the effect of HAART; (**C**): the effect of HAART-SP (created with BioRender.com).

In the presence of HAART, Akt mRNA (Figure 1B) expression varied, elevated in the presence of 3TC, and reduced in the presence of TDF and FTC. TDF is associated with the inhibition of Akt [72,73]. The presence of FTC and TDF is linked to an increased eNOS expression and a decreased Akt expression in adult rats treated with FTC-TDF-EFV [74]. In addition, this agrees with the significantly increased *eNOS* expression (Figure 2B) observed in this present study. ENOS increases NO, and breaks the Keap1/NRF-2 complex, phosphorylating NRF-2 [62]. This is evident by the elevated NRF-2 protein expression (Figure 8D) and reduced *Keap1* mRNA expression (Figure 5B). However, the *NRF-2* mRNA (Figure 4B) non-significantly decreased and the pNRF-2 expression varied, with only FTC inducing an increase in the pNRF-2 expression (Figure 8B). HAART exhausted the NRF-2 response after chronic exposure in HepG2 cells, which may explain the downregulation of pNRF-2 (Figure 8B), but the upregulation of NRF-2 at the protein level (Figure 8D) and not at the gene level (Figure 4B). Post-HAART, *CAT* was upregulated (Figure 7B), but *NQO-1* was downregulated (Figure 6B), which means HAART induced a limited antioxidant response in HepG2 cells. The upregulation of CAT makes sense, since HAART has been linked to the restoration and expression of CAT in HIV-seropositive individuals on HAART [75] (Figure 9B).

HAART-SP exposure reduced Akt and eNOS expression (Figures 1B and 2B); this is an indication that there is reduced or no cellular stress [47], which may be due to post-exposure to *Spirulina platensis*. NRF-2 is a master regulator of the transcriptional response to oxidative stress [76]. Post-treatment with *Spirulina platensis* after HAART upregulated total NRF-2 and pNRF-2 (Figures 4B, 8D and 8B, respectively), which is the key to the activation of detoxification antioxidant genes. Hence, *CAT* and *NQO-1* were also upregulated (Figures 6B and 7B). The increase in NRF-2 expression is a sign of antioxidant response and a good indication that the balance is restored, or in the process of restoration, reducing the oxidative stress. NRF-2, apart from being an antioxidant, plays a huge role in metabolic homeostasis, eliminating lipid accumulation and oxidative stress [77,78]. Eliminating oxidative stress may be the key to inhibiting hypertension and type 2 diabetes, as oxidative stress is suspected to be a major contributor to the development of metabolic syndromes [79]. Some studies have demonstrated that NRF-2 activation prevents ROS-induced damage in the pancreatic β cells [80,81], and this protective characteristic can inhibit diabetes mellitus. NRF-2 controls downstream genes, including NQO-1 antioxidant. NQO-1 reduction or knockout can result in an increase in insulin resistance in mice [82]. The presence of FTC-SP upregulated NRF-2 and pNRF-2 at the protein level, however, it

downregulated *NRF-2* at the gene level. This may be due to high FTC toxicity compared to the other ARVs; more exposure time to *Spirulina platensis* may be required to ameliorate FTC toxicity or to trigger an NRF-2 response. It is noteworthy that fluoride toxicity may add to FTC adverse drug reactions [83]. The FTC persisted with the same pattern as in the cells, where *Spirulina platensis* was not introduced. In both events, the FTC reduced *NRF-2* at the gene level and downregulated *NQO-1*. NQO-1 is a phase-II detoxifying enzyme that uses the pyridine nucleotides NADPH or NADH as an electron donor to reduce quinones to hydroquinones. Additionally, NQO-1 reduces ROS production via direct scavenging superoxide [62] (Figure 9C). The data above implies that the *Spirulina platensis* and HAART synergy may be via an NRF-2 pathway response.

4. Materials and Methods

4.1. Materials

Spirulina platensis, extracted from *Spirulina platensis* (Shewal) capsules, was obtained from HeriCure Healthcare Ltd. (Pune, India); A 10 mg/mL aqueous stock solution of the extract was prepared from the capsule content (the *Spirulina platensis* from the capsules was dissolved in distilled water (dH_2O)), and the solution was filtered (0.45 µm) and used to prepare the concentrations of *Spirulina platensis* extract required for the study). The extract was then incubated at −80 °C for 24 h and lyophilized for 48 h using the SP Vir Tis Scientific Freeze Dryer (Warminster, PA, USA) (−46 °C, 79 mT,). The final weight of the extracts was obtained, and the extracts were stored in the dark at 4 °C until further use. For heat-sensitive cyanobacteria, such as *spirulina*, freeze-drying is one of the best treatment options since it causes the least amount of changes to their nutritive, sensory, and physicochemical characteristics, leaving the lyophilized products similar to the fresh biomass [40]. The antiretroviral drug compounds were purchased from Pharmed Pharmaceuticals and extracted using dichloromethane, which was then removed using a standard laboratory rotary evaporator. The identity of the extracted compounds was confirmed using NMR analysis and showed a purity of >98%. The resultant antiretroviral drugs were obtained from the NIH AIDS Reagents Program. The HepG2 cell line was acquired from Highveld Biologicals (Johannesburg, South Africa). The cell culture reagents and supplements were purchased from Lonza BioWhittaker (Basel, Switzerland). Unless otherwise stated, all the other used reagents were purchased from Merck (Darmstadt, Germany).

4.2. Cell Culture

The HepG2 cells were cultured in a monolayer (10^6 cells per 25 cm^3 culture flask) with complete culture media [CCM: Eagle's Essential Minimal Media (EMEM) supplemented with 10% foetal calf serum, 1% penstrepfungizone and 1% L-glutamine] at 37 °C in a humidified incubator. The cells were allowed to reach 80% confluence in 25 cm^3 flasks before treatment with only antiretrovirals (ARVs), using the plasma peak values from the literature that represent the physiological concentrations of ARVs in humans (3TC: 6.6 µM (1.51 µg/mL), TDF: 0.3 µg/mL, and FTC: 1.8 µg/mL) [37–39] in CCM for 96 h [84]. For the 96 h of treatment, fresh cell culture medium containing ARVs treatment was replenished every 48 h. Thereafter, the ARVs were removed, and the cells were gently rinsed with 0.1 mol/L phosphate buffer saline (PBS) and treated with only 1.5 µg/mL *Spirulina platensis* (SP) in CCM for 24 h. An untreated control, containing only CCM, was also prepared. A treatment for a 24-h time period was also conducted, containing only ARVs [85] and SP separately.

4.3. Protein Expression

The protein expressions of nuclear factor-erythroid 2-related factor 2 (NRF-2) and phosphorylated NRF-2 (p NRF-2) were determined by Western blotting. Standardised protein samples were boiled in Laemmeli buffer [dH_2O, 0.5 mol/L Tris-HCl (pH 6.8), glycerol, 10% sodium dodecyl sulphide (SDS), and b-mercaptoethanol, 1% bromophenol blue] for

5 min. The proteins (25 µL) were separated by electrophoresis on SDS-polyacrylamide electrophoresis gels (4% stacking gel; 10% resolving gel) and electrotransferred to nitrocellulose membranes. The membranes were blocked with 5% BSA in Tween 20-Tris buffer saline (TTBS 150 mmol/L NaCl, 3 mmol/L KCl, 25 mmol/L Tris, 0.05% Tween 20, and dH_2O, pH 7.5) for 1 h and incubated with a primary antibody [pNRF-2 (ab76026); NRF-2 (ab31163)] in 5% BSA in TTBS (1:1000 dilution) overnight at 4 °C. Following the overnight incubation, the membranes were equilibrated to room temperature (RT) and washed with TTBS (5 times, 10 min). The membranes were subsequently probed with a horseradish peroxidase (HRP)-conjugated secondary antibody [Rabbit (7074S)] in 5% BSA in TTBS (1:5000) for 1 h at RT. Thereafter, the membranes were washed with TTBS (5 times, 10 min) and immunoreactivity was detected (Clarity Western ECL Substrate) with the Bio-Rad ChemiDoc gel documentation system. After detection, the membranes were quenched with 5% H_2O_2 for 30 min, then rinsed once in TTBS and incubated in a blocking solution (5% BSA for 1 h at RT), rinsed thrice in TTBS, and probed with HRP-conjugated anti-β-actin (housekeeping protein). The protein expression was analysed by the Image Lab Software version 5.0 (Bio-Rad) and the results were expressed as the relative band density (RBD). The expression of proteins of interest was normalized against β-Actin.

4.4. RNA Analysis

Total ribonucleic acid (RNA) was isolated according to the method described by Chuturgoon, Phulukdaree and Moodley [86]. Isolated RNA was quantified (Nanodrop 2000, Thermo Scientific, Waltham, MA, USA) and standardized to 1000 ng/µL. Complementary deoxyribonucleic acid (cDNA) was synthesized from the standardized RNA using the iScript cDNA synthesis kit (Bio-Rad). The thermocycler conditions for the cDNA synthesis were 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, and a final hold at 4 °C [85]. The gene expression was analysed using the SsoAdvanced Universal SYBR Green Supermix kit (Bio-Rad). The messenger RNA (mRNA) expressions of *NRF-2*, *Kelch-like ECH-associated protein 1 (Keap1)*, *catalase (CAT)*, *NADH quinone oxidoreductase 1 (NQO-1)*, *Endothelial nitric oxide synthase* (*eNOS)*, and *protein kinase B (Akt)* were investigated using specific forward and reverse primers (Table 1). The reaction volumes, which consisted of the following, were prepared: SYBR green (5 µL), forward primer (1 µL), reverse primer (1 µL), nuclease-free water (2 µL), and the cDNA template (1 µL). All the reactions were carried out in triplicate. The samples were amplified using a CFX96 TouchReal-Time PCR Detection System (Bio-Rad). The initial denaturation occurred at 95 °C (4 min). Thereafter, 37 cycles of denaturation (15 s, 95 °C), annealing (40 s; temperatures—Table 1), and extension (30 s, 72 °C) occurred. The method described by Livak and Schmittgen [87] was employed to determine the changes in relative mRNA expression, where $2^{-\Delta\Delta Ct}$ represents the fold change relative to the untreated control. The expression of the gene of interest was normalized against the housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was amplified simultaneously under the same conditions.

4.5. Statistical Analysis

All the experiments were conducted independently in triplicate. GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA) was used to perform all the statistical analyses. The one-way analysis of variance (ANOVA), followed by a Bonferroni test for multiple group comparison (data are presented as 95% CI), was used to determine statistical significance. All the results were represented as the mean ± standard deviation unless otherwise stated. A value of $p < 0.05$ was considered statistically significant.

Table 1. The annealing temperatures and primer sequences for the genes of interest.

Gene	Annealing Temperature	Primer	Sequence	Length	Accession Number	Product Length
Nrf-2	60 °C	Forward Reverse	5′-CACATCCAGTCAGAAACCAGTGG-3′ 5′-GGAATGTCTGCGCCAAAAGCTG-3′	2446	NM_006164.5	772 bp
Keap1	59.3 °C	Forward Reverse	5′-CTGGAGGATCATACCAAGCAGG-3′ 5′-GGATACCCTCAATGGACACCAC-3′	2524	NM_012289.4	1599 bp
CAT	53 °C	Forward Reverse	5′-GTGCGGAGATTCAACACTGCCA-3′ 5′-CGGCAATGTTCTCACACAGACG-3′	2291	NM_001752.4	1043 bp
NQO-1	59.7 °C	Forward Reverse	5′-GAAGAGCACTGATCGTACTGGC-3′ 5′-GGATACTGAAAGTTCGCAGGG-3′	2407	NM_001025434.2	1907 bp
eNOS	59.7 °C	Forward Reverse	5′-GTGGCTGTCTGCATGGACCT-3′ 5′-CCACGATGGTGATTTGGCT-3′	2058	NM_001160109.2	1788 bp
Akt	59.7 °C	Forward Reverse	5′-TGGACTACCTGCACTCGGAGAA-3′ 5′-GTGCCGCAAAAGGTCTTCATGG-3′	2922	NM_001382431.1	1468 bp
GAPDH	Same as the gene of interest	Forward Reverse	5′-TCCACCACCCTGTTGCTGTA-3′ 5′-ACCACAGTCCATGCCATCAC-3′	1285	NM_002046.7	231 bp

5. Conclusions

Spirulina platensis, as an antioxidant and anti-inflammatory agent, possesses various corrective properties against HAART adverse drug reactions. The corrective properties of *Spirulina platensis* shown in this study highlight its potential to mitigate HAART adverse drug reactions. This study investigated the antioxidant properties of the potent antioxidant, *Spirulina platensis*. In addition, the study also explored how *Spirulina platensis* supplementation may benefit HAART-dependent individuals. The results revealed that *Spirulina platensis* ameliorated HAART toxicity via the induction of an antioxidant response. In addition, the study showed a significant response to the positive synergistic HAART-SP effect theory. Individuals on HAART may benefit from *Spirulina platensis* supplementation. However, due to the fluoride content of FTC, a well-known mitochondrial toxicity-inducing compound, we recommend more studies on FTC toxicity. Furthermore, we suggest the need for further studies on the *Spirulina platensis* and HAART synergy.

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Abbreviations

acquired immunodeficiency syndrome	AIDS
antioxidant response element	ARE
antiretrovirals	ARVs
Catalase	CAT
Complementary deoxyribonucleic acid	cDNA
Complete culture media	CCM
distilled water	dH_2O

Eagle's Essential Minimal Media	EMEM
Emtricitabine/2′,3′-dideoxy-5fluoro-3′-thiacytidine	FTC
Endothelial nitric oxide synthase	eNOS
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
highly active antiretroviral therapy	HAART
human immunodeficiency virus	HIV
Human hepatoma/Human liver cells	HepG2
horseradish peroxidase	HRP
hydroxyl	OH·
hydrogen peroxide	H_2O_2
inducible NO synthase	iNOS
tenofovir disoproxil fumarate	TDF
Kelch-like ECH-associated protein 1	Keap1
Lamivudine/(-)-L-2′,3′-dideoxy-3′-thiacytidine	3TC
Messenger RNA	mRNA
nuclear factor erythroid 2-related factor 2	NRF-2
nucleoside/nucleotide reverse transcriptase inhibitors	NRTIs
NADH quinone oxidoreductase 1	NQO-1
NADPH oxidase	NOX
nitric oxide	NO·
Nuclear Magnetic Resonance	NMR
One-way analysis of variance	ANOVA
Phosphate buffered saline	PBS
phosphorylated NRF-2	pNRF-2
potential exposure prophylaxis	PEP
Pre-exposure prophylaxis	PrEP
protein kinase B	Akt
Quantitative polymerase chain reaction	qPCR
Reactive oxygen species	ROS
relative band density	RBD
ribonucleic acid	RNA
room temperature	RT
Spirulina platensis	SP
Sodium dodecyl sulphide	SDS
tenofovir disoproxil fumarate	TDF
World Health Organization	WHO

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CHAPTER FOUR

Spirulina Platensis mitigates the inhibition of selected miRNAs that promote inflammation in HAART treated HepG2 cells

Thabani Sibiya[1], Terisha Ghazi[1*], Jivanka Mohan[1], Savania Nagiah[1,2], Anil A. Chuturgoon[1*]

[1]Department of Medical Biochemistry and Chemical Pathology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Howard College Campus, Durban 4013, South Africa

[2]Department of Human Biology, Medical Programme, Faculty of Health Sciences, Nelson Mandela University Missionvale, Bethelsdorp, Port Elizabeth, South Africa, 6059

*Corresponding authors: Professor Anil A Chuturgoon and Dr Terisha Ghazi, Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Howard College Campus, Durban 4013, South Africa. Emails: chutur@ukzn.ac.za; terishaghazi@gmail.com

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Abstract

The introduction of highly active antiretroviral therapy (HAART) in the treatment of HIV/AIDS has recently gained popularity. Also, the significant role of microRNA expression in HIV pathogenesis cannot be overlooked, hence, the need to explore the mechanisms of microRNA expression in the presence of HAART and *Spirulina Platensis* (SP) in HepG2 cells. This study investigates the biochemical mechanisms of microRNA expression in HepG2 cells in the presence of HAART, SP, and the potential synergistic effect of HAART-SP. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to determine cell viability following SP treatment. Cellular redox status was assessed using the quantification of intracellular reactive oxygen species (ROS), lipid peroxidation, and lactate dehydrogenase (LDH) assay. The fluorometric, JC-1 assay was used to determine mitochondrial polarisation. Quantitative Polymerase Chain Reaction (qPCR) was also employed for micro-RNA and gene expressions. The result show that MiR-146a ($p < 0.0001$) and miR-155 ($p < 0.0001$) levels increased in SP treated cells. However, only miR-146a ($p < 0.0001$) in HAART-SP indicated an increase, while miR-155 ($p < 0.0001$) in HAART-SP treatment indicated a significant decrease expression. Further inflammation analysis revealed that Cox-1 mRNA expression was reduced in SP treated cells ($p = 0.4129$). However, Cox-1 expression is significantly increased in HAART-SP treated cells ($p < 0.0001$). The investigation reveal that HepG2 cells exposed to HAART-SP treatment showed a significant decrease Cox-2 ($p < 0.0001$) expression, mRNA expression also decreased in SP treated cells ($p < 0.0001$), therefore, *Spirulina Platensis* potentially controls inflammation by regulating microRNA expressions. Moreover, the positive synergistic effect is indicated by normalised intracellular ROS levels ($p < 0.0001$) in HAART-SP treatment. We hereby recommend further investigation on the synergistic roles of SP and HAART in the expression of microRNA with more focus on inflammatory and oxidative pathway.

Keywords: Highly active antiretroviral therapy (HAART); *Spirulina Platensis*; Oxidative stress; Antioxidant; Micro-RNA; Inflammation

Introduction

HAART is a combination of drugs used to combat human immunodeficiency virus (HIV) that continues to be a global public concern due to its alarming infection rate and mortality rate [66]. Following a recent report from Joint United Nations Programme on HIV/AIDS (UNAIDS) in November 2021, an estimated figure of about 37.7 million people globally are living with HIV. It was also reported that about 1.5 million new HIV-infected persons were recorded with about 680 000 deaths in 2020 [1-4]. South Africa has one of the highest infection rate, approximately 8.2 million South Africans are living with HIV in the year 2021 [4]. The above statistic could have been worse without the availability of antiretrovirals (ARVs) that have also helped in the lifespan elongation of the persons with HIV-AIDS and reducing the number of people infected with the virus. Globally, about 27.5 million HIV-infected persons had access

to ARVs in 2020 while approximately 5.6 million infected South Africans accessed ARVs in 2020 [3, 4, 67, 68].

HAART prolong HIV-infected patients' lifespan through regulation of the viral load and prevention of the associated symptoms from progressing to AIDS. Despite its success, the use of HAART promotes metabolic syndrome through inflammatory pathway, excess production of reactive oxygen species (ROS), and mitochondrial dysfunction [13-19]. There are antioxidant agents capable of ameliorating metabolic syndromes, cyanobacteria such as *Spirulina Platensis* (SP) have been well documented for this ability [24, 30].

Spirulina Platensis (SP) possesses various medicinal properties which include building the humoral and cellular mechanisms of the immune system when consumed [26]. Interestingly, SP is linked with metabolic syndrome lowering properties such as hypoglycemic [69], hypolipidemic [28], antihypertensive [29]. Studies in some rodent species suggested that SP is mainly useful in the prevention of metabolic syndromes [29]. SP contains bioactive substances such as carotenoids, phenols, chlorophylls, phycocyanin, polyunsaturated fatty acids (PUFAs), glycosides, flavonoids, and alkaloids, according to studies [70-74]. SP contains oxidative stress inhibitors, phycocyanin, and phycocyanobilin [24, 75, 76]. Phycocyanin is responsible for reducing oxidative stress via inhibition of NADPH oxidase and suppresses the activation of Inflammation [30]. SP also inhibits oxidative stress [24, 25, 30, 31], promotes mitochondrial health [32-35], and inhibits inflammation [23, 24]. Furthermore, it has been found to be useful in prevention of atherosclerosis [30], and diabetes development [24].

Biological processes such as cell proliferation and apoptosis, require small non-coding RNAs called microRNA (miRNA) for gene regulation [77]. MiRNA are approximately 22 nucleotides in length and are generated from long primary miRNA transcripts. The main function of miRNA is to control gene expression at the post-transcriptional level through degrading or repressing target mRNAs [78]. It is estimated that 30% of all human genes expression are regulated by miRNAs [79]. MiRNAs are important in the coordination of many cellular processes such as regulating apoptosis, proliferation, differentiation, development, and metabolism [80-82]. MiRNA play important regulatory roles in a variety of biological processes including metabolic processes (metabolic integration, insulin resistance and appetite regulation) [83]. There is evidence supporting the role of miRNAs as an important inflammatory mediator by regulating both the adaptive and innate immunity.[84].

Oxidative stress results in the dysregulation of signalling pathways associated with metabolism and epigenetics, including microRNAs, which are biomarkers of metabolic disorders. Studies have proven that different sources of oxidative stress change the expression of numerous microRNAs in organs involved in the regulation of glucose and lipid metabolism and endothelium. Dysregulated microRNAs either directly or indirectly affect the expression and activity of molecules of antioxidative signalling

pathways, as well as genes of numerous signalling pathways connected with inflammation, insulin sensitivity, and lipid metabolism, thus promoting the progression of metabolic imbalance.[85].

