

Article

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

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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-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 [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).

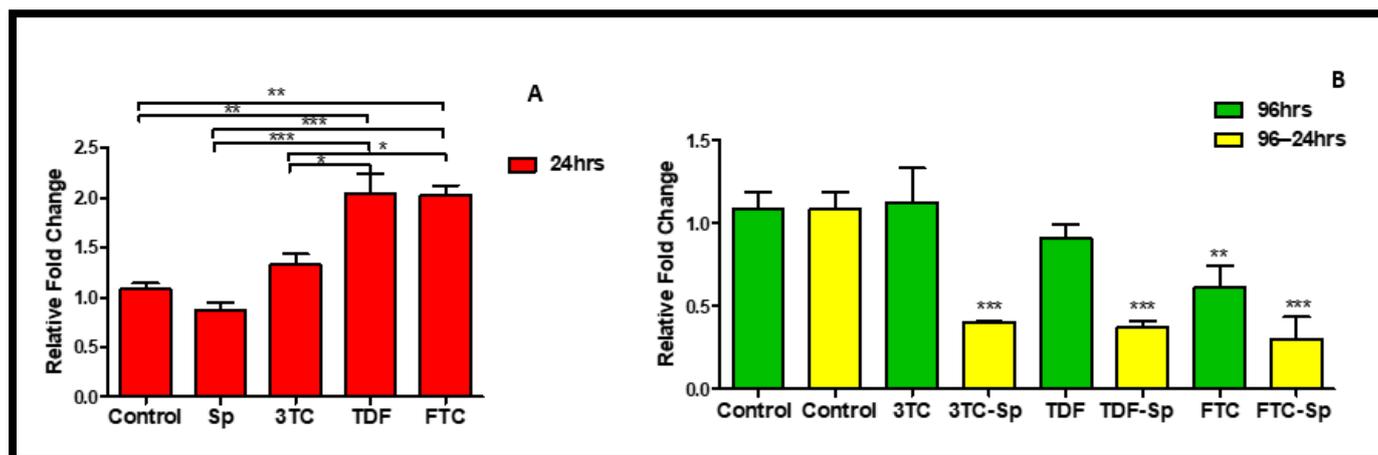


Figure 1. Effects of SP and HAART (3TC, TDF, and FTC) on *Akt* mRNA levels. *Akt* mRNA levels after exposure of HepG2 cells to (A): SP and HAART for 24 h, (B): HAART (96 h), and HAART (96 h) followed by SP (24 h); * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