Specific miRNAs, such as miR-155 and miR-146a, were initially linked with the inflammatory response by virtue of their potent up-regulation in multiple immune cell lineages by Toll-like receptor ligands, inflammatory cytokines, and specific antigens. However, the increased expression of miR-155 and miR-146a in metabolic syndromes was found to contribute to inflammation-mediated glomerular endothelial injury [86]. Due to the alarmingly increasing number of HIV-infected people and their high dependence on HAART, this study investigates micro-RNA involvement in the inflammation pathway.

Materials and methods

Materials

Spirulina Platensis, extracted from *Spirulina Platensis* (Shewal) capsules, were obtained from HeriCure Healthcare Ltd (Pune, India); Antiretroviral drugs were obtained from the NIH AIDS reagents program: The antiretroviral drug compounds were purchased from Pharmed Pharmaceuticals and extracted using dichloromethane, which was then removed using a standard laboratory rotary evaporator. The identity of the extracted compounds was confirmed using NMR analysis and showed a purity of >98%. The HepG2 cell line was acquired from Highveld Biologicals (Johannesburg, South Africa). Cell culture reagents and supplements were purchased from Lonza Bio-Whittaker (Basel, Switzerland) while all other reagents were purchased from Merck (Darmstadt, Germany).

Cell culture

HepG2 cells were cultured in monolayer (10^6 cells per 25 cm^3 culture flask) with complete culture media [CCM: Eagle's Essential Minimal Media (EMEM) supplemented with 10% foetal calf serum, 1% penstrepfungizone and 1% L-glutamine] at 37 °C in a humidified incubator. Cells were allowed to reach 80% confluence in 25 cm^3 flasks before treatment with only antiretrovirals (ARVs) using the plasma peak values from literature that represent the physiological concentrations of ARVs in humans (3TC: 6.6 µM (1.51 µg/ml), TDF: 0.3 µg/ml, FTC: 1.8 µg/ml) [36-38] in CCM for 96 hours [88]. For the 96 hours treatment, fresh cell culture medium containing ARVs treatment was replenished every 48 hours. Thereafter ARVs were removed, and cells were gently rinsed with 0.1 mol/l phosphate buffer saline (PBS) and treated with only 1.5 µg/ml *Spirulina platensis* (SP) on its own in CCM for 24 hours. The 1.5 µg/ml SP concentration falls within the range that has been used in other studies [89], and MTT results supported this concentration. An untreated control, containing only CCM, was also prepared. Treatment for 24 hours' time period was also conducted, containing only ARVs [40] and SP separately.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

The MTT assay was used to determine the cell viability. Cells (20 000 cells/well) were seeded in triplicate for each treatment in a 96-well microtitre plate and allowed to attach over 24 hours period (37°C, 5% CO_2). Thereafter, the treatment medium (SP) was added to the relevant wells from 0-5 µg/ml. After 24 hours the treatment medium (SP) was removed and replaced with a solution containing 4 mg MTT salt, 800 µl PBS and 4ml CCM. The solution was incubated for 4 hours and replaced with DMSO for 1 hour. The absorbance was then read at 570 nm with a reference wavelength of 690 nm (BioTek µQuant spectrophotometer, USA). The absorbance values were used to calculate the cell viability [90]. The log concentration and cell viability were analysed using GraphPad Prism 5 and Microsoft excel.

Reactive oxygen species analyses

Intracellular ROS was quantified using the fluorometric 2′,7′-dichlorodihydrofluorescein-diacetate (H_2DCF-DA) assay. Control and treated cells (50,000 cells per treatment) were incubated in 500µl of 5µmol/l H_2DCF-DA stain (30 mins, 37 °C). Thereafter, the stain was removed through centrifugation (400xg, 10 mins, 24 °C) and cells were washed twice with 0.1 mol/l phosphate buffer saline (PBS). Cells were resuspended in 400 µl of 0.1 mol/l PBS and seeded in triplicate (100 µl/well) in a 96 well opaque microtiter plate. Fluorescence was measured with Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, CA) using a blue filter with an excitation wavelength (λex) of 488 nm and emission wavelength (λem) of 529 nm. Results were expressed as Relative Fluorescence Units (RFU).

Lactate dehydrogenase (LDH) activity

The LDH cytotoxicity detection kit (Roche, Mannheim, Germany) was used to measure cell death/damage. To measure LDH activity, supernatant (100 µl) was transferred into a 96-well microtitre plate in triplicate. Thereafter, substrate mixture (100 µl) containing catalyst (diaphorase/NAD^+) and dye solution (INT/sodium lactate) was added to the supernatant and allowed to react at RT for 25 mins. Optical density was measured at 500 nm (microplate reader – Bio-Tek µQuant). Results are presented as mean optical density.

Mitochondrial membrane potential

The mitochondrial membrane potential (Δψm) was measured by the JC-1 stain [91]. All samples, both control and treated cells (50,000 cells per treatment) were incubated in 200 µl of 5 µg/ml JC-1 stain (BD Biosciences, San Jose, NJ, USA) (20 mins, 37 °C). The stain was removed via centrifugation (400xg, 10 mins, 24 °C) and the cells were washed twice with JC-1 staining buffer. Cells were re-suspended in 400 µl of JC-1 staining buffer and seeded in an opaque 96-well plate in triplicate (100 µl/well). Fluorescence was quantified on a Modulus™ microplate reader (Turner Biosystems, Sunnyvale, CA). JC-1 monomers were measured with a blue filter (λex = 488 nm, λem = 529 nm) and JC-1 aggregates were measured

with a green filter (λex = 524 nm, λem = 594 nm). The Δψm of the HepG2 cells was expressed as the fluorescence intensity ratio of JC-1 aggregates and JC-1 monomers [91].

Lipid peroxidation assessment

The thiobarbituric acid reactive substances (TBARS) assay measured lipid peroxidation by-products malondialdehyde (MDA) and other TBARS as a measure of oxidative damage to lipids. TBARS assay was conducted as per the method described by Abdul, Nagiah [92]. Absorbance of the samples was read using a spectrophotometer, λ = 532/600 nm. Results were expressed as MDA concentration (μM).

RNA analysis

Total RNA was isolated according to the method described by Chuturgoon, Phulukdaree [41]. Isolated RNA was quantified (Nanodrop 2000, Thermo Scientific, Waltham, USA) and standardised to 1000 ng/μl. cDNA was synthesised from standardised RNA using the iScript cDNA synthesis kit (Bio-Rad). Thermocycler conditions for cDNA synthesis were 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min and a final hold at 4 °C. Gene expression was analysed using the SsoAdvanced™ Universal SYBR® Green Supermix kit (Bio-Rad). The mRNA expressions of Cox-1, Cox-2, Akt, and JNK were investigated using specific forward and reverse primers (Table 1). Reaction volumes which consisted of the following were prepared: SYBR green (5 μl), forward primer (1 μl), reverse primer (1 μl), nuclease free water (2 μl) and cDNA template (1 μl). All reactions were carried out in triplicate. The samples were amplified using a CFX96 Touch™Real- Time PCR Detection System (Bio-Rad). The initial denaturation occurred at 95 °C (4 min). Thereafter, 37 cycles of denaturation (15 s, 95 °C), annealing (40 s; temperatures— Table 1) and extension (30 s, 72 °C) occurred. The method described by Livak and Schmittgen [42] was employed to determine the changes in relative mRNA expression, where $2^{-\Delta\Delta Ct}$ represents the fold change relative to the untreated control. The expression of the gene of interest was normalised against the housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was amplified simultaneously under the same conditions.

Table 1 The annealing temperatures and primer sequences for the genes of interest

Gene	Annealing temperature	Primer	Sequence
Cox-1	50°C	Forward Reverse	5'-CGCCAGTGAATCCCTGTTGTT-3' 5'-AAGGTGGCATTGACAAACTCC-3'
Cox-2	53°C	Forward Reverse	5'-TAAGTGCGATTGTACCCGGAC-3' 5'-TTTGTAGCCATAGTCAGCATTGT-3'
JNK	59,7°C	Forward Reverse	5'-GACGCCTTATGTAGTGACTCGC-3' 5'-TCCTGGAAAGAGGATTTTGTGGC-3'

GAPDH		Forward Reverse	5′-TCCACCACCCTGTTGCTGTA-3′ 5′-ACCACAGTCCATGCCATCAC-3′

Micro-RNA analysis

The total RNA extracted (as previously described above) was reverse transcribed using the miScript ® II RT Kit (Qiagen, Hilden, Germany; catalogue number 218160) as per manufacturer's instructions. To quantify miRNA levels, *miR-155* (MS00031486) and *miR-146a* (MS00033740) miScript Primer Assays was used, while *RNU6* (MS00033740) was used as an internal control (Qiagen, Hilden, Germany). Experimental protocol was performed as per manufacturer's instructions. The reaction was carried out with an initial activation step (95ºC, 15 min), followed by 40 cycles of denaturation (94ºC, 15 sec), annealing (55ºC, 30 sec), extension (70ºC, 30 sec) and a plate read. Assays were conducted using CFX Touch™ Real Time PCR Detection System (Bio-Rad). The analysis of data was conducted using the method described by Livak and Schmittgen ($2^{-\Delta\Delta CT}$) [42].

Statistical analysis

GraphPad Prism version 5.0 (GraphPad Software Inc., California) was used to perform all statistical analyses. The one-way analysis of variance (ANOVA) followed by a Bonferroni test for multiple group comparison (data is presented as 95% CI) was used to determine statistical significance. All results were represented as the mean ± standard deviation unless otherwise stated. A value of $p<0.05$ was considered statistically significant.

Results

Cell viability

The MTT assay was used to determine cell viability and to confirm the suitable concentration for SP treatment. 1.5 µg/ml SP concentration is supported by range from other studies [89]. Figure 1A shows that cell viability mostly increased with increased SP concentrations. Figure 1B indicates that 1.5µg/ml is more beneficial to HepG2 cell viability. Moreover, an IC_{50} value was calculated using GraphPad Prism 5.0 and was determined to be 11.75 µg/ml for SP in HepG2 cells.

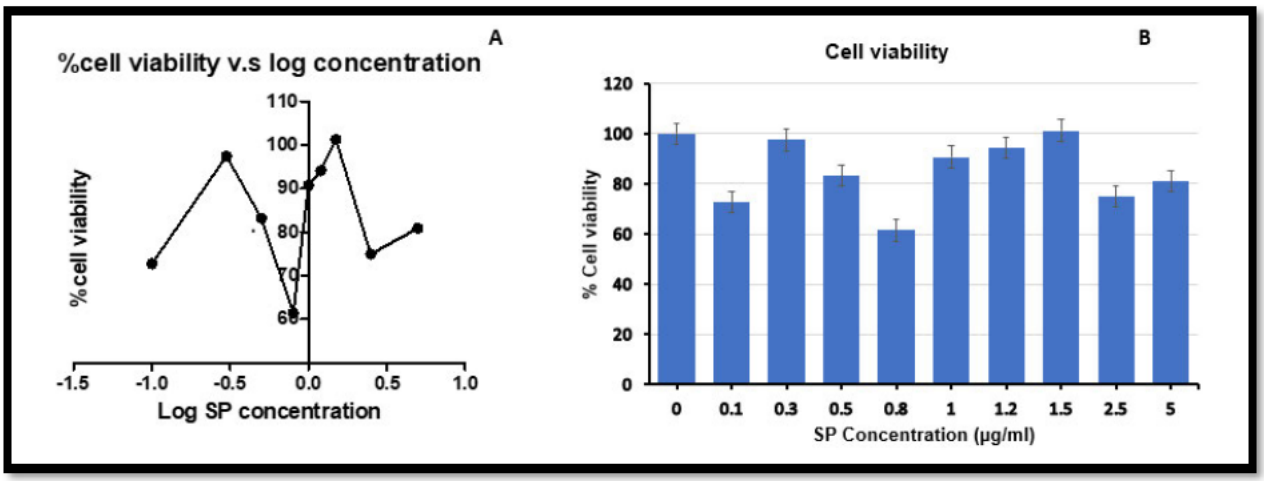

Figure 1. The effects of increased SP treatment concentration on the cell viability in HepG2 cells after 24 hours. A: Overall SP increased the cell viability above that of control cells; B: SP concentration of 1.5 µg/ml showed to be more favourable in maintaining cell viability.

MicroRNA Response

The main function of miRNA is to control gene expression at the post-transcriptional level through degrading or repressing target mRNAs. MiR-146a levels A: ($p < 0.0001$), B: ($p < 0.0001$) increased in SP and HAART treated cells except 3TC. HAART-SP also indicated an increase miR-146a level except FTC-SP B: ($p < 0.0001$). (Figure 2). The miR-155 levels increased in SP and HAART treated cells except 3TC and TDF A: ($p < 0.0001$), B: ($p < 0.0001$). HAART-SP treated cells indicated a significant decrease in miR-146a levels B: ($p < 0.0001$) (Figure 3).

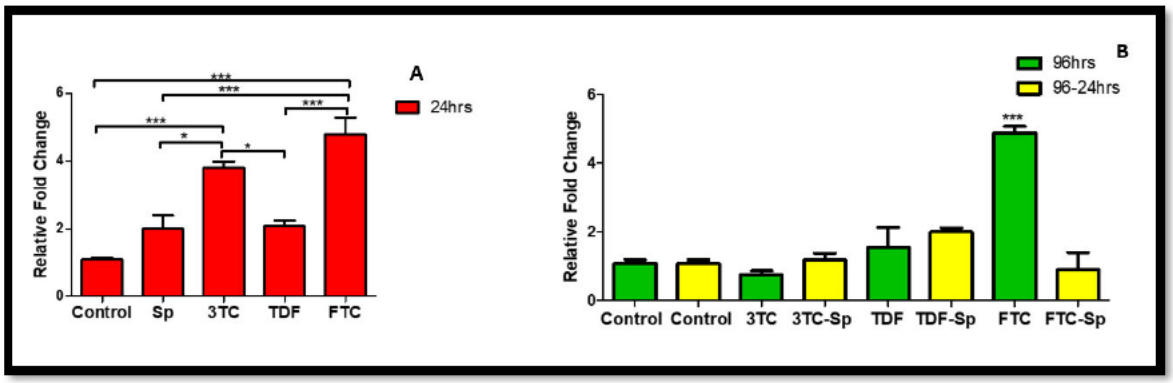

Figure 2. Effects of SP and HAART (3TC, TDF, and FTC) on MiR-146a levels. MiR-146a levels after exposure of HepG2 cells to A: SP and HAART for 24 hours, B: HAART (96 hrs), and HAART (96 hrs) followed by SP (24 hrs); *$p<0.05$, **$p<0.005$, ***$p<0.0001$

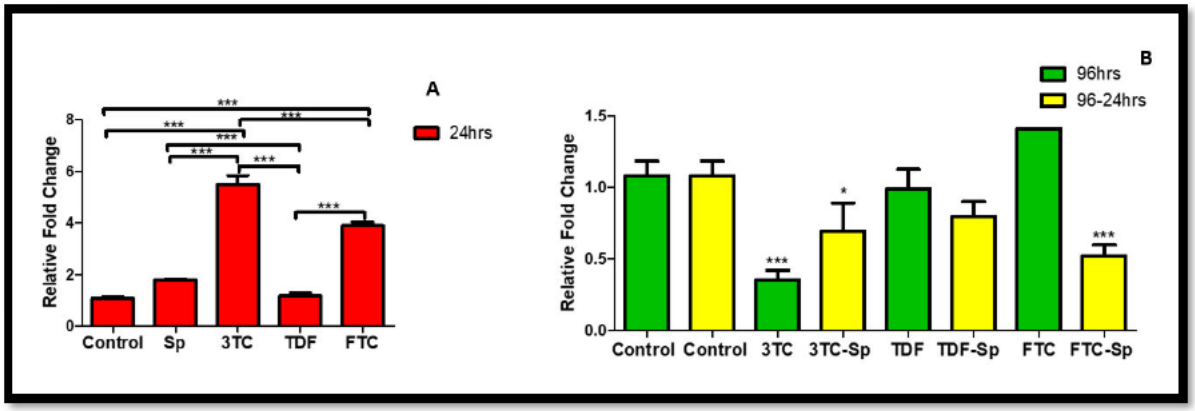

Figure 3. Effects of SP and HAART (3TC, TDF, and FTC) on the miR-155 levels. MiR-155 levels after exposure of HepG2 cells to A: SP and HAART for 24 hours, B: HAART (96 hrs), and HAART (96 hrs) followed by SP (24 hrs); *$p<0.05$, ***$p<0.0001$.

Cyclooxygenase (Cox) family Response

Cyclooxygenase-2 (Cox-2) is expressed by inflammatory cells, such as macrophages, and can be induced by TNF. Cox-2 is a central link to various inflammatory processes [93]. Cox-2 has been associated with inflammation, whereas the constitutively expressed Cyclooxygenase-1 (Cox-1) is generally considered as a housekeeping enzyme. However, recent evidence suggests that COX-1 can also be upregulated and may play a prominent role in the brain during neuroinflammation [94]. Cox-1 mRNA expression was reduced in SP treated cells and mostly decreased in HAART (except TDF) treated cells A: ($p = 0.0003$), B: ($p < 0.0001$). However, Cox-1 expression is significantly increased in HAART-SP treated cells B: ($p < 0.0001$) (Figure 4). Cox-2 mRNA expression is decreased in SP treated cells at 24 hrs exposure A: ($p < 0.0001$), and mostly reduced in HAART (except 3TC) treated cells after 24 hrs exposure B: ($p < 0.0001$) (Figure 5). However, cells exposed to HAART-SP treatment showed a significant decrease B: ($p < 0.0001$) (Figure 5).

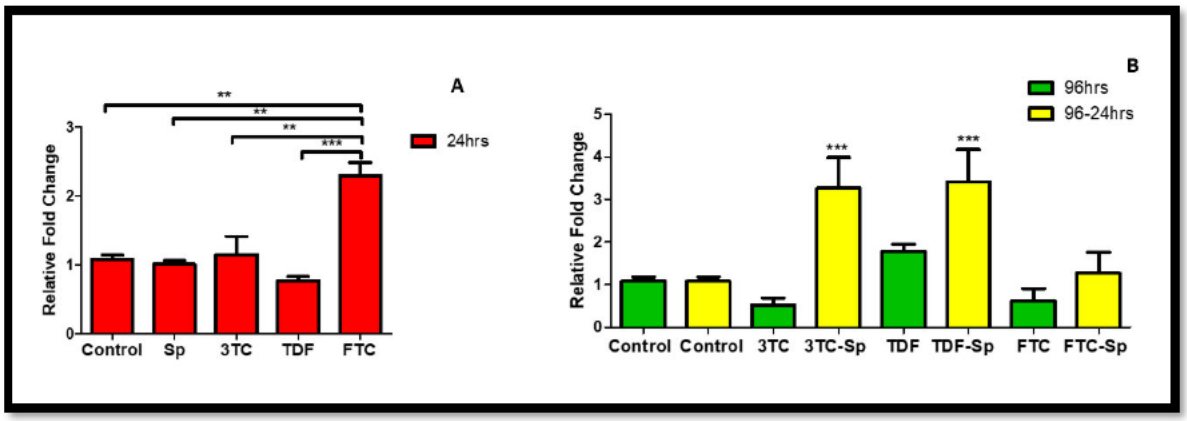

Figure 4. Effects of SP and HAART (3TC, TDF, and FTC) on Cox-1 mRNA expression. Cox-1 mRNA expression after exposure of HepG2 cells to A: SP and HAART for 24 hours, B: HAART (96 hrs), and HAART (96 hrs) followed by SP (24 hrs); **$p<0.005$, ***$p<0.0001$

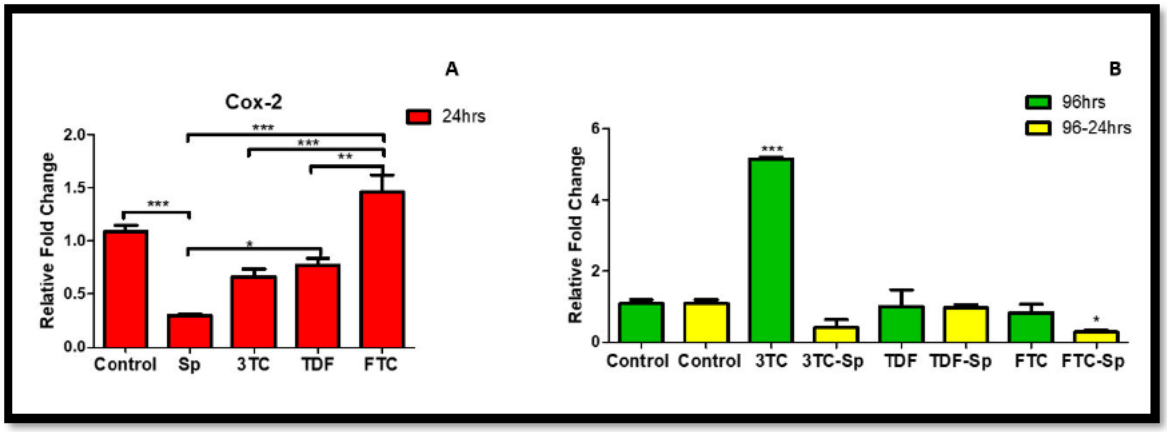

Figure 5. Effects of SP and HAART (3TC, TDF, and FTC) on Cox-2 mRNA expression. Cox-2 mRNA expression after exposure of HepG2 cells to A: SP and HAART for 24 hours, B: HAART (96 hrs), and HAART (96 hrs) followed by SP (24 hrs); *$p<0.05$, **$p<0.005$, ***$p<0.0001$

Jun N-terminal kinases (JNK)

Jun N-terminal kinases (JNK) belong to the superfamily of MAP kinases that are involved in the regulation of cell proliferation, differentiation, and apoptosis [95]. JNK mRNA expression decreased in SP and HAART treated cells A: ($p < 0.0001$), B: ($p < 0.0001$). HAART-SP treated cells showed a decrease in the expression of JNK mRNA except TDF-SP B: ($p < 0.0001$) (Figure 6).

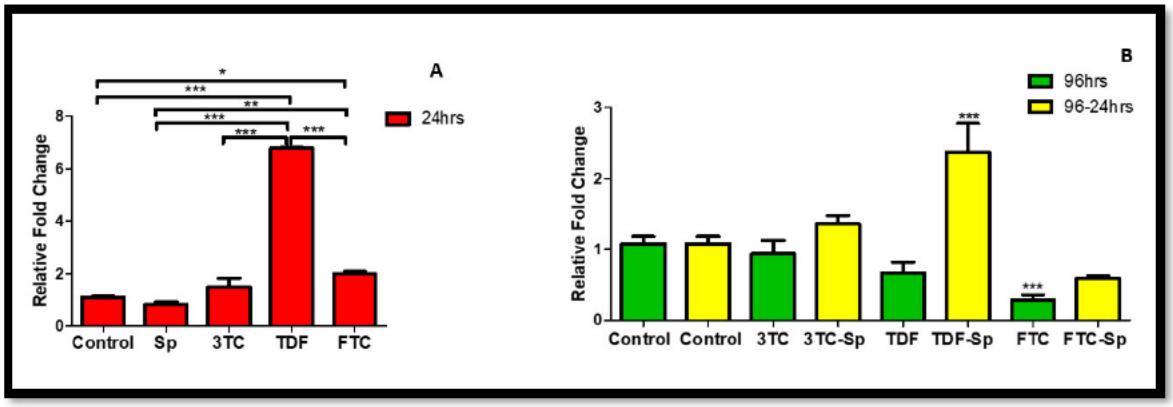

Figure 6. Effects of SP and HAART (3TC, TDF, and FTC) on JNK mRNA expression. JNK mRNA expression after exposure of HepG2 cells to A: SP and HAART for 24 hours, B: HAART (96 hrs), and HAART (96 hrs) followed by SP (24 hrs); *$p<0.05$, **$p<0.005$, ***$p<0.0001$

Assessment of oxidative stress

Oxidative stress parameters were quantified in HepG2 cells via H_2DCF-DA assay. SP treated cells displayed significant increased levels of intracellular ROS, while HAART also induced a significantly abnormal increase in intracellular ROS following acute and prolonged exposure, with only 3TC (96hrs) indicating a significant decrease A: ($p < 0.0001$), B: ($p < 0.0001$). Interestingly, SP managed to reduce

access ROS induced by prolonged exposure to HAART, specifically there was a positive synergistic effect B: ($p < 0.0001$) (Figure 7).

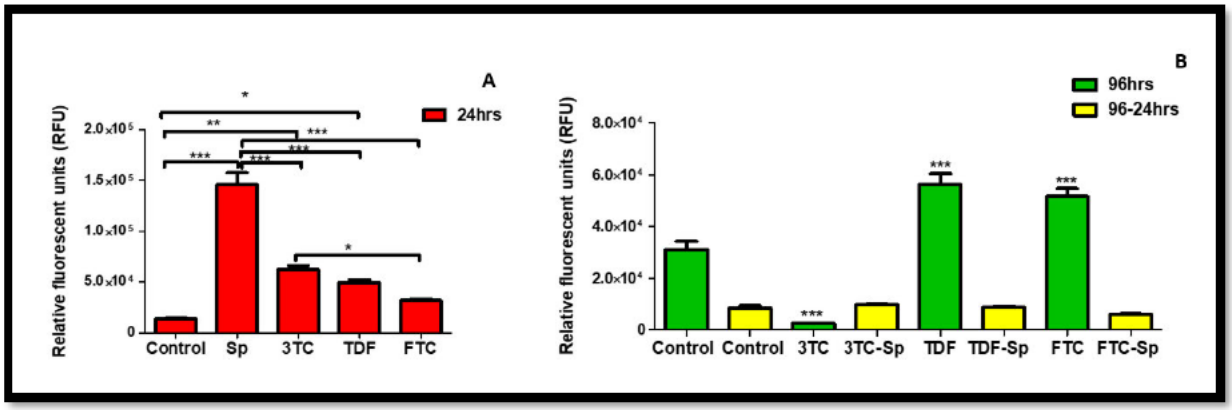

Figure 7. Intracellular ROS levels represented as relative light units (RLU) produced after H_2DCF-DA staining in HepG2 cells. Intracellular ROS levels after exposure of HepG2 cells to A: SP and HAART for 24 hours, B: HAART (96 hrs), and HAART (96 hrs) followed by SP (24 hrs); *$p<0.05$, **$p<0.005$, ***$p<0.0001$

Mitochondrial stress responses

Mitochondrial membrane potential (Δmψ) was measured to determine mitochondrial health and function. The JC-1 assay was used to determine Δmψ. SP and HAART treated HepG2 cells showed healthy Δψm A: ($p < 0.0001$), B: ($p < 0.0001$), and HAART-SP also showed healthy Δψm results B: ($p < 0.0001$) (Figure 8). Extracellular levels of LDH were quantified using a colorimetric assay to assess the integrity of the cell membrane, since LDH is exclusively found in the cytoplasm and only exits the cell through damaged membranes [96]. The increase in LDH release suggests increased cell damage [97], and can be an early indicator of increased necrotic cell death. SP and HAART mostly indicated significant elevated LDH levels after acute exposure A: ($p < 0.0001$). However, prolonged exposure of HepG2 cells to HAART followed by acute exposure to SP mostly reduced LDH levels B: ($p < 0.0001$) (Figure 9). Unfavourably, FTC-SP indicated a significant increase (Figure 9).

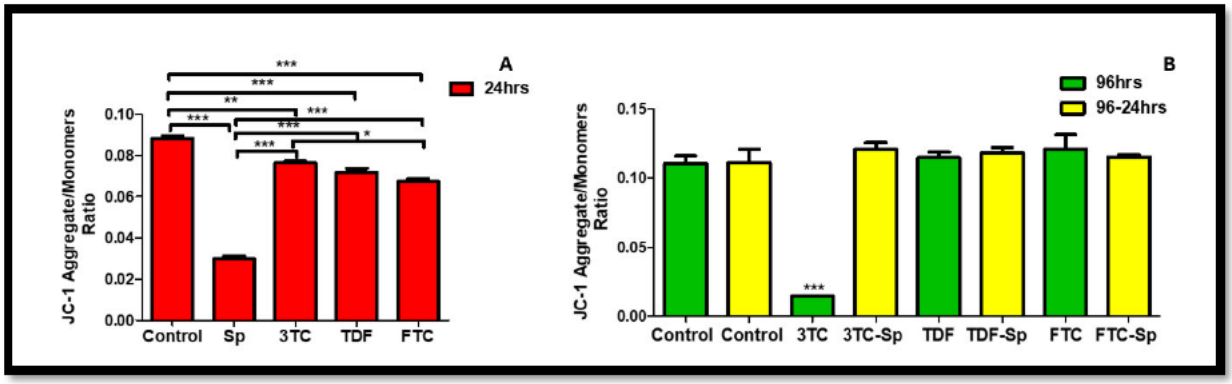

Figure 8. Mitochondrial response. Δmψ represented as a ratio of JC-1 aggregates and JC-1 monomers. The Δmψ after exposure of HepG2 cells to A: SP and HAART for 24 hours, B: HAART (96 hrs), and HAART (96 hrs) followed by SP (24 hrs); *$p<0.05$, **$p<0.005$, ***$p<0.0001$

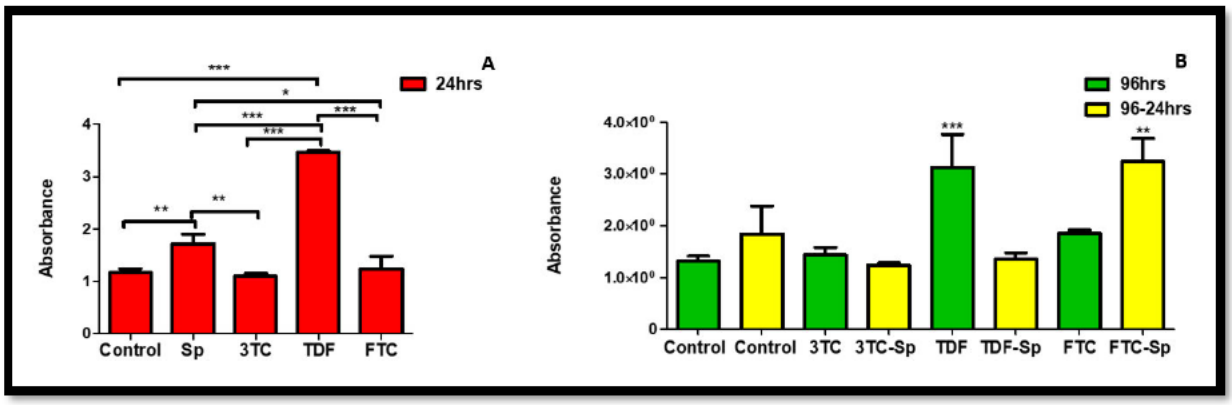

Figure 9. Effects of SP and HAART (3TC, TDF, and FTC) on Intracellular LDH levels. Intracellular LDH levels after exposure of HepG2 cells to A: SP and HAART for 24 hours, B: HAART (96 hrs), and HAART (96 hrs) followed by SP (24 hrs); *$p<0.05$, **$p<0.005$, ***$p<0.0001$

The MDA levels were quantified in HepG2 cells post chronic exposure to ARVs and acute exposure to *Spirulina platensis*. The MDA levels were significantly decreased in SP treated cells and significantly increased for 3TC and TDF after acute exposure A: ($p < 0.0001$), while decreased in HAART-SP treated HepG2 cells compared to the untreated cells B: ($p < 0.0001$), except for FTC-SP which showed a significant increase (Figure 10).

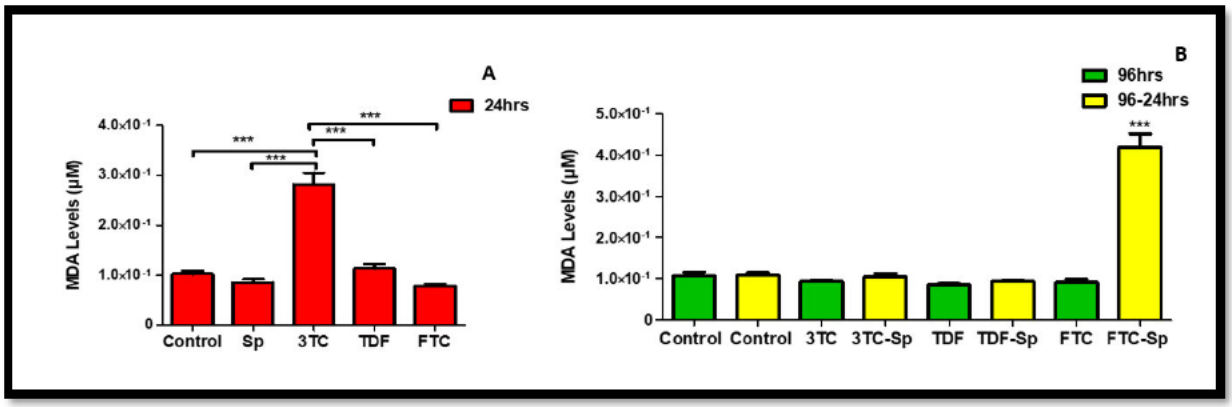

Figure 10. Extracellular MDA levels in SP and HAART (3TC, TDF, and FTC) treated HepG2 cells. Extracellular MDA levels after exposure of HepG2 cells to A: SP and HAART for 24 hours, B: HAART (96 hrs), and HAART (96 hrs) followed by SP (24 hrs); ***$p<0.0001$

Discussion

Studies have shown that the possible therapeutic effects of antioxidants may provide strategies in suppressing oxidative stress- and inflammation-induced comorbidities that emerge with the use of HAART therapy in HIV-infected individuals [19]. The combination of HIV and HAART has been

associated with increased oxidative stress and lipid peroxidation [98]. *Spirulina Platensis* (SP) is a potent antioxidant [75, 76] with anti-inflammatory activities [23], which makes it a potential supplement in the mitigation of oxidative stress induced by HAART adverse drug reactions. SP can inhibit NADPH oxidase which is considered as one of the main sources of reactive oxygen species (ROS) and free radicals [23], resulting in reduced oxidative stress [30]. Coincidentally, HAART is known to induce oxidative stress [17, 18, 99]. SP increased cell viability of HepG2 cell upon acute exposure. The ability of SP was supported by our data from measuring intracellular ROS, where SP managed to bring normalcy (Figure 7). SP only treated cells displayed increased levels of intracellular ROS, while HAART induced a significantly abnormal increase (Figure 7). However, SP managed to reduce access ROS induced by prolonged exposure to HAART (Figure 7). SP is rich with antioxidant properties [26, 75], also contains phycocyanin commonly known for reducing oxidative stress and NADPH oxidase [30]. Hence, the oxidative stress and NADPH oxidase inhibition ability by SP may explain the observed reduction levels of intracellular ROS (Figure 7) following SP exposure in HepG2 cells treated with HAART.

The ETC is responsible for ROS production and complications in this process may result in oxidative stress and depolarisation of the mitochondria, consequently causing a decrease in mitochondrial membrane potential ($\Delta\psi m$) and mitochondrial production of ATP [100]. SP prevented mitochondrial membrane depolarisation of HepG2 cells, this is demonstrated by the $\Delta\psi m$ data (Figure 8). SP and HAART treated cells showed healthy $\Delta\psi m$, and HAART-SP also showed healthy $\Delta\psi m$ (Figure 8). Studies *in vitro* showed that SP can scavenge nitric oxide and prevent DNA damage [101], also can enhance cell nucleus enzyme function and DNA repair synthesis [102]. Moreover, it can enhance mitochondrial health [32-35], this agrees with the results observed in this present study.

ROS induced lipid peroxidation is responsible for oxidative damage and reduction of cell membrane function [103]. LDH is exclusively found in the cytoplasm and only exits the cell through damaged membranes [96]. The ETC is responsible for ROS production and complications in this process may result in oxidative stress and depolarisation of the mitochondria, consequently causing a decrease in mitochondrial membrane potential and mitochondrial production of ATP [100]. SP and HAART mostly indicated significant elevated LDH levels after acute exposure (Figure 9). However, prolonged exposure of HepG2 cells to HAART followed by acute exposure to SP mostly reduced LDH levels (Figure 9).

Abnormal production of ROS results in peroxidation of lipids, which produces by-products such as MDA [104]. The MDA levels were significantly decreased in SP treated cells, while decreased in HAART-SP treated HepG2 cells compared to the untreated cells (Figure 10). However, there was an increase MDA levels for FTC-SP (Figure 10), this could be due to the fact that FTC is fluorinated NRTI [105], and fluoride on its own have been linked to oxidative stress, mitochondrial damage, and alteration of gene expression upon prolonged exposure [106], this could lead to SP requiring more exposure period to mend or bring balance to HepG2 cells that have been exposed to FTC.

The evidence supporting the function of microRNAs (miRNAs) in the control of inflammatory diseases is growing. Dysregulated microRNAs either directly or indirectly affect the expression and activity of molecules of inflammation [85]. The increased expression of miR-155 and miR-146a in metabolic syndromes was found to contribute to inflammation-mediated glomerular endothelial injury [86]. Together, SP and HAART were able to significantly lower miR-155, which may be a sign of that the medication is reducing antiinflammation (Figure 3). The main function of miRNA is to control gene expression at the post-transcriptional level through degrading or repressing target mRNAs. SP and HAART together managed to significantly reduce miR-155, this is the sign of reduction of inflammation due to the treatment (Figure 3). MiR-146a levels increased in SP and HAART treated cells (Figure 2). However, HAART-SP also indicated an increase miR-146a level (Figure 2), This might be due to limited exposure time or SP might be using another favourable path to combat inflammation.

Increasing evidence suggests the involvement of microRNA (miR-146a) in the pathogenesis of multiple diseases, including atherosclerosis, bacterial infection, and cancer [107]. MiR-146a levels increased in SP and HAART treated cells except 3TC (Figure 2). HAART-SP also indicated an increase miR-146a level except FTC-SP (Figure 2). The miR-155 levels increased in SP and HAART treated cells except 3TC and TDF. HAART-SP treated cells indicated a significant decrease in miR-155 levels (Figure 3). It is noteworthy, that the expression of miR-146a in HepG2 cells after exposure to SP and HAART is being tested for the first time in this present study. Studies revealed that miR-146a expression was deceased when c-jun N-terminal kinase (JNK) or nuclear factor (NF)-κB signaling was inhibited, suggesting that there is a corelation between the expression of JNK and miR-146a. Moreover, It has been demonstrated that miR-146a might be useful to inhibits inflammatory activation [107]. In the present study, miR-146a expression decreased in HepG2 cells exposed to HAART, following up with SP.

It has been demonstrated that miR-146a expression levels are significantly lower in lung cancer cells as compared with normal lung cells. Conversely, lung cancer cells have higher levels of Cyclooxygenase-2 (Cox-2) protein and mRNA expression [108]. According to Cornett and Lutz [108], introduction of miR-146a can specifically ablate Cox-2 protein and the biological activity of Cox-2, they proposed that decreased miR-146a expression contributes to the up-regulation and overexpression of Cox-2 in lung cancer cells [108].

Cox-2 is expressed by inflammatory cells, such as macrophages, and can be induced by tumor necrosis factor (TNF). Cox-2 is a central link to various inflammatory processes [93]. Cox-2 has been associated with inflammation, whereas the constitutively expressed Cyclooxygenase-1 (Cox-1) is generally considered as a housekeeping enzyme. However, recent evidence suggests that Cox-1 can also be upregulated and may play a prominent role in the brain during neuroinflammation [94]. Cox-1 mRNA expression was reduced in SP treated cells and mostly decreased in HAART (except TDF) treated cells (Figure 4). However, Cox-1 expression is significantly increased in HAART-SP treated cells (Figure 4). Continuing Cox-family investigation, Cox-2 mRNA expression is decreased in SP treated cells upon acute exposure (Figure 5). However, cells exposed to HAART-SP treatment showed a significant

decrease in Cox-2 mRNA expression (Figure 5). SP has been proven to inhibit Cox-2 expression. In addition, SP exerts regulatory effects on mitogen-activated protein kinase (MAPK) activation pathways, such as c-Jun N-terminal kinase (JNK) [109-111]. The data indicate that JNK mRNA was reduced by SP, which agrees with previous studies. Moreso, SP and HAART showed synergy except TDF. Jun N-terminal kinases (JNK) belong to the superfamily of MAP kinases that are involved in the regulation of cell proliferation, differentiation, and apoptosis [95]. JNK mRNA expression decreased in SP and HAART treated cells. HAART-SP treated cells showed a decrease in the expression of JNK mRNA except TDF-SP (Figure 6).

COX is a key enzyme for conversion of arachidonic acid to prostaglandins and has two isozymes Cox-1 and Cox-2. It has been found that overexpression of Cox-2 in cancer cell lines promotes their ability to invade surrounding tissues as well as increases cell invasion in gastric cancer. Some miRNAs downregulated the expression of *Cox-1* and *Cox-2* genes and thereby inhibited cell invasion [112]. This study investigated the expression of miRNAs that target *Cox-1/* 2 mRNAs and evaluated the effect of SP on the expression of the *Cox-1/* 2 mRNAs in HepG2 cells. In the current study, miRNA and mRNA expression was performed to find the corelation in the expression of miRNAs (miR-146a and miR-155) and *Cox-1/* 2 mRNA [112]. The present study shows a significant reduction of Cox-2, this is an indication that SP might be targeting Cox-2 as one of the many mechanisms to inhibit inflammation.

Cox-1 is known to be present in most tissues that involve maintenance of tissue homeostasis and cell signalling. Also, Cox-1 is shown in angiogenesis in endothelial cells. Cox-2 is a well-known gene associated with inflammatory mediation and participated in numerous biological processes such as pain, inflammation, cancer, angiogenesis, carcinogenesis or development of immunity [113]. According to Cheng, Zhao [114], inhibition of miR-155 and Cox-2 provides a protective effect in high glucose conditions [114]. MiR-155 enhances Cox-2 expression and is an established regulator of epithelial-mesenchymal transition and inflammation [115]. Some natural compounds suppress inflammatory activity, especially, those that are found in traditional medicine and dietary supplements have the potential to be developed as Cox-2 inhibitor [113]. Cox-1 expression increased post-treatment HAART-SP in this present study is a sign of protective function and successful synergy between SP and HAART (Figure 4). This study found that SP potentially mitigates metabolic syndrome characteristics via the regulation of inflammatory miRNAs. We hereby recommend further exploration on the synergistic roles of SP and HAART in the expression of microRNA with more focus on inflammatory pathway.

Conclusion

SP mitigates metabolic syndrome characteristics via the inhibition of miRNA that promotes inflammation. Moreover, HAART-SP promotes ROS balance, which is important for mitochondrial quality control. SP maintains intracellular balance, reduces excess ROS, protects mitochondrial potential, prevents necrotic cell death, and enhance mitochondrial quality. Most of these SP qualities worked most favourably with HAART. We hereby recommend further investigation of *Spirulina*

Platensis' ability to inhibit chronic negative effects of highly active antiretroviral therapy (HAART) *in vitro* via gene knockouts.

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CHAPTER FIVE

Spirulina platensis mitigates inflammation induced by highly active antiretroviral therapy (HAART) in HepG2 liver cells

Thabani Sibiya[1], Terisha Ghazi[1], Jivanka Mohan[1], Savania Nagiah[1,2], Anil A. Chuturgoon[1], *

[1]Discipline of Medical Biochemistry and Chemical Pathology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Howard College Campus, Durban 4013, South Africa

[2]Department of Human Biology, Medical Programme, Faculty of Health Sciences, Nelson Mandela University Missionvale, Bethelsdorp, Port Elizabeth, South Africa, 6059

*Corresponding author: Professor Anil A Chuturgoon, Discipline of Medical Biochemistry and Chemical Pathology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Howard College Campus, Durban 4013, South Africa. Telephone: +27312604404. Email: chutur@ukzn.ac.za

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Abstract

The human immunodeficiency virus (HIV) is responsible for acquired immune deficiency syndrome (AIDS), one of the prevalent communicable diseases on the global burner since its emergence in the 1980s. It is estimated that about 37.7 million people are infected with HIV globally, and 8.2 million infected persons in South African. Highly active antiretroviral therapy (HAART) inhibits viral replication thus slowing the development of AIDS. The use of HAART causes metabolic syndrome via an inflammatory pathway, excessive formation of reactive oxygen species (ROS), and mitochondrial dysfunction, despite its success in extending the lives of HIV-infected patients. *Spirulina platensis* (SP) has been proven to mitigate metabolic syndrome (MetS) through the regulation of oxidative and inflammatory pathways. This study aims to examine the protective role of SP acute exposure against prolonged HAART-induced inflammation in HepG2 cells. Protein expression was determined using western blots. Quantitative Polymerase Chain Reaction (qPCR) was also employed for mRNA quantification. SP significantly decreased *iNOS* ($p < 0.0001$), *IκB-α* ($p < 0.0001$), *NF-κB* ($p < 0.0001$), IL-1β ($p = 0.0002$) and *TNF-α* ($p = 0.0074$) mRNA levels. The result demonstrated by HAART-SP post treatment indicated a decreased inflammation NF-κB ($p < 0.0001$), IL-1β ($p < 0.0001$), IL-12 ($p < 0.0001$), TNF-α ($p < 0.0001$) at mRNA level. The finding proves that HAART-SP post treatment downregulated NF-κB ($p < 0.0001$) protein expression and shows SP potential to inhibit inflammation induced by HAART (3TC, TDF and FTC) in HepG2 cells.

Keywords: highly active antiretroviral therapy (HAART); *Spirulina platensis* (SP); Inflammation; antioxidant; Metabolic Syndrome (MetS); nuclear transcription factor kappa B (NF-κB)

Introduction

It is estimated that 37.7 million people globally were infected with HIV in 2021 [1-4], with 8.2 million infected persons in South Africa [4]. HIV replicates and results in a wide range of immunological abnormalities, including depleting CD4+ T helper cells, which raises the risk of infectious and oncological consequences [5]. HAART reduces viral load and subsequently prevents HIV progressing to AIDS. By far, HAART remains a widely implemented and effective therapy in reducing mortality among AIDS patients [6-8]. Despite its success in viral load inhibition, the use of HAART promotes the persistence of elevated inflammatory markers such as interleukin IL-6 [9], and tumour necrosis factors-alpha (TNF-α) [10, 11], which are mediated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [12]. Inflammation is one of the major contributors in MetS. This is accompanied by excess production of ROS, and mitochondrial dysfunction [13-19]. Studies have suggested that HAART, in addition to HIV-related complications, actively induces MetS in persons with HIV [20-22]. The latter highlights a need for an anti-inflammatory agent with the ability to inhibit MetS, especially with the high number of people living with HIV. Studies on MetS inhibitory compounds such as SP have demonstrated significant anti-inflammatory properties [23, 24].

SP is a nutritious blue-green microalga and a traditional food for some Mexican and African people [25, 26]. It is mostly found in alkaline water of volcanic lakes. SP helps in building up the humoral and cellular immunity [26]. There is solid evidence that SP inhibits MetS through hypoglycemic [27], hypolipidemic [28], antihypertensive [29]. Previous studies have also demonstrated protective properties by SP. SP composition includes chlorophyll, phycocyanin, and carotenoid which may be major contributors to it protective effects. Moreover, SP inhibits oxidative stress [24, 25, 30, 31], and promotes mitochondrial health [32-35], subsequently, inhibiting inflammation [23, 24]. In view of the above concerns, the present study was designed to evaluate the anti-inflammatory action of SP in ameliorating inflammation induced by HAART.

Materials and methods

Materials

SP, extracted from *Spirulina Platensis* (Shewal) capsules, were obtained from HeriCure Healthcare Ltd (Pune, India). Antiretroviral drugs 2',3'-dideoxy-5fluoro-3'-thiacytidine (FTC), (-)-L-2',3'-dideoxy-3'-thiacytidine (3TC), and tenofovir disoproxil fumarate (TDF) were obtained from the NIH AIDS reagents program: The antiretroviral drug compounds were purchased from Pharmed Pharmaceuticals and extracted using dichloromethane, which was then removed using a standard laboratory rotary evaporator. The identity of the extracted compounds was confirmed using Nuclear magnetic resonance (NMR) analysis and showed a purity of >98%. The HepG2 cell line was acquired from Highveld Biologicals (Johannesburg, South Africa). Cell culture reagents and supplements were purchased from Lonza Bio-Whittaker (Basel, Switzerland). Unless otherwise stated, all other reagents were purchased from Merck (Darmstadt, Germany).

Cell culture

HepG2 cells were cultured in monolayer (10^6 cells per 25 cm^3 culture flask) with complete culture media [CCM: Eagle's Essential Minimal Media (EMEM) supplemented with 10% foetal calf serum, 1% penstrepfungizone and 1% L-glutamine] at 37 °C in a humidified incubator. Cells were allowed to reach 80% confluence in 25 cm^3 flasks before treatment with only antiretrovirals (ARVs) using the plasma peak values from literature that represent the physiological concentrations of ARVs in humans (3TC: 6.6 µM (1.51 µg/ml), TDF: 0.3 µg/ml, FTC: 1.8 µg/ml) [36-38] in CCM for 96 hours [39]. For the 96 hours treatment, fresh cell culture medium containing ARVs treatment was replenished every 48 hours. Thereafter ARVs were removed, and cells were gently rinsed with 0.1 mol/l phosphate buffer saline (PBS) and treated with only 1.5 µg/ml *Spirulina Platensis* (SP) on its own in CCM for 24 hours. An untreated control, containing only CCM, was also prepared. Treatment for 24 hours' time period was also conducted, containing only ARVs [40] and SP separately.

Protein expression

The protein expression of NF-κB was determined by western blotting. Standardised protein samples were boiled in Laemmeli buffer [dH_2O, 0.5 mol/l Tris-HCl (pH 6.8), glycerol, 10% sodium dodecyl sulphide (SDS), b-mercaptoethanol, 1% bromophenol blue] for 5 mins. Proteins (25µl) were separated by electrophoresis on SDS-polyacrylamide electrophoresis gels (4% stacking gel; 10% resolving gel) and electrotransferred to nitrocellulose membranes. Membranes were blocked with 5% BSA in Tween 20-Tris buffer saline (TTBS 150 mmol/l NaCl, 3 mmol/l KCl, 25 mmol/l Tris, 0.05% Tween 20, dH_2O, pH 7.5; RT) for 1 hour, and incubated with primary antibody [NF-κB (8242S)] in 5% BSA in TTBS (1:1000 dilution) overnight at 4°C. Following overnight incubation, membranes were equilibrated to room temperature (RT) and washed with TTBS (5 times, 10 mins). Membranes were subsequently probed with horseradish peroxidase (HRP)-conjugated secondary antibody [Rabbit (7074P2)] in 5% BSA in TTBS (1:5000) for 1 hour at RT. Thereafter, membranes were washed with TTBS (5 times, 10 mins) and immunoreactivity was detected (Clarity Western ECL Substrate) with the Bio-Rad Chemidoc gel documentation system. After detection, membranes were quenched with 5% H_2O_2 for 30 mins, incubated in blocking solution (5% BSA for 1 hour at RT), rinsed thrice in TTBS, and probed with HRP-conjugated anti-β-actin (housekeeping protein). Protein expression was analysed by the Image Lab Software version 5.0 (Bio-Rad) and the results were expressed as relative band density (RBD). The expression of proteins of interest was normalised against β-Actin.

Ribonucleic acid (RNA) analysis

Total RNA was isolated according to the method described by Chuturgoon, Phulukdaree [41]. Isolated RNA was quantified (Nanodrop 2000, Thermo Scientific, Waltham, USA) and standardised to 1000 ng/µl. Complementary deoxyribonucleic acid (cDNA) was synthesised from standardised RNA using the iScript cDNA synthesis kit (Bio-Rad). Thermocycler conditions for cDNA synthesis were 25 °C for 5 mins, 42 °C for 30 mins, 85 °C for 5 mins and a final hold at 4 °C [40]. Gene expression was analysed using the SsoAdvanced™ Universal SYBR® Green Supermix kit (Bio-Rad). The messenger RNA (mRNA) expressions of inducible nitric oxide synthase (*iNOS*), NF-κB inhibitor-alpha (*IκB-α*), *NF-κB*, Interleukin-1 beta (*IL-1β*), Interleukin-12 (*IL-12*), tumor necrosis factor alpha (*TNF-α*) were investigated using specific forward and reverse primers (Table 1). Reaction volumes which consisted of the following were prepared: SYBR green (5µl), forward primer (1µl), reverse primer (1µl), nuclease free water (2µl) and cDNA template (1µl). All reactions were carried out in triplicate. The samples were amplified using a CFX96 Touch™Real- Time PCR Detection System (Bio-Rad). The initial denaturation occurred at 95 °C (4 mins). Thereafter, 37 cycles of denaturation (15 seconds, 95 °C), annealing (40 seconds; temperatures— Table 1) and extension (30 seconds, 72 °C) occurred. The method described by Livak and Schmittgen [42] was employed to calculate the changes in relative mRNA expression, where $2^{-\Delta\Delta Ct}$ represents the fold change relative to the untreated control. The expression of the gene of interest was

normalised against the housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), which was amplified simultaneously under the same conditions.

Table 1 The annealing temperatures and primer sequences for the genes of interest

Gene	Annealing temperature	Primer	Sequence
IκB-α	60°C	Forward Reverse	5'-TCCACTCCATCCTGAAGGCTAC-3' 5'-CAAGGACACCAAAAGCTCCACG-3'
NF-κB	50°C	Forward Reverse	5'-TGAACCGAAACTCTGGCAGCTG-3' 5'-CATCAGCTTGCGAAAAGGAGCC-3'
iNOS	59,7°C	Forward Reverse	5'-GCTCTACACCTCCAATGTGACC-3' 5'-CTGCCGAGATTTGAGCCTCATG-3'
IL-12	59,7°C	Forward Reverse	5'-GACATTCTGCGTTCAGGTCCAG-3' 5'-CATTTTTGCGGCAGATGACCGTG-3'
IL-1β	53°C	Forward Reverse	5'-CAGCTACGAATCTCCGACCAC-3' 5'-GGCAGGGAACCAGCATCTTC-3'
TNF-α	53°C	Forward Reverse	5'-CTCTTCTGCCTGCTGCACTTTG-3' 5'-ATGGGCTACAGGCTTGTCACTC-3'
GAPDH		Forward Reverse	5′-TCCACCACCCTGTTGCTGTA-3′ 5′-ACCACAGTCCATGCCATCAC-3′

Statistical analysis

All experiments were conducted independently in triplicate. GraphPad Prism version 5.0 (GraphPad Software Inc., California) was used to perform all statistical analyses. The one-way analysis of variance (ANOVA) followed by a Bonferroni test for multiple group comparison (data is presented as 95% CI) was used to determine statistical significance. All results were represented as the mean ± standard deviation. A value of $p<0.05$ was considered statistically significant.

Results

Assessment of oxidative stress

Nitric oxide (NO), which is potently increased in response to proinflammatory stimuli, is produced by a crucial enzyme in the macrophage inflammatory response called iNOS [43]. SP treated cells displayed a significant decreased levels of *iNOS* A: ($p < 0.0001$) after acute exposure, while HAART induced a significant increase in *iNOS* levels in HAART treated HepG2 cells B: ($p < 0.0001$) after prolonged exposure (Figure 1). HAART-SP on the other hand displayed varying results B: ($p < 0.0001$), with only TDF-SP and FTC-SP showing a significant increase of iNOS mRNA levels (Figure 1).

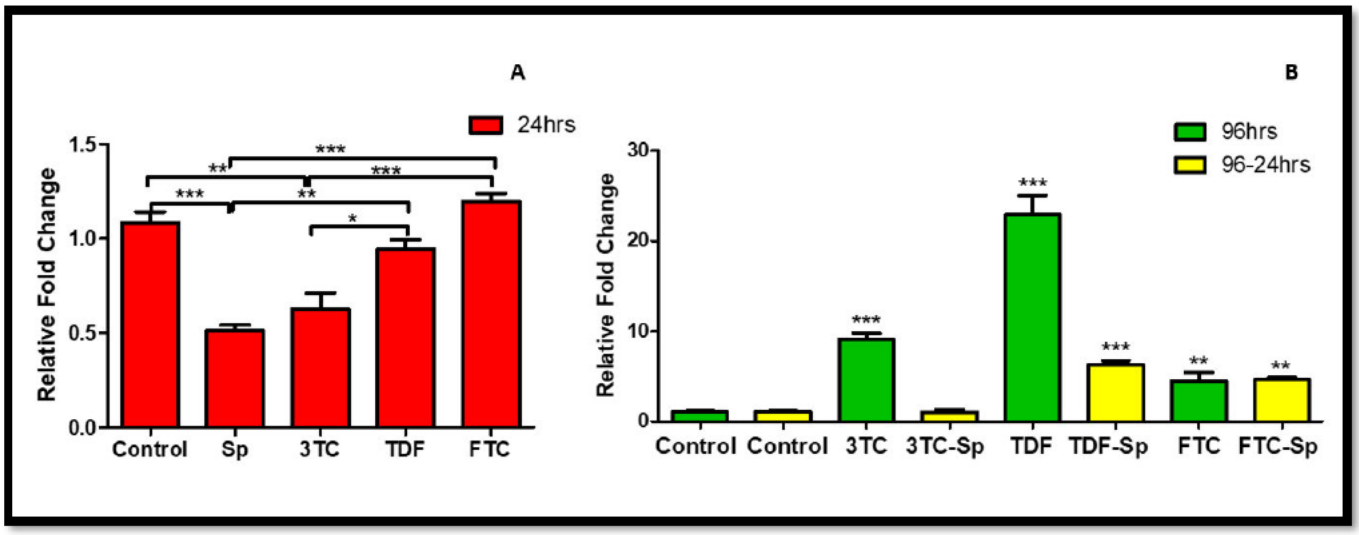

Figure 1. Effects of SP and HAART (3TC, TDF, and FTC) on *iNOS* mRNA levels. *iNOS* mRNA levels after exposure of HepG2 cells to A: SP and HAART for 24 hours, B: HAART (96 hrs), and HAART (96 hrs) followed by SP (24 hrs); *$p<0.05$, **$p<0.005$, ***$p<0.0001$

Inflammatory Response

Activation of NF-κB is triggered by signal-induced degradation of IκB-α [44]. NF-κB attaches to specific DNA regions in the nucleus, causing the transcription of many genes that are involved in pro-inflammatory processes [45]. *IκB-α* mRNA expression is decreased in SP and HAART treated cells A: ($p < 0.0001$) after acute exposure, while HAART prolonged exposure showed no significant difference B: ($p = 0.0002$). HAART-SP treated cells increased IκB-α mRNA expression B: ($p = 0.0002$) with only FTC-sp exception (Figure 2). *NF-κB* mRNA expression of SP treated cells decreased compared to the control A: ($p < 0.0001$) post-acute exposure and increased in HAART treated cells B: ($p < 0.0001$) following prolonged exposure (Figure 3). However, HAART-SP treated cells showed an increased

expression of *NF-κB* mRNA levels compared to the control with only FTC-SP indicating a decrease B: ($p < 0.0001$) (Figure 3). The NF-κB protein expression A: ($p = 0.0003$), B: ($p < 0.0001$) decreased in almost all treatments compared to the control, except FTC (24 hrs) (Figure 4).

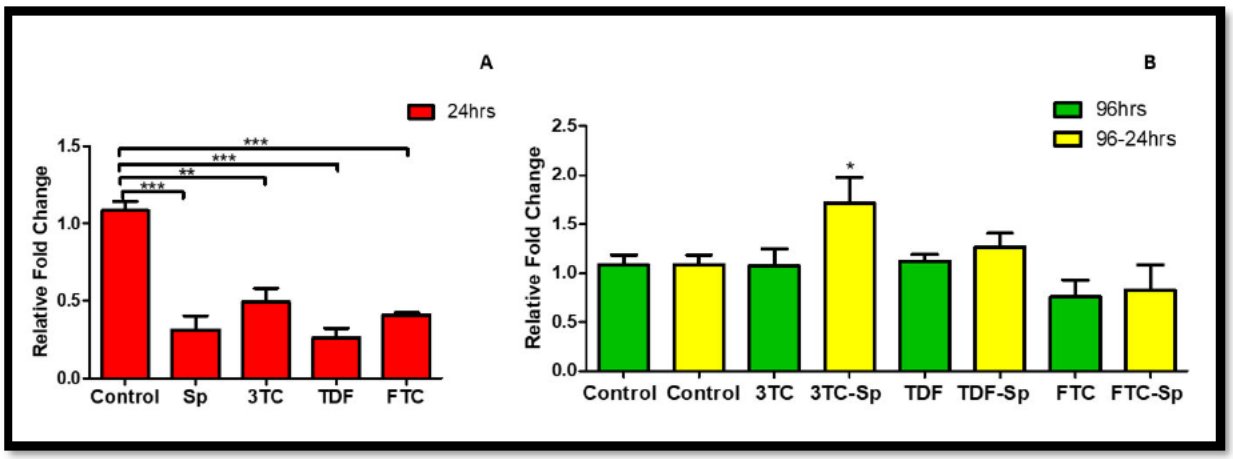

Figure 2. Effects of SP and HAART (3TC, TDF, and FTC) on *IκB-α* mRNA expression. *IκB-α* mRNA expression after exposure of HepG2 cells to A: SP and HAART for 24 hours, B: HAART (96 hrs), and HAART (96 hrs) followed by SP (24 hrs); *$p<0.05$, **$p<0.005$, ***$p<0.0001$

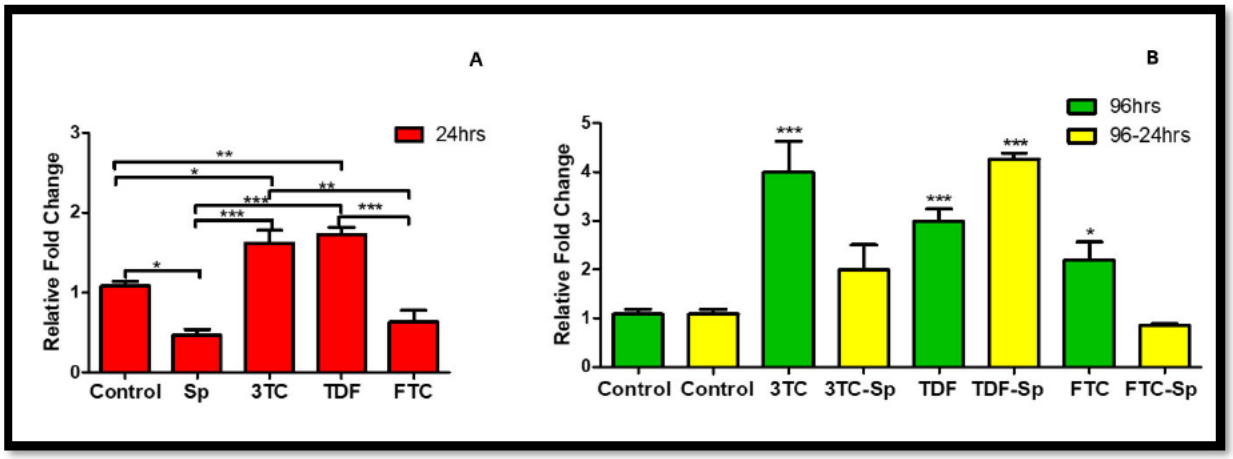

Figure 3. Effects of SP and HAART (3TC, TDF, and FTC) on *NF-κB* mRNA expression. *NF-κB* mRNA expression after exposure of HepG2 cells to A: SP and HAART for 24 hours, B: HAART (96 hrs), and HAART (96 hrs) followed by SP (24 hrs); *$p<0.05$, **$p<0.005$, ***$p<0.0001$

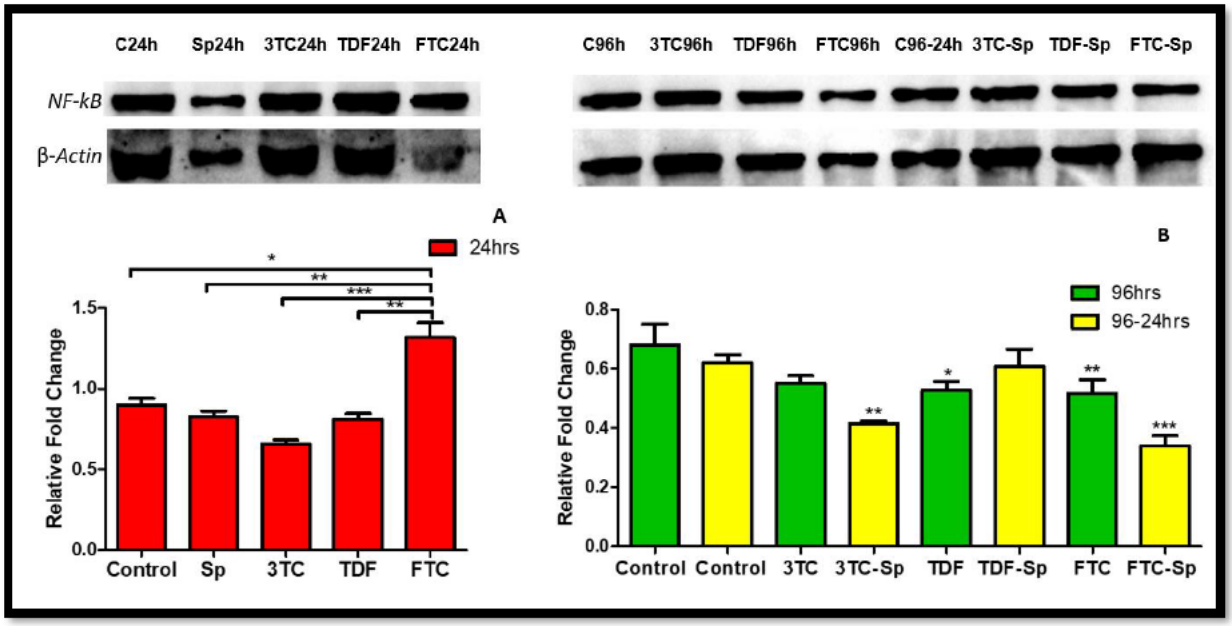

Figure 4. Effects of SP and HAART (3TC, TDF, and FTC) on NF-κB protein expression. NF-κB protein expression after exposure of HepG2 cells to A: SP and HAART for 24 hours, B: HAART (96 hrs), and HAART (96 hrs) followed by SP (24 hrs); *$p<0.05$, **$p<0.005$, ***$p<0.0001$

Inflammatory cytokines

IL-1β is considered one of the key cytokines involved in the induction of inflammatory reactions and is one of the 11 representatives of the IL-1 family (IL-1F) [46]. *IL-1β* mRNA expression decreased in SP treated cells A: ($p = 0.0002$) after acute exposure and there is a partial significant increased expression in HAART (3TC and FTC to be specific) treated cells following prolonged exposure, while TDF indicated a decreased expression on *IL-1β* mRNA B: ($p < 0.0001$) (Figure 5). However, HAART-SP treated cells showed a significant decrease expression of *IL-1β* mRNA when compared to the control and HAART only B: ($p < 0.0001$) (Figure 5). The heterodimeric cytokine interleukin-12 (IL-12) is essential for stimulating type 1 T helper cell (Th1) responses and, consequently, cell-mediated immunity [47]. *IL-12* mRNA expression is significantly increased in SP A: ($p = 0.0014$) and HAART treated cells B: ($p < 0.0001$) (Figure 6). However, HAART-SP treated cells showed decreased expression of *IL-12* mRNA B: ($p < 0.0001$) (Figure 6). TNF-α, one of the 19 ligands in the tumour necrosis factors superfamily, is regarded as a major inflammatory cytokine alongside IL-1β. There is a clear synergism between the two cytokines, and in the majority of cases, TNF-α effect and IL-1β effect are complementary [46]. *TNF-α* mRNA expression is decreased in SP treated cells A: ($p = 0.0074$) following acute exposure and mostly decreased in HAART (except 3TC) treated cells B: ($p < 0.0001$) following prolonged exposure, while HAART-SP treated cells indicate a significant decrease in *TNF-α* mRNA expression B: ($p < 0.0001$) (Figure 7).

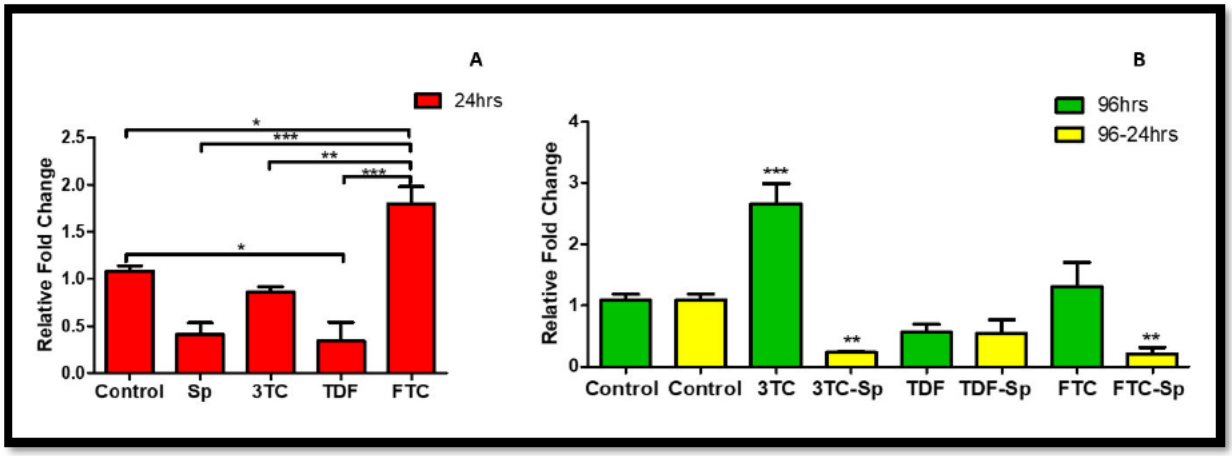

Figure 5. Effects of SP and HAART (3TC, TDF, and FTC) on *IL-1β* mRNA expression. *IL-1β* mRNA expression after exposure of HepG2 cells to A: SP and HAART for 24 hours, B: HAART (96 hrs), and HAART (96 hrs) followed by SP (24 hrs); *$p<0.05$, **$p<0.005$, ***$p<0.0001$

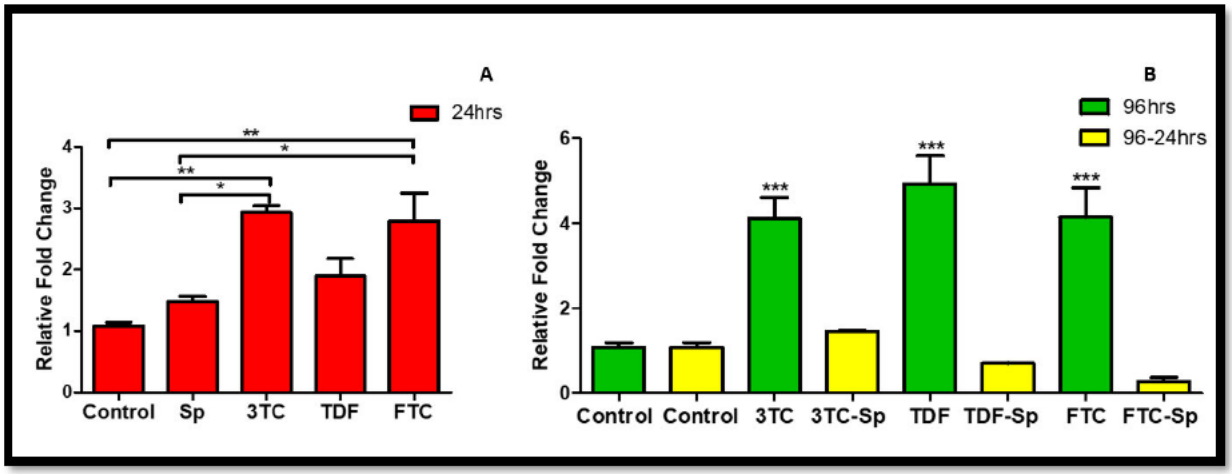

Figure 6. Effects of SP and HAART (3TC, TDF, and FTC) on *IL-12* mRNA expression. *IL-12* mRNA expression after exposure of HepG2 cells to A: SP and HAART for 24 hours, B: HAART (96 hrs), and HAART (96 hrs) followed by SP (24 hrs); *$p<0.05$, **$p<0.005$, ***$p<0.0001$

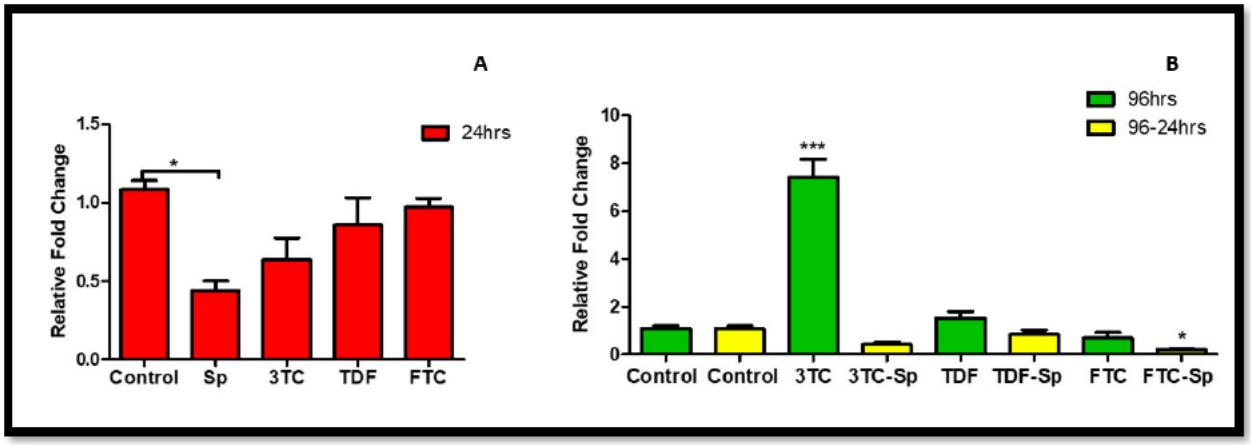

Figure 7. Effects of SP and HAART (3TC, TDF, and FTC) on *TNF-α* mRNA expression. *TNF-α* mRNA expression after exposure of HepG2 cells to A: SP and HAART for 24 hours, B: HAART (96 hrs), and HAART (96 hrs) followed by SP (24 hrs); *$p<0.05$, ***$p<0.0001$

Discussion

We investigated the effect of SP on the inflammatory pathway after exposure of HepG2 cells to three antiretroviral drugs that form the backbone of HAART (3TC, TDF, and FTC separately). Inflammation is advantageous because it encourages the regeneration of cells following liver injuries. Furthermore, it triggers an immune response that targets and destroys aberrant cells. Chronic inflammation, however, is harmful because it promotes the progression of fibrosis, cirrhosis, and ultimately hepatocellular cancer as a result of continuous hepatic injury and the regenerative damage healing responses that are linked to it [48-50]. The NF-κB is one of the key mediators of inflammation [12].

NF-κB is a dimeric complex which regulates the transcription of genes involved in cell survival, growth and differentiation, immune responses, and inflammation [12]. Under normal physiological conditions, NF-κB is cloistered in the cytoplasm by binding to the IκB proteins which consists catalytic subunits, IkappaB Kinase alpha (IKKα) and IkappaB Kinase beta (IKKβ) and a regulatory subunit IkappaB Kinase gamma NEMO (IKK γ/NEMO) [51]. SP reduced *IκB-α* mRNA expression (Figure 2A), and *NF-κB* mRNA expression compared to the control (Figure 3A). Down regulation of IκB-α was not expected as previous study by Cherng, Cheng [52] demonstrated that SP contains compounds that promotes IκB-α expression. However, NF-κB protein expression (Figure 4A) decreased in SP treated cells, which aligned with the reduction of NF-κB mRNA expression. SP reducing NF-κB is not surprising since the data agrees with previous reports [52, 53]. The present study did not investigate the degradation of cytosolic IκB-α, which suppresses the activation of NF-κB [30]. However, SP downregulated NF-κB at transcriptional level, and this is one of the indications of anti-inflammation. Furthermore, NF-κB protein expression (Figure 4B) significantly decreased in HAART-SP treated cells when compared to the

control, supporting anti-inflammatory ability of SP post-exposure to HAART. As it has been proven that SP promotes IκB-α to suppress NF-κB activation [30], this is supported by an increase in IκB-α mRNA expression (Figure 2B) in HAART-SP treated cells.

IκB is phosphorylated in response to a particular stimulus, such as chemotherapy, stressors, or cytokines, which causes it to be ubiquitinated and degraded by the 26S proteasome. Liberated NF-κB dimer translocate to the nucleus and binds to the kappaB site, which is also known as the consensus DNA sequence 5'-GGGRNNYYCC-3' where N is any nucleotide, Y is cytosine or thymine, and R is adenine or guanine [54]. NF-κB mRNA expression increased in HAART treated cells (Figure 3B), which is an indication of inflammation activation. Interestingly, *IκB-α* mRNA expression is decreased in HAART treated cells (Figure 2B), leaving NF-κB to be activated. Consequently, HAART-SP treated cells showed an increased *NF-κB* mRNA expression compared to the control with only FTC-SP indicating a decrease (Figure 3B). However, NF-κB protein expression (Figure 4B) decreased in HAART and HAART-SP treated cells. SP in this study may be using NF-κB pathway to inhibit inflammation. More so, SP has been proven to inhibit the formation of the pro-inflammatory cytokine, TNF-α expression [30, 52, 55-57]. Furthermore, it has been demonstrated by the previous studies to possess antioxidant, and anti-inflammatory activities [58-60].

SP inhibits the production of nitric oxide and suppresses the expression of iNOS, TNF-α, and IL-1β. TNF-α, together with IL-1β, is considered a key inflammatory cytokine. The effect of TNF-α in most cases coincides with the action of IL-1β [46]. TNF-α (Figure 7A) and IL-1β (Figure 5A) mRNA expression decreased in SP treated cells, and this agrees with anti-inflammatory abilities of SP reported in the previous studies [30, 61, 62]. Moreover, HAART-SP treated cells indicate a significant decrease of TNF-α mRNA expression (Figure 7B). The TNF-α significant decrease in the presence of SP in cells that were chronically exposed to HAART may be a justifiable clue to the mechanism of anti-inflammatory pathway followed by SP. TNF-α mRNA expression mostly increased in HAART treated cells (Figure 7B), which is an indication of inflammatory cytokine promotion. IL-1β is considered one of the key cytokines involved in the induction of inflammatory reactions [46]. *IL-1β* mRNA increased expression in HAART (3TC and FTC to be specific) treated cells, while TDF indicated a decreased expression on *IL-1β* mRNA (Figure 5B) shows that HAART mostly promotes inflammation. However, HAART-SP treated cells showed a significant decrease expression of IL-1β mRNA when compared to the control (Figure 5B) proving anti-inflammation ability of SP post-treatment.

SP contains phycocyanin, which scavenges free radicals, decreases nitrite production, and suppresses inducible nitric oxide synthase (iNOS) expression [30, 61, 62]. SP treated cells displayed decreased levels of iNOS (Figure 1A), this data agrees with previous studies. However, SP indicated limited ability to decrease *iNOS* mRNA (Figure 1B) expression following chronic HAART exposure, with only 3TC-SP indicating a downregulation of iNOS mRNA (Figure 1B) and a significant increase in iNOS mRNA

levels in TDF-SP and FTC-SP exposed cells. As expected, HAART (Figure 1B) induced a significant increase of iNOS mRNA levels in treated cells. SP also contains vitamin D3, vitamin C and magnesium, and these components have been suspected to stimulate nitric oxide (NO) production [63].

The suppression of inflammatory mediators by SP is justified by its' ability to inhibit NF-κB activation by preventing nuclear translocation of NF-κB p65 subunit [59, 64, 65], this is supported by a significant decrease in NF-κB protein expression (Figure 4). Studies have indicated that SP properties suppressed the transcription of inflammatory cytokines such as IL-1β, and IL-12 *in vitro* [60], this is in line with the present study indicating a significant decrease in IL-1β at transcript level by SP in HepG2 cells exposed to HAART for 96 hours. Interleukin-12 (IL-12) is a heterodimeric cytokine that plays a central role in promoting type 1 T helper cell (Th1) responses and, also cell-mediated immunity [47]. Unexpectedly, *IL-12* mRNA expression is significantly increased in SP and HAART treated cells (Figure 6A & 6B). However, HAART-SP (except 3TC-SP) treated cells showed a significant decreased expression of IL-12 mRNA (Figure 6B), this is an indication of positive synergistic outcome.

This study found that SP can attenuate inflammation induced by HAART (3TC, TDF and FTC) in HepG2 cells, as noted by a decrease in inflammatory key regulator NF-κB and the corresponding decrease in inflammatory cytokines (Figure 8).

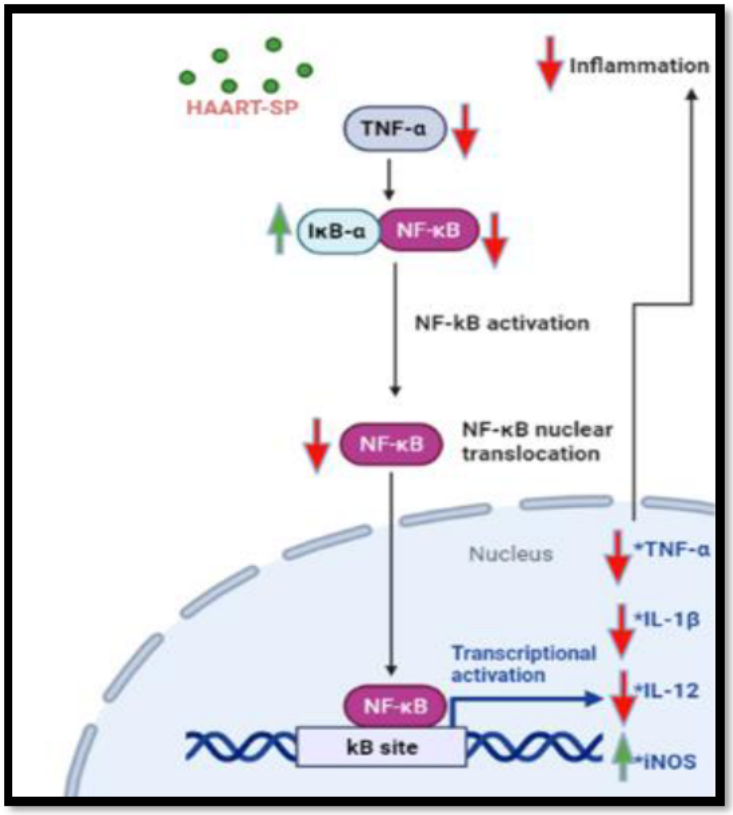

Figure 8. Effects of SP post HAART (3TC, TDF, and FTC) exposure (HAART-SP) on inflammation pathway in HepG2 cells (by author, created with BioRender.com).

Conclusion

SP is an anti-inflammatory agent - this is supported by the findings in this present study showing that HAART-SP post treatment downregulates NF-κB protein expression. Furthermore, reduction of inflammatory cytokines solidifies SP and HAART positive synergy. This study shows that SP has the potential to inhibit inflammation induced by HAART (3TC, TDF and FTC) in HepG2 cells. At a molecular level, this study explored the potential of SP supplementation in the mitigation of inflammation induced by HAART. Moreso, the results unveil that SP can play a significant role in the amelioration of HAART toxicity through inflammatory pathway. Furthermore, we suggest the need for further exploration on SP and HAART favourable synergy at a molecular level.

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CHAPTER SIX

CONCLUSION

HIV is a leading cause of death worldwide. HAART drugs currently used to treat HIV have been reported to have adverse effects, which lead to MetS including oxidative stress, inflammation, and mitochondrial dysfunction (Mondal, Pradhan et al. 2004, Ngondi, Oben et al. 2006, Masiá, Padilla et al. 2007, Blas-Garcia, Apostolova et al. 2011, Manda, Banerjee et al. 2011). SP is an antioxidant and anti-inflammatory with the potential to ameliorate MetS induced by HAART. The present study evaluated the potential of SP to ameliorate HAART-induced oxidative stress and inflammation in HepG2 liver cells. This study provides novel and useful insight into SP's potential as a supplement to ameliorate HAART induced adverse outcomes in a human liver cell culture model. The results supported the hypothesis of the study and showed that SP ameliorated oxidative stress and inflammation associated with HAART in HepG2 cells. Further, SP induced a potent antioxidant response that not only helped curb oxidative stress but also reduced HAART associated inflammation.

SP induced antioxidant response in HepG2 cells by increasing *NRF-2*, *CAT*, and *NQO-1* mRNA expression. Moreover, NRF-2 and pNRF-2 protein expression was upregulated in the HepG2 cells post-exposure to HAART and SP. This data agrees with the previous study that demonstrated SP has direct antioxidant activity and modulation of the ARE/NRF-2 pathway in HepG2 cells (Vigliante, Mannino et al. 2019). Furthermore, exposure to HAART followed by exposure to SP reduced LDH levels in HepG2 cells. The ability of SP to reduce ROS was supported by SP inhibiting ROS induced by prolonged exposure to HAART, indicating a positive synergistic effect. This study also found that SP potentially mitigates MetS characteristics via the regulation of inflammatory miRNAs. SP attenuated inflammation induced by HAART (3TC, TDF and FTC) in HepG2 cells, as noted by a decrease in inflammatory key regulator NF-κB and the corresponding decrease in inflammatory cytokines.

Taken together, this study indicates that SP has the potential to mitigate HAART induced adverse drug reactions, albeit in an *in vitro* HepG2 model. Furthermore, this study provides insight for future molecular studies at a longer SP exposure time as well as the elucidation of SP as a safer supplement for individuals on life-long HAART. Although this study offers insight to molecular events in the presence of acute SP and prolonged HAART exposure in liver cells, there are limitations. The limitation of a unicellular model is always present in an *in vitro* model, which leaves out the possibility of a systemic interaction in a multiorgan setting. However, the results of this study offer a strong basis for identifying molecular targets at the clinical level. The pathways investigated can be adapted to an *in vivo* model (animal study) to further refine molecular targets of SP in the inhibition of HAART (3TC, TDF and FTC) toxicity. Herein, gene knockout studies will further solidify the favourable synergy at a molecular level

as observed in this present study. The implications of these findings can next be tested in patients who show HAART-related negative health outcomes such as MetS.

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APPENDIX A

The Potential of *Spirulina platensis* to Ameliorate the Adverse Effects of Highly Active Antiretroviral Therapy (HAART)

Thabani Sibiya[1], Terisha Ghazi[1], Anil A. Chuturgoon[1], *

[1]Department of Medical Biochemistry and Chemical Pathology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Howard College Campus, Durban 4013, South Africa

*Corresponding author: Professor Anil A Chuturgoon, Department of Medical Biochemistry and Chemical Pathology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Howard College Campus, Durban 4013, South Africa. Telephone: +27312604404. Email: chutur@ukzn.ac.za

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Review

The Potential of *Spirulina platensis* to Ameliorate the Adverse Effects of Highly Active Antiretroviral Therapy (HAART)

Thabani Sibiya, Terisha Ghazi and Anil Chuturgoon *

Discipline of Medical Biochemistry and Chemical Pathology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Howard College Campus, Durban 4013, South Africa; thabani585@gmail.com (T.S.); terishaghazi@gmail.com (T.G.)

* Correspondence: chutur@ukzn.ac.za

Abstract: The human immunodeficiency virus (HIV) is one of the most prevalent diseases globally. It is estimated that 37.7 million people are infected with HIV globally, and 8.2 million persons are infected with the virus in South Africa. The highly active antiretroviral therapy (HAART) involves combining various types of antiretroviral drugs that are dependent on the infected person's viral load. HAART helps regulate the viral load and prevents its associated symptoms from progressing into acquired immune deficiency syndrome (AIDS). Despite its success in prolonging HIV-infected patients' lifespans, the use of HAART promotes metabolic syndrome (MetS) through an inflammatory pathway, excess production of reactive oxygen species (ROS), and mitochondrial dysfunction. Interestingly, *Spirulina platensis* (SP), a blue-green microalgae commonly used as a traditional food by Mexican and African people, has been demonstrated to mitigate MetS by regulating oxidative and inflammatory pathways. SP is also a potent antioxidant that has been shown to exhibit immunological, anticancer, anti-inflammatory, anti-aging, antidiabetic, antibacterial, and antiviral properties. This review is aimed at highlighting the biochemical mechanism of SP with a focus on studies linking SP to the inhibition of HIV, inflammation, and oxidative stress. Further, we propose SP as a potential supplement for HIV-infected persons on lifelong HAART.

Keywords: HAART/ARVs; *Spirulina platensis*; oxidative stress; HIV; antioxidant; inflammation; HAART toxicity; MetS

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1. Introduction

The human immunodeficiency virus (HIV) has continued to be a global public concern due to its widespread infection rate and alarming mortality rate [1]. The Joint United Nations Programme on HIV/AIDS (UNAIDS), in its most recent report in November 2021, estimated that 37.7 million people globally are living with HIV. It was also reported that about 1.5 million new HIV infections and 680,000 AIDS-related deaths have occurred in the year 2020 [1–4]. In South Africa, approximately 8.2 million people were living with HIV in the year 2021 [4]. According to the South African mid-year population statistics 2021, there has been an unprecedented increase from 79,420 to 85,154 HIV-AIDS-related deaths in 2021 [4]. Recently, the easy availability of antiretrovirals (ARVs) has tremendously changed the pattern of death. ARVs have also helped prolong the lifespan of HIV-infected people in South Africa. Globally, about 27.5 million HIV-infected persons had access to ARVs in 2020, while approximately 5.6 million infected South Africans accessed ARVs in 2020 [1,4,5].

The highly active antiretroviral therapy (HAART) entails combining three or more antiretroviral drugs that are subject to the HIV-infected person's viral load. HAART assists in regulating viral loads and preventing the progression to AIDS. Despite its success in prolonging HIV-infected patients' lifespans, the use of HAART promotes metabolic syndrome (MetS) through an inflammatory pathway, excess production of reactive oxygen species (ROS), and mitochondrial dysfunction. Over three decades since its discovery, HAART has

significantly improved the diagnosis and management of persons with HIV [6–12]. The persistence of MetS before and during HAART treatment further highlights the need for more studies on MetS inhibitory compounds, such as *Spirulina platensis* (SP) [13], *Moringa oleifera* [14,15], Curcumin [16], and Mangiferin [17]. However, there are other alternatives to combat MetS and these include exercise training [18], life style changes, and properly balanced healthy food choices [19,20].

SP is a blue-green microalgae commonly used as a traditional food by some Mexican and African people [21,22]. SP is mostly found in the alkaline water of volcanic lakes. In addition to its popular nutritional value, SP possesses various medicinal properties. It can induce both the humoral and cellular mechanisms of the immune system when consumed [22]. Interestingly, SP was linked with MetS-lowering properties, such as hypoglycemia [23], hypolipidemia [24], and hypotension [13]. Studies in rodents suggested that SP is particularly useful in preventing MetS [13]. SP contains oxidative stress inhibitors, phycocyanin and phycocyanobilin [25–27]. Previous studies have also demonstrated that SP inhibits oxidative stress [21,25,28,29] and promotes mitochondrial health [30–33], thereby inhibiting inflammation [25,34]. Furthermore, SP can prevent the development of atherosclerosis [28] and diabetes [25]. The Food and Agriculture Organization (FAO) of the United Nations recommends *Spirulina* as a daily dietary supplement [35]. Microalgaes including *Spirulina* are environmentally friendly and have a high rate of yield in large-scale production under controlled conditions [36]. *Spirulina*, in addition to its nutritional and medicinal security, has the potential to eliminate poverty. The considerable potential for sustainable financial development in a small-scale crop for nutritional enhancement was evident in China, where the production increase resulted in a dramatic increase in profit from USD 7.6 million to USD 16.6 million. *Spirulina* production is possible for small and marginal farmers as well as enthusiastic urban gardeners; this makes it easily accessible to the population [37]. Moreover, the health system can provide SP as medication to control adverse effects in people living with HIV on HAART with minimal costs.

Due to the increasing number of HIV-infected people and their high dependence on HAART, it is imperative to explore the anti-inflammatory and antioxidant mechanisms of SP against HIV and MetS. This review explores the anti-inflammatory and antioxidant mechanisms of SP in the inhibition of MetS and its potential as a supplement against HIV and ARV-induced MetS.

2. The Roles of HIV and HAART in MetS

HIV has often been associated with MetS [38], which results in cardiovascular diseases. Recent reports have linked some HIV-related features to MetS. These characteristic features include escalated cases of cardiovascular diseases, type 2 diabetes mellitus, dyslipidemia, immunodeficiency, high viral load, and atherosclerosis [7]. Recently, studies have suggested that HAART, in addition to the above-mentioned HIV-related features, actively induces MetS in persons with HIV [7,39–41]. Earlier studies by Palios et al. (2011) on arterial stiffness, displayed by pulse wave velocity (PWV), and markers of MetS, reported that persons with HIV exhibited an increased degree of PWV when compared with the healthy controls. Subsequently, persons on HAART have been shown to exhibit a similar PWV as persons with hypertension [39,40]. The prevalence of MetS in HIV-infected people receiving antiretroviral (ARV) treatment was higher when compared to the general population. This prevalence was attributed to age, physical inactivity, and a low cluster of differentiation 4 (CD4) count [42–44]. The patient response to HAART varies; some antiviral drugs can successfully suppress the plasma viral load without increasing the CD4 count; this allows the risks of opportunistic infections and abnormalities. Failure to increase the CD4 count during HAART may be due to several factors, including drug resistance, low CD4+ T-cell count at the initiation of HAART, the advanced stage of the disease, and a low adherence to HAART [45].

The nuclear factor-kappa-light chain-enhancer of the activated B cell (NF-κB) is a protein complex that is responsible for DNA and cytokine (IL-1β, IL-6, and TNF-α) tran-

scription, chemokine activation, and the survival of cells [7]. NF-κB is also a well-known mediator of inflammation that promotes HIV transcription [7,46].

Inflammation linked to HAART is evidenced by the persistently high levels of interleukin 6 (IL-6), C-reactive proteins, and D-dimers [47]. HIV creates chronic pro-inflammatory conditions that promote MetS [7]. Studies have shown that HIV is associated with inflammation, apoptosis, and mitochondrial dysfunction [7,48]; however, the mechanism that links HIV with MetS remains unclear [7]. Herein, we highlight the significance of SP as an anti-inflammatory supplement for HIV-infected people on lifelong HAART and its mechanism of inhibition on MetS.

3. Spirulina Species

Spirulina has three commonly investigated species due to their potential therapeutic nature and high nutritional content. These *Spirulina* species include *Spirulina platensis* (SP) (otherwise known as *Arthrospira platensis*), *Spirulina maxima* (*Arthrospira maxima*), and *Spirulina fusiformis* (*Arthrospira fusiformis*). These *Spirulina* species are also classified as oxygenic photosynthetic bacteria under Cyanobacteria and Prochlorales [49–54]. SP is found in alkaline water with abundant bicarbonate and saline [22,55]. *Spirulina* species are generally three-dimensional helix microstructures [56] protected by a cell wall composed of complex sugars and proteins [22]; however, helical transformation results after mature trichomes divide into hormogonia, binary fission, and undergo length elongation [57]. SP is considered an antioxidant and anti-inflammatory agent [58]. It is also considered as a nutraceutical food supplement due to its high content of proteins, vitamins, and minerals. Moreover, its composition includes chlorophyll, phycocyanin, and carotenoid. Chlorophyll has antioxidant and antimutagenic properties [59,60], carotenoids are vitally important antioxidants with cancer-inhibiting abilities [53], and phycocyanin is a Bili protein with antioxidant and radical scavenging properties [61]. Moreso, SP has also been credited for its cancer- and viral infection-suppressing abilities [62].

3.1. Role of Spirulina in the Inhibition of Oxidative Stress

Recently, research has unveiled the important roles of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the production of reactive oxygen species (ROS). NADPH is contained in the nervous system and its assemblage and activation generate free radicals (FR) which subsequently destroy cells. *Spirulina* is a potent inhibitor of NADPH oxidase, which has been one of the proteins responsible for the production of ROS and subsequent oxidative stress [54,63,64]. SP has the potential to inhibit oxidative stress by blocking NADPH oxidase [21,25,28,29] and to enhance (Figure 1) mitochondrial health by promoting an antioxidant response [30–33]. Furthermore, SP prevents FR-induced apoptotic cell death through the inhibition of oxidative stress [65].

3.2. Multitargeted Therapeutic Roles of SP

SP has a therapeutic effect against vascular diseases, cancer, diabetes, neurodegenerative diseases, and inflammatory disorders [66]. *Spirulina* treatment enhances the NF-kappa B-directed luciferase expression [67]. It has antiallergic effects [54], including against allergic rhinitis in humans [68]. *Spirulina* is an immune booster [22]. It prevents cellular aging, infectious diseases, and promotes a strong immune system [57]. *Spirulina* has central neuroprotective effects in rodents [69]. It is also associated with the inhibitory effects against numerous viruses, such as HIV-1, herpes simplex virus 1 and 2 (HSV-1 and HSV-2), human cytomegalovirus (HCMV), influenza type A, measles, and other enveloped viruses [53,70–73]. Moreover, it has antimutagenic and anticancer effects [57]. *Spirulina* is an effective treatment against chronic arsenic poisoning with melanosis and keratosis [74]. SP shares similar chemical structures and physiological activities with bilirubin [25–28,75]. SP antioxidant properties are due to its composition and the presence of phycobilins, phycocyanin, and phycocyanobilin [25–27] (Figure 2). Phycobilins are similar in structure to bile pigments such as bilirubin, a well-known ROS scavenger [22,76]. Phycocyanin has

been proven to possess antioxidant and anti-inflammatory activities [34,54,63,64,77,78]. Phycocyanin is also structurally similar to biliverdin, a strong inhibitor of NADPH oxidase and inflammation-induced radicals [25,34]. A study conducted by Zheng et al. (2013) indicated that phycocyanin normalized urinary and renal oxidative stress markers and the expression of NADPH oxidase components. Furthermore, phycocyanobilin, bilirubin, and biliverdin inhibited NADPH-dependent superoxide production in renal mesangial cells [25]. The study also demonstrated that SP may be used in a therapeutic approach to prevent diabetic nephropathy through the inhibition of oxidative stress [25]. Thus far, phycocyanine, the most powerful natural antioxidant, is only present in cyanobacteria and thus in *spirulina*.

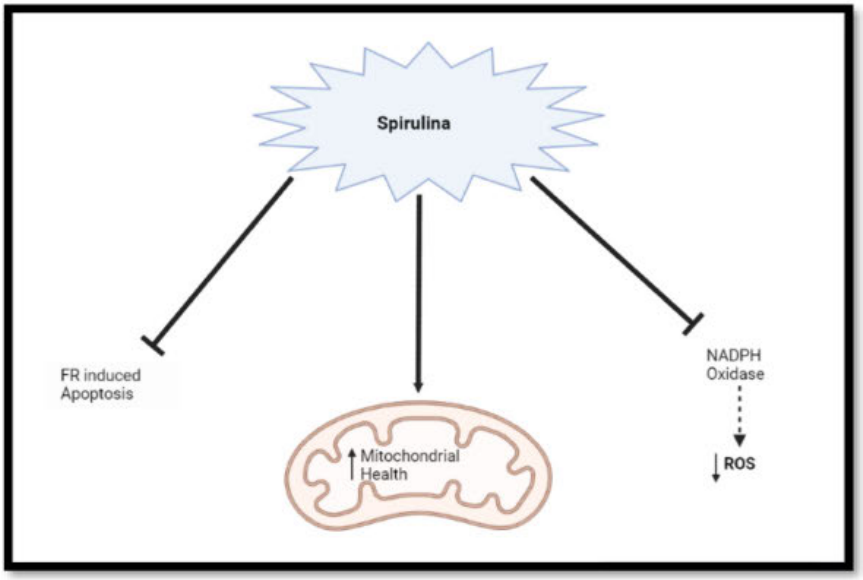

Figure 1. Diagrammatic representation of *Spirulina* reduction of oxidative stress via several pathways. *Spirulina* inhibits NADPH oxidase, reduces ROS, blocks FR-induced apoptosis, and promotes mitochondrial health (Created with BioRender.com, accessed on 17 November 2021).

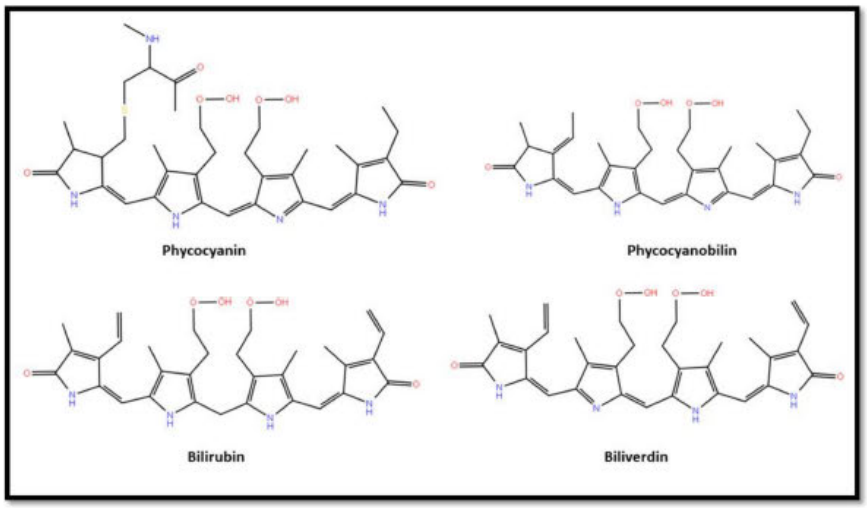

Figure 2. The 2D chemical structures of phycocyanin, phycocyanobilin, bilirubin, and biliverdin (prepared by author using maestro 11.2).

SP exhibited neuroprotective activities through antioxidant and anti-inflammatory effects [79]. It has also shown significant antioxidant activity in vitro by scavenging nitric oxide and preventing DNA damage by scavenging hydroxyl radical (Figure 3) [80]. Antidiabetic and anti-inflammatory properties of SP [81] are based on its significant free-radical scavenging activities. SP contains compounds that fall under a broad spectrum of antioxidant agents, such as alkaloids, flavonoids [82], and phycocyanin [28,83–86]. Moreso, it provides trace minerals for the synthesis of antioxidant enzymes, demonstrated by the antidiabetic response in rats [87]. It has potential benefits in assisting with the reduction in chronic inflammatory conditions [88]. *Spirulina* incorporated into skin cream showed promising results as an anti-inflammatory and a wound-healing agent [89].

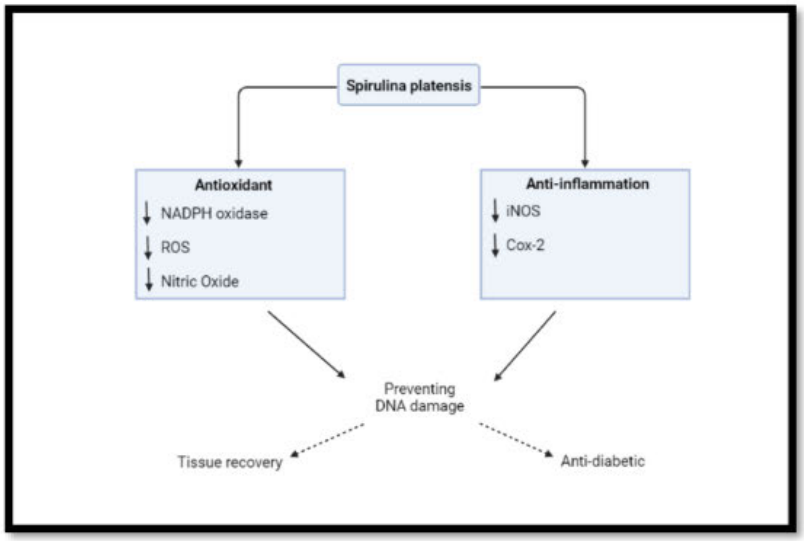

Figure 3. Antioxidant and anti-inflammatory effects of SP (created with BioRender.com, accessed on 17 November 2021).

Spirulina against HIV-1 demonstrated its ability as an antiviral compound. Studies have demonstrated the ability of SP in the inhibition of HIV-1 replication in human T-cell lines, peripheral blood mononuclear cells (PBMC), and Langerhans cells (LC). The inhibition of the viral production by *spirulina* extract (between 0.3 and 1.2 µg/mL) was found to be approximately 50% in PBMCs [57]. More studies are needed to fully understand the mechanism behind the inhibition of HIV by SP.

3.3. Mechanism of Action

SP contains several vital antioxidant and anti-inflammatory compounds as mentioned above, such as chlorophyll, phycocyanin, and carotenoids (β-carotene). The antioxidant and anti-inflammatory properties of phycocyanin have been determined in numerous studies [28,83–86,90–97]. Phycocyanin is responsible for reducing oxidative stress and NADPH oxidase [28]. It scavenges free radicals, such as alkoxy, hydroxyl, and peroxyl radicals, and decreases nitrite production and inducible nitric oxide synthase (iNOS) expression. Phycocyanin also inhibits liver microsomal lipid peroxidation [28,83–86,90–97].

Phycocyanin has been proven to inhibit the formation of the pro-inflammatory cytokine TNF-α and cyclooxygenase-2 (COX-2) expression. Additionally, it decreases prostaglandin E(2) production [28,83–86]. Phycocyanin prevents the degradation of cytosolic IκB-α, which suppresses the activation of nuclear factor-κB (NF-κB) [28]. Furthermore, the inhibitory activity of phycocyanin is associated with the suppression of TNF-α formation in the macrophages [86]. In addition, phycocyanin exerts regulatory effects on mitogen-activated protein kinase (MAPK) activation pathways, such as the p38, c-Jun N-terminal

kinase (JNK), and extracellular-signal-regulated kinase (ERK1/2) pathways [98–100]. The second compound of SP, carotenoids, β-carotene to be specific, is an antioxidant that has anti-carcinogenic, antioxidant, and anti-inflammatory activities [101–103]. As a membrane antioxidant, β-carotene protects against singlet oxygen-mediated lipid peroxidation [101]. Beta carotene inhibits the production of nitric oxide and prostaglandin E(2) and suppresses the expression of inducible nitric oxide synthase (iNOS), COX-2, TNF-α, and IL-1β. The suppression of inflammatory mediators by β-carotene results from its' ability to inhibit NF-κB activation by preventing nuclear translocation of the NF-κB p65 subunit [102,104,105]. Studies have shown that β-carotene suppressed the transcription of inflammatory cytokines such as IL-1β, IL-6, and IL-12 in vitro [103]; this takes place in the macrophages. The third compound of *spirulina*, chlorophyll, can perform antioxidant and antimutagenic activities [59,60]. *Spirulina*'s mechanism of action is a concert of compounds, but it is not clear whether they all act simultaneously during demanding events.

SP promotes the activation and expression of heme oxygenase 1 (HO-1) and endothelial nitric oxide synthase (eNOS) [104,105]. HO-1 is suggested to play an important part in the adaptive reprogramming which could result in Nrf-2 activation, but this pathway is unclear, and more studies are required. Moreso, SP causes the activation of the Nrf2/HO-1 pathway [106]. Nrf-2 activation by SP results in the production and increased expression of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) [100].

4. Common Highly Active Antiretroviral Therapy (HAART) Combinations

HAART has several classes: nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) [48,107–114], protease inhibitors (PIs) [114–117], integrase strand transfer inhibitors (INSTIs) [118–123], fusion inhibitors (FIs) [124], and chemokine receptor antagonists (CCR5 antagonists) [125–127]. HAART is a specifically selected combination of NRTI and NNRTI, PI, or INSTI drugs responsible for the inhibition of viral replication by multiple virus targets [113,128–136]. However, HAART can cause adverse drug reactions. Furthermore, 2′,3′-dideoxy-3′-thiacytidine (3TC), 2′,3′-dideoxy-5-fluoro-3′-thiacytidine (FTC), TDF (Tenofovir Disoproxil Fumarate), ZDV (Zidovudine), and d4T (Stavudine) are associated with mitochondrial toxicity and oxidative stress [137–140]. Additionally, NNRTIs are linked to toxic hepatitis, and PIs are implicated in insulin resistance and hyperlipidemia [114]. Chronic side effects linked to HAART include ROS-induced insulin resistance [141,142], lipodystrophy, gastrointestinal disorders [143], and cardiovascular disease [11,144]. Primarily, HAART is used for the treatment and prevention of HIV-1. These primary functions of HAART are achieved by attacking different components of the virus lifecycle which ensures inhibition regardless of the virus being resistant to one of the drugs [113,128–136]. A combination of two NRTIs (mostly FTC and TDF) and one NNRTI (e.g., EFV; Efavirenz) is a more favorable choice due to the convenience to dose, effectiveness, and less toxic effects compared to other drug combinations [114,145,146]. A combination of three NRTIs is less effective than two NRTIs with an NNRTI [147]. The d4T/ddI combination is associated with high toxicity and hence it is not often recommended [148]. The most popular NRTIs are cytidine analogs (XTC), FTC, 3TC, and TDF, which form part of the first-line therapy [149–153]. FTC and 3TC are similar in chemical structures with different pharmacokinetic and pharmacodynamic properties, and they have required deoxynucleosides for HIV DNA synthesis. They undergo phosphorylation through intracellular kinases to become FTC 50 -triphosphate (FTC-TP) and 3TC-TP; triphosphate metabolites with FTC-TP are more efficiently incorporated during HIV DNA synthesis than 3TC-TP [145,154–157]. Moreover, 3TC-TP has a shorter intracellular half-life compared to FTC-TP [155]. The TDF and FTC combination has a synergistic effect, increasing intracellular metabolites, and they are recommended for pre-exposure prophylaxis (PrEP) [155,158–161]. Contraindications (Table 1) on HAART treatment are antiretroviral medication-specific and can be overcome by a change in the HAART combination to suit the individuals own treatment [107,108,119–121,131,162–165] or the supplementation and treatment of the symptoms.

Table 1. HAART drugs mechanism and their adverse effects.

HAART	Mechanism	Example	Adverse Effect
Nucleoside/Nucleotide reverse transcriptase inhibitors (NRTIs)	NRTIs require intracellular phosphorylation via host enzymes before they can inhibit viral replication. These agents are nucleoside or nucleotide analogs with an absent hydroxyl at the 3′ end that are incorporated into the growing viral DNA strand. They competitively bind to reverse transcriptase and cause premature DNA chain termination as they inhibit 3′ to 5′ phosphodiester bond formation.	abacavir, didanosine, lamivudine, stavudine, tenofovir, emtricitabine, atazanavir, and zidovudine	Mitochondrial toxicity, bone marrow suppression, anemia, and lipodystrophy. NRTIs inhibit mitochondrial DNA polymerase. Tenofovir may cause kidney injury or decreased bone mineral density or osteoporosis. Abacavir is associated with a CD8-mediated hypersensitivity reaction in patients with the HLA-B*5701 mutation. Didanosine is associated with a high risk of pancreatitis and hepatomegaly.
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	NNRTIs bind to HIV reverse transcriptase at an allosteric, hydrophobic site. These agents cause a stereochemical change within reverse transcriptase, thus inhibiting nucleoside binding and inhibition of DNA polymerase.	delavirdine, efavirenz, nevirapine, rilpivirine	Temporary rashes but may progress to Stevens–Johnson's syndrome. Hepatitis may progress to liver failure. Efavirenz may cause teratogenicity Risk of neural tube defects. NNRTIs (mostly rilpivirine) may result in QT prolongation. Numerous interactions with hepatic cytochrome P450 enzymes. Efavirenz is linked to various psychiatric and CNS effects, including, but not limited to: vivid dreams, delusions, sleep disturbances, dizziness, headaches, increased suicidality, psychosis-like behavior, and mania.
Protease inhibitors (PIs)	PIs competitively inhibit the proteolytic cleavage of the gag/pol polyproteins in HIV-infected cells. These agents result in immature, noninfectious virions. PIs are administered with boosting agents such as ritonavir or cobicistat to patients that are failing their initial HAART combination.	atazanavir, darunavir, indinavir	Hepatotoxicity, insulin resistance, hyperglycæmia, hyperlipidemia, lipodystrophy, and PR interval prolongation. Other PIs are inefficient and have a high resistance and increased risk of nephrolithiasis, hence indinavir and saquinavir are no longer used.
Integrase strand transfer inhibitors (INSTIs)	Integrase inhibitors bind viral integrase and prevent viral DNA from being incorporated into the host cell chromosome.	dolutegravir, elvitegravir, raltegravir	Some patients may experience dizziness, sleep disturbances, or depression. Raltegravir and dolutegravir can cause rhabdomyolysis and myopathy. Dolutegravir can block the secretion of creatinine and occasionally cause a decrease in the GFR. It can also have interactions with several medications, including those that inhibit/induce CYP3A4 enzymes, metformin, rifampin, and antiepileptics.
Fusion inhibitors (FIs)	Fusion inhibitors bind to the envelope glycoprotein gp41 and prevent viral fusion to the CD4 T-cells.	enfuvirtide	Enfuvirtide is generally well tolerated though some patients may experience injection site reactions.
Chemokine receptor antagonists (CCR5 antagonists)	CCR5 antagonists selectively and reversibly block entry into the CD4 T-cells by preventing interaction between CD4 cells and the gp120 subunit of the viral envelope glycoprotein.	maraviroc	Some patients may experience dizziness or skin rashes. There is a risk of hepatotoxicity with allergic features, high risk of hepatic dysfunction. Drug–drug interactions should be a consideration if patients are taking concurrent CYP3A4 inhibitors or inducers.

Abbreviations: HLA—human leucocyte antigen; CNS—central nervous system; GFR—glomerular filtration rate.

5. Mechanism of HAART-Induced Oxidative Stress, Inflammation, and Mitochondrial Dysfunction

The exact mechanism of HAART-induced oxidative stress has not been completely explored; however, studies have demonstrated the link between HAART use and oxidative stress. HAART is linked with lipid metabolism dysfunction through the induction of peripheral lipodystrophy. Lipodystrophy results from the impaired cytoplasmic retinoic-acid binding protein type 1 (CRABP1)-mediated cis-9-retinoic acid stimulation of peroxisome proliferator-activated receptor type gamma (PPAR-γ), leading to impaired differentiation and increased apoptosis of peripheral adipocytes. HIV-1 protease-inhibitors further inhibit the cytochrome P450 3A-mediated synthesis of cis-9-retinoic acid, one of the key activators of PPAR-γ [166]. Insulin resistance occurs following impaired fat storage and lipid release [141,142,166], which impacts the oxidant profile. The depletion of ATP production and mitochondrial dysfunction [8,9], and the depletion of mitochondrial DNA [167–169], are some of the ways HAART induces oxidative stress. HIV increases oxidative stress, and HAART increases lipid oxidation, which amplifies the ROS imbalance leading to increased oxidative stress complications [10,170].

Chronic exposure to HAART induces increased oxidative stress in endothelial cells and mononuclear cell recruitment, which leads to cardiovascular diseases in HIV patients on ARV therapy [12]. Inducing oxidative stress is common for protease and reverse transcriptase inhibitors [171]. HAART drugs induce oxidative stress in various ways, which include inhibiting DNA pol-γ activity and leading to mitochondrial dysfunction, and also through the depletion of mitochondrial DNA [167–169]. Studies have shown that patients on HAART have abnormally high levels of free oxygen radicals in sera compared to untreated HIV patients and HIV-uninfected participants [10,11,168,170,172,173].

Adverse drug reactions vary; TDF and lopinavir cause acute and chronic renal dysfunction [174–178]. TDF inhibits mitochondrial DNA-polymerase gamma, hence leading to the impaired function of energy-dependent transporters [179,180]. NRTIs are associated with the inhibition of mitochondrial DNA polymerase, lactic acidosis, subcutaneous lipoatrophy, peripheral neuropathy, and pancreatitis. The level of mitochondrial toxicity depends on the drugs; it is low with 3TC, FTC, and TDF, followed by ZDV, and higher with d4T. NNRTIs are associated with life-threatening skin reactions and toxic hepatitis. PIs are associated with insulin resistance and hyperlipidemia [7,114].

6. Combined and Synergistic Therapeutic Actions of HAART and SP

Studies have shown that the possible therapeutic effects of antioxidants may provide strategies in suppressing oxidative stress-induced comorbidities that emerge with the use of HAART therapy in HIV-infected individuals [12]. The combination of HIV and HAART has been associated with increased oxidative stress and lipid peroxidation. Furthermore, HIV or HAART induces ROS by inducing NADPH oxidase [181,182]. Interestingly, SP is a potent antioxidant [26,27] with anti-inflammatory activities [34], which makes it a potential supplement in the mitigation of oxidative stress induced by HAART adverse drug reactions. Moreover, SP can inhibit NADPH oxidase which is considered one of the main sources of ROS and free radicals in HIV-infected persons on HAART [34,181,182], resulting in reduced oxidative stress [28]. Moreover, β-carotene from SP protects against singlet oxygen-mediated lipid peroxidation [101]. Among HAART complications, TDF and lopinavir cause acute and chronic renal dysfunction [174–178]. Herein, phycocyanin from SP can normalize urinary and renal oxidative stress markers and inhibit NADPH-dependent superoxide production in renal mesangial cells [25], ameliorating renal dysfunction. Lately, SP has been an effective therapeutic approach to preventing diabetic nephropathy through the inhibition of oxidative stress [25]. These properties indicate SP as a potential agent to mitigate renal dysfunction caused by HAART. As mentioned above, NRTIs can inhibit mitochondrial DNA polymerase [179,180]. Studies in vitro showed that SP can enhance cell nucleus enzyme function, repair DNA synthesis [57], and enhance mitochondrial health [30–33,80]. Mitochondrial toxicity presented as peripheral neuropathy and lactic

acidosis can be corrected by SP through providing trace minerals for the synthesis of antioxidant enzymes [87] and reducing chronic inflammatory conditions [88].

NNRTIs are associated with life-threatening skin reactions and toxic hepatitis [114], these conditions may be ameliorated by SP. Phycocyanin from SP can inhibit liver microsomal lipid peroxidation [28,83–86,90–97], and hence reducing toxic hepatitis. Moreso, SP incorporated into skin creams showed promising results as an anti-inflammatory and a wound-healing agent [89]; this can be beneficial in the mitigation of NNRTI-induced skin reactions. PI therapy induces insulin resistance and hyperlipidemia [7]. Additionally, HAART may be associated with a higher risk of myocardial infarction [114,183,184]. SP has a therapeutic effect against vascular diseases, cancer, diabetes, and neurodegenerative diseases [66]. In addition, the *Spirulina* family has also shown central neuroprotective effects in rodents [69] and may exert its neuroprotective activities through antioxidant and anti-inflammatory effects [79]. Therefore, SP is a recommended antioxidant to use as a supplement; the list of benefits is evident. It also has antiallergic effects [54], prevents cellular aging and infectious diseases, and promotes a strong immune system [57]. Herein, promotion of a strong immune system by SP can help increase CD4 cell counts, lower HIV viral loads, and slow down the progression to AIDS. Moreover, SP prevents FR-induced apoptotic cell death [65]; this may help decrease apoptosis of peripheral adipocytes induced by HAART. Chemically, SP is a recommended source of proteins, vitamins, and minerals [57], important nutrients for individuals on the HAART program. Finally, SP can assist HAART in the inhibition of HIV-1 replication because it has been shown to inhibit viral production in PBMCs.

There has been a number of clinical studies to investigate whether SP improves the quality of life in HIV-infected individuals. Marcel (2011) reported that insulin sensitivity in HIV patients improved more when a spirulina nutritional supplement was used instead of soybean [185]. Another study demonstrated for the first time that spirulina improves antioxidant capacity in people living with HIV [186]. *Spirulina* supplementation combined with a qualitative balanced diet showed potential to inhibit lipid abnormalities [187], significantly increase CD4 cells, and reduce the viral load in HIV-infected antiretroviral-naive patients [187–189]. However, there is limited information on the investigation of SP confirming the mechanism of antioxidant and anti-inflammatory effects and the impact on the quality of life in the HIV-positive population taking HAART. Thus far, it has been shown that supplementation with *Spirulina* platensis could improve the immune status of HIV patients on ART and decrease inflammatory and pro-oxidant levels [190]. The development of more clinical studies to confirm the SP protective effect in this population will answer many questions. The recommended concentrations of SP for daily supplementation varies, as studies have successfully used 19 g [185], 5 g [186], and 10 g [190,191].

7. Conclusions

HIV continues to be a major global cause of mortality. Besides the therapeutic benefits of HAART in HIV treatment, HAART has been linked to numerous adverse drug reactions which include oxidative stress, inflammation, and the disruption of mitochondrial function. SP as an antioxidant, anti-inflammatory, anticancer, and nutritional supplement possesses various corrective properties against attacks from viruses and bacteria. The corrective health properties of SP are largely attributed to antioxidant pigments found in SP. These pigments include chlorophyll, carotenoids, and phycocyanin which facilitate antioxidant, anti-inflammatory, and anticancer properties. The corrective properties of SP indicated in this review highlight its potential in the mitigation of HAART adverse drug reactions and MetS. The SP supplement potential is also supported by its ability to assist HAART in the inhibition of HIV-1. This review highlighted the corrective properties of potent antioxidant SP potential as a supplement for individuals on lifelong HAART experiencing MetS. Furthermore, this review highlights the need for more studies on SP and HAART synergy.

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APPENDIX B

Apoptosis assessment (Caspase activity)

Caspase activity was assessed via luminometry. ARVs/*Spirulina* treatment varied in terms of response of all caspases measured: Caspase-9, showed a non-significant decrease in all ARVs/Spirulina treated cells (p = 0.1444; 95% CI, (3TC: -652800 to 1445000), (TDF: -870400 to 1228000), (FTC: -283400 to 1815000)) (Figure 1). As shown in Figure 2, Caspase-8, 3TC and FTC were increased, while TDF was decreased (p = 0.0186; 95% CI, (3TC: -1783000 to 601700), (TDF: -607900 to 1777000), (FTC: -1905000 to 480100)). Caspase-3, FTC increased while 3TC and TDF decreased (p = 0.0024; 95% CI, (3TC: -496400 to 2211000), (TDF: -452700 to 2254000), (FTC: -2261000 to 445400)) (Figure 3).

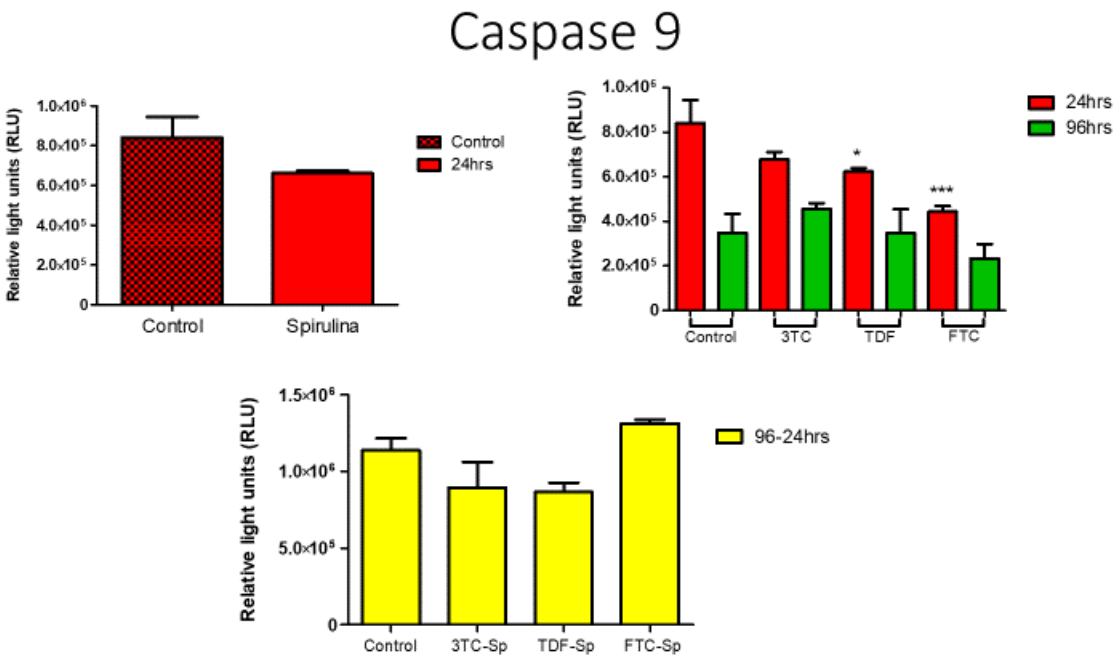

Figure 1 Caspase 9 expression. expression of caspase 9 after exposure to SP, ARVs, and expression of caspase 9 after exposure to ARVs followed by SP, where a single asterisk represents significance $p < 0.05$

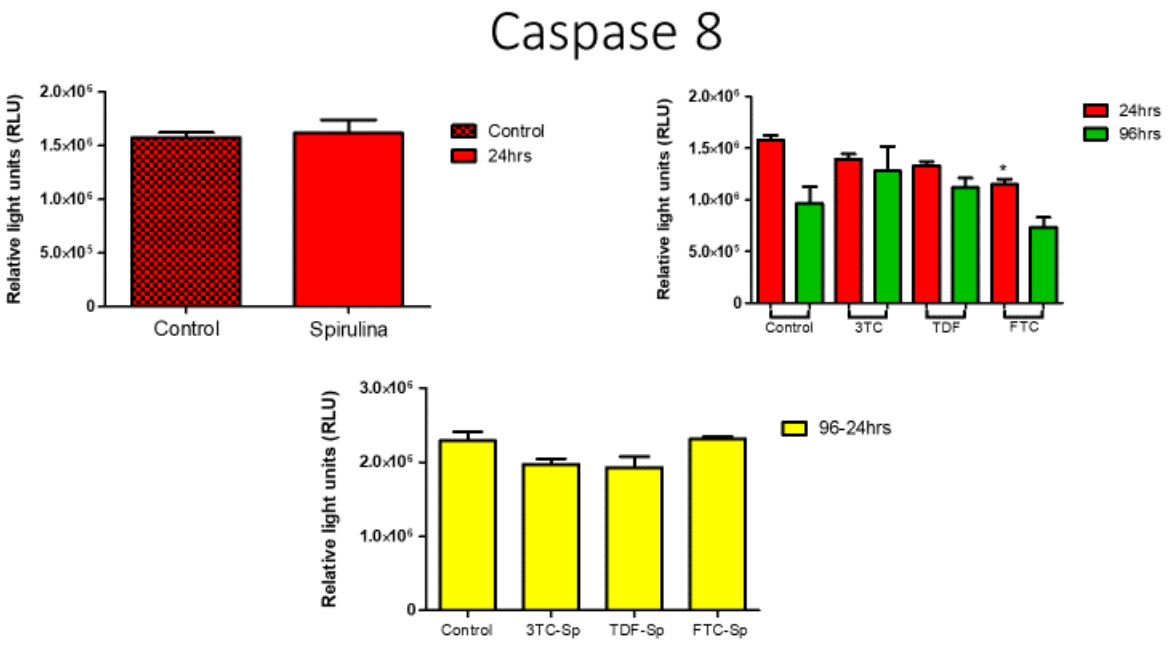

Figure 2 Caspase 8 expression. expression of caspase 8 after exposure to SP, ARVs, and expression of caspase 8 after exposure to ARVs followed by SP, where a single asterisk represents significance $p < 0.05$

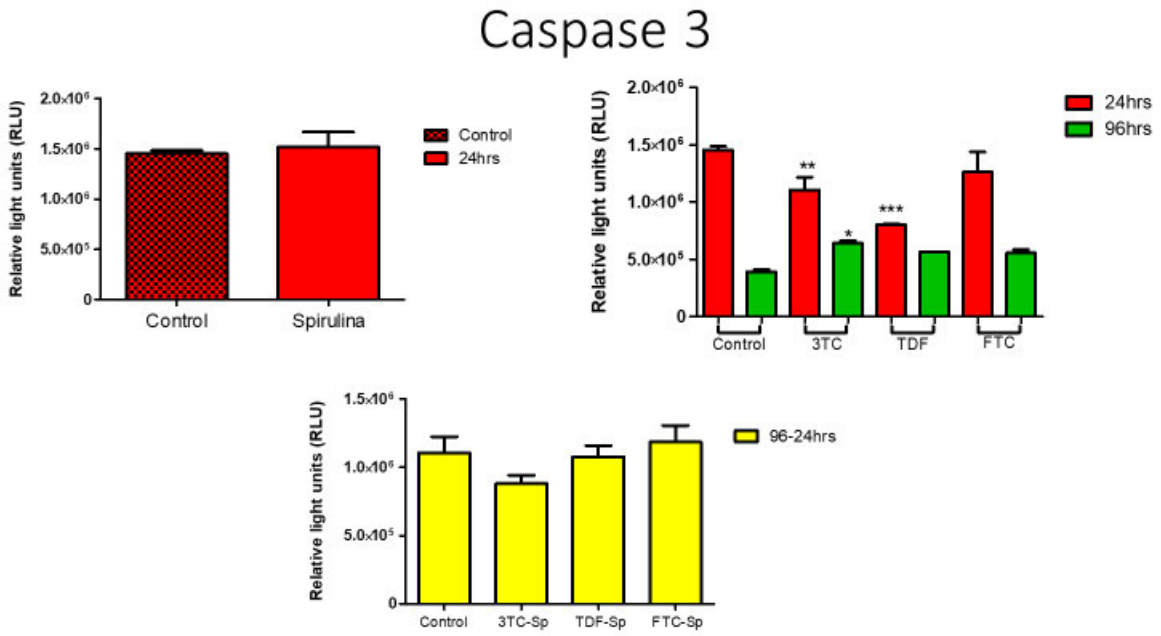

Figure 3 Caspase 3 expression. expression of caspase 3 after exposure to SP, ARVs, and expression of caspase 3 after exposure to ARVs followed by SP, where a single asterisk represents significance $p < 0.05$

APPENDIX C

Mitochondrial stress responses

Extracellular levels of LDH were quantified using a colorimetric assay, following 4hrs and 24hrs exposure periods. The increase in LDH release suggests increased cell damage [97], and can be an early indicator of increased necrotic cell death. SP and HAART indicated low LDH levels A: ($p = 0.0288$), B: ($p < 0.0001$), C: ($p < 0.0001$) (Figure 1). ROS induced lipid peroxidation is responsible for oxidative damage and reduction of cell membrane function [103]. LDH is exclusively found in the cytoplasm and only exits the cell through damaged membranes [96].

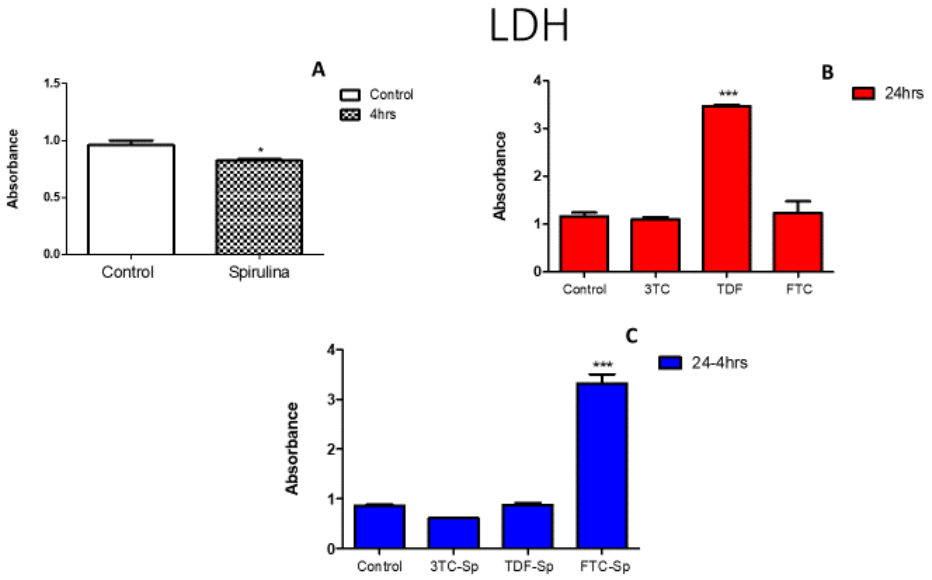

Figure 1. Intracellular LDH levels A: ($p = 0,0288$), B: ($p < 0.0001$), C: ($p < 0.0001$).

APPENDIX D

Micro-RNA Response

The main function of miRNA is to control gene expression at the post-transcriptional level through degrading or repressing target mRNAs. MiR-146a levels A: (p = 0.0002), B: (p < 0.0001) increased in SP and HAART treated cells. However, HAART-SP indicated a decrease C: (p = 0.1778) (Figure 1). The miR-155 levels increased in SP and HAART treated cells A: (p = 0.0924), B: (p < 0.0001). HAART-SP indicated a decrease C: (p = 0.0149) (Figure 2).

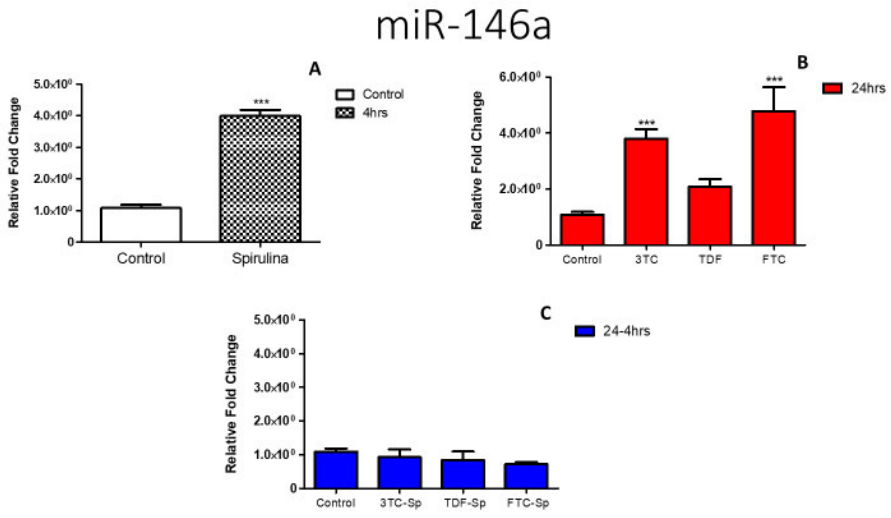

Figure 1. MiR-146a levels A: (p = 0.0002), B: (p < 0.0001), C: (p = 0.1778).

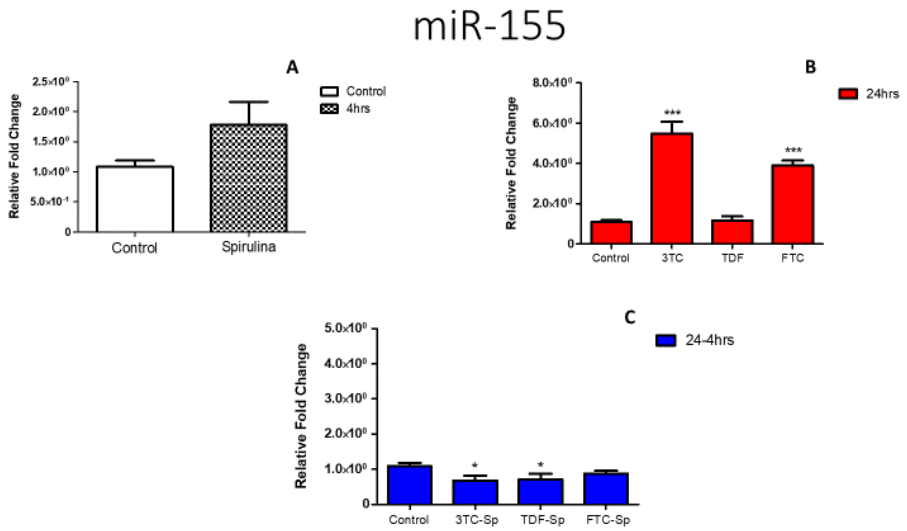

Figure 2. The miR-155 levels A: (p = 0.0924), B: (p < 0.0001), C: (p = 0.0149).

APPENDIX E

Detoxification of peroxides

Catalase (CAT) maintains the redox balance. CAT mRNA expression increased in SP treated cell and mostly decreased in HAART treated cells A: (p = 0.0054), B: (p = 0.0341) (Figure 1). However, CAT mRNA expression increased in HAART-SP exposed cells C: (p = 0.0069) (Figure 1). Glutathione Peroxidase (GPx) uses low molecular weight thiols such as glutathione as cofactors, and converts H_2O_2 to H_2O, GPx also converts lipid peroxides to H_2O. GPx mRNA expression increased in SP treated cells and mostly decreased in HAART treated cells A: (p = 0.0007), B: (p = 0.0001) (Figure 2). HAART-SP treated cells indicated an increase in comparison to the control C: (p = 0.0005) (Figure 2).

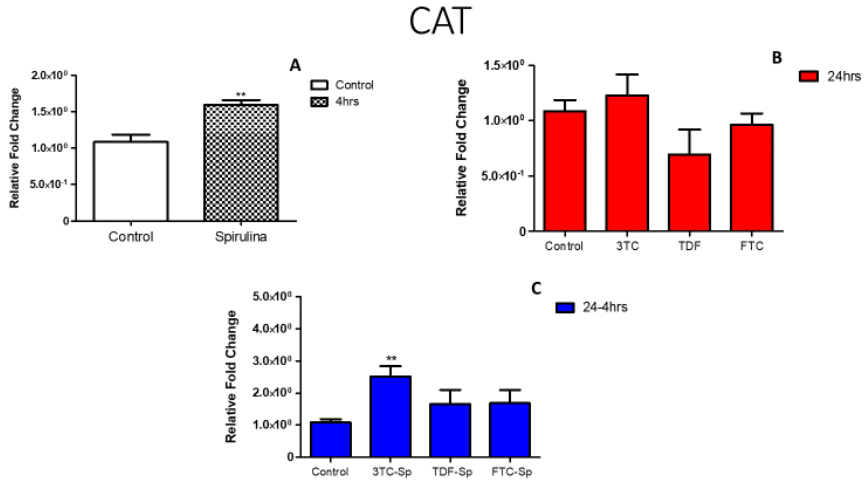

Figure 1. CAT mRNA expression A: (p = 0.0054), B: (p = 0.0341), C: (p = 0.0069).

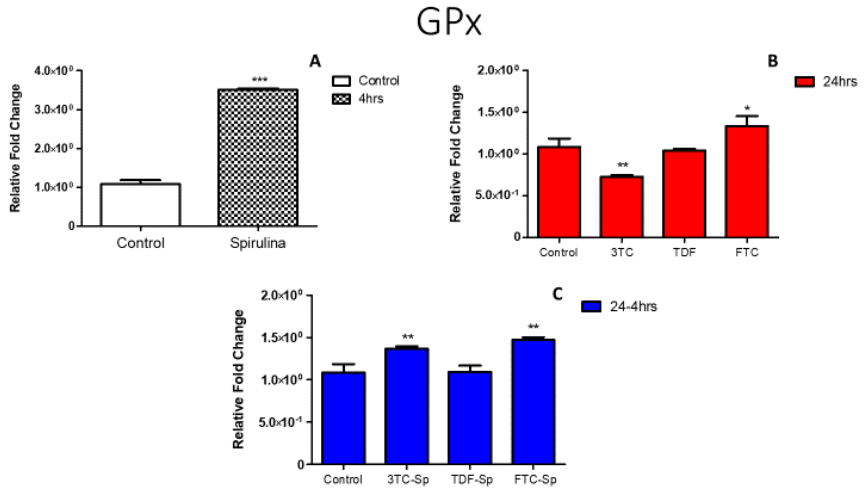

Figure 2. GPx mRNA expression A: (p = 0.0007), B: (p = 0.0001), C: (p = 0.0005).

APPENDIX F

Inflammatory Response

NF-κB in the nucleus, it binds to specific DNA regions thus inducing gene transcription of several genes that contribute to pro-inflammatory functions [45]. NF-kB mRNA expression of SP treated cells is normal compared to the control and mostly increased in HAART treated cells A: (p = 0.8275), B: (p = 0.0011). However, HAART-SP treated cells showed a reduced expression compared to the control C: (p < 0.0001) (Figure 1).

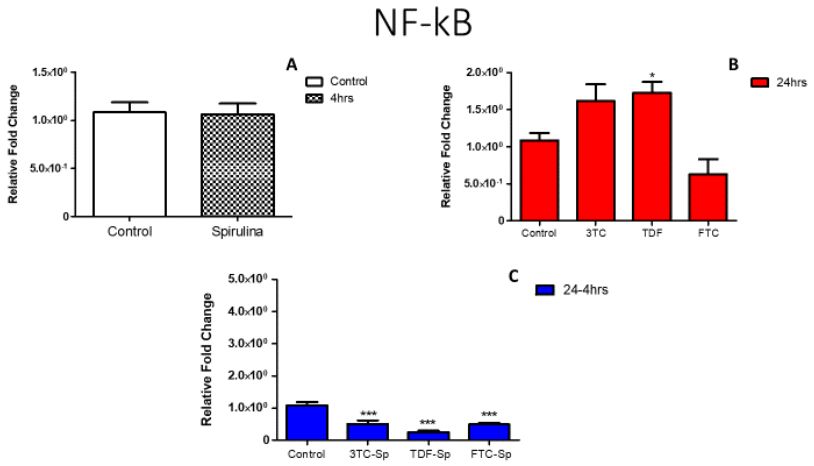

Figure 1. NF-kB mRNA expression A: (p = 0.8275), B: (p = 0.0011), C: (p < 0.0001).

APPENDIX G

Cell Death

Caspase activity was assessed via luminometry. Caspase 9 levels increased in cells exposed to SP for 4 hrs and decreased in HAART (24hrs) treated cells A: (p = 0.0205), B: (p = 0.0002). However, HAART-SP treated cells indicated an increased level of Caspase 9 C: (p = 0.0178) (Figure 1). Caspase 8 levels followed the same pattern as caspase 9 levels A: (p = 0.2138), B: (p < 0.0001), C: (p = 0.0034) (Figure 2). Levels of caspase 3 A: (p = 0.3680), B: (p = 0.0004), C: (p < 0.0001) (Figure 3) followed the same pattern observed in Caspase 9 and caspase 8.

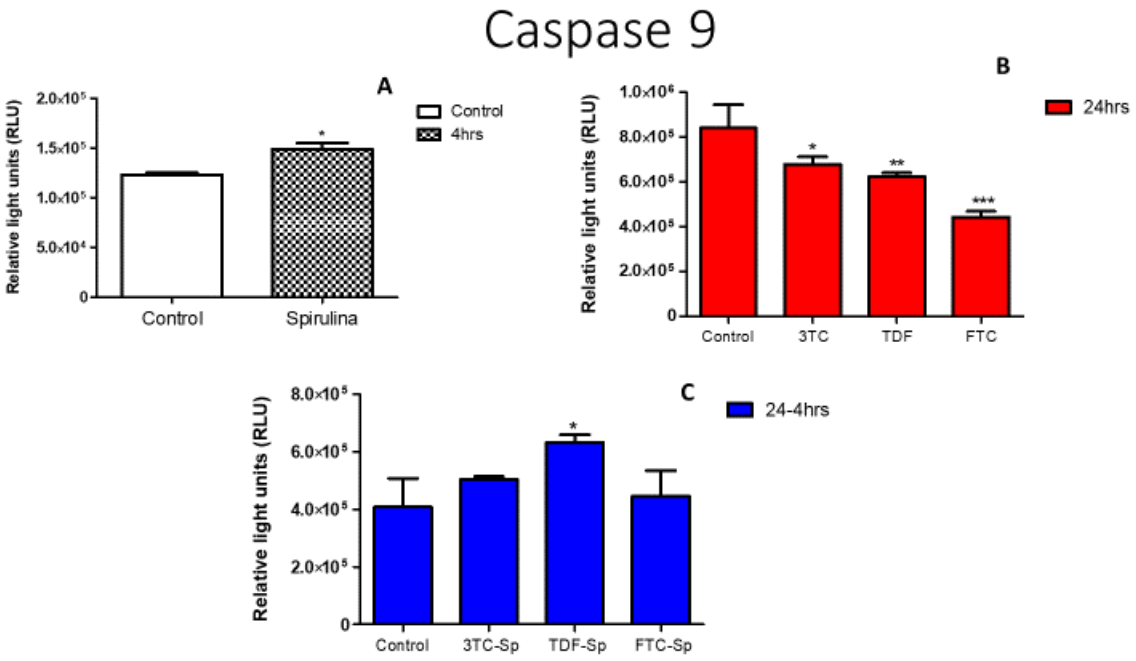

Figure 1. Caspase 9 levels A: (p = 0.0205), B: (p = 0.0002), C: (p = 0.0178). RLU: relative light units.

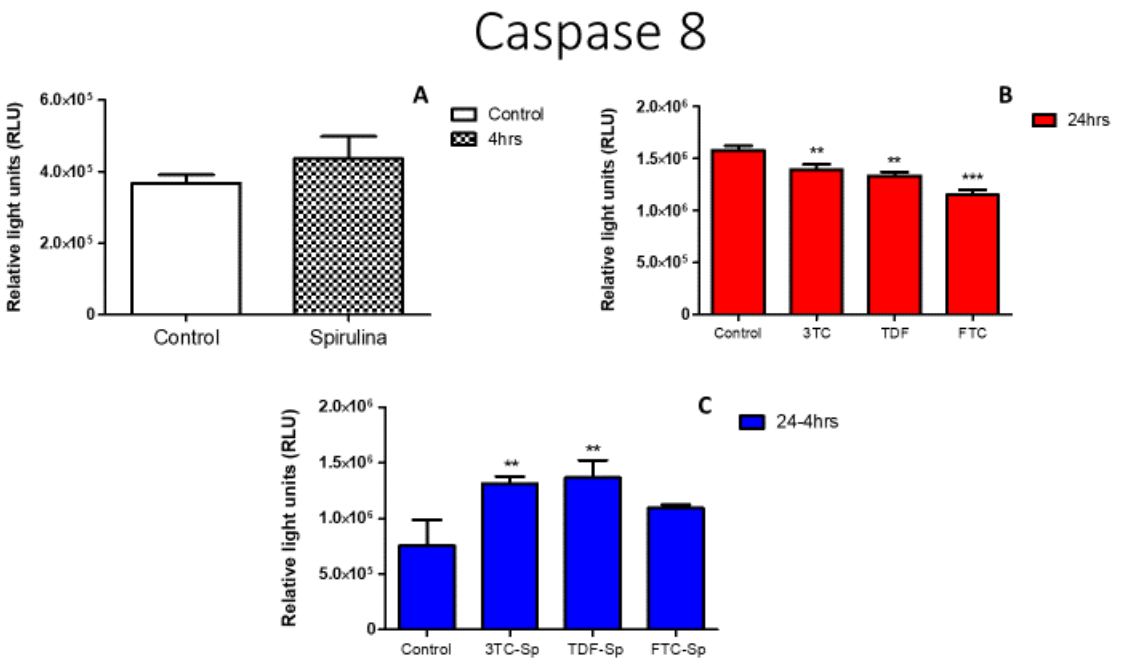

Figure 2. Caspase 8 levels A: (p = 0.2138), B: (p < 0.0001), C: (p = 0.0034). RLU: relative light units.

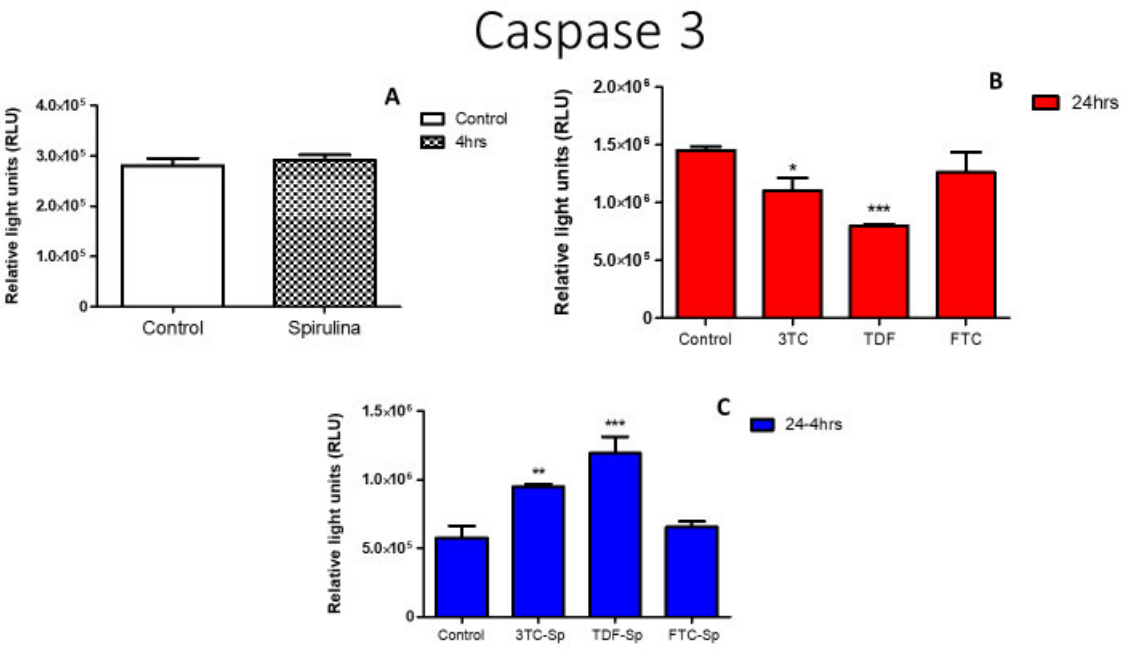

Figure 3. Levels of caspase 3 A: (p = 0.3680), B: (p = 0.0004), C: (p < 0.0001). RLU: relative light units.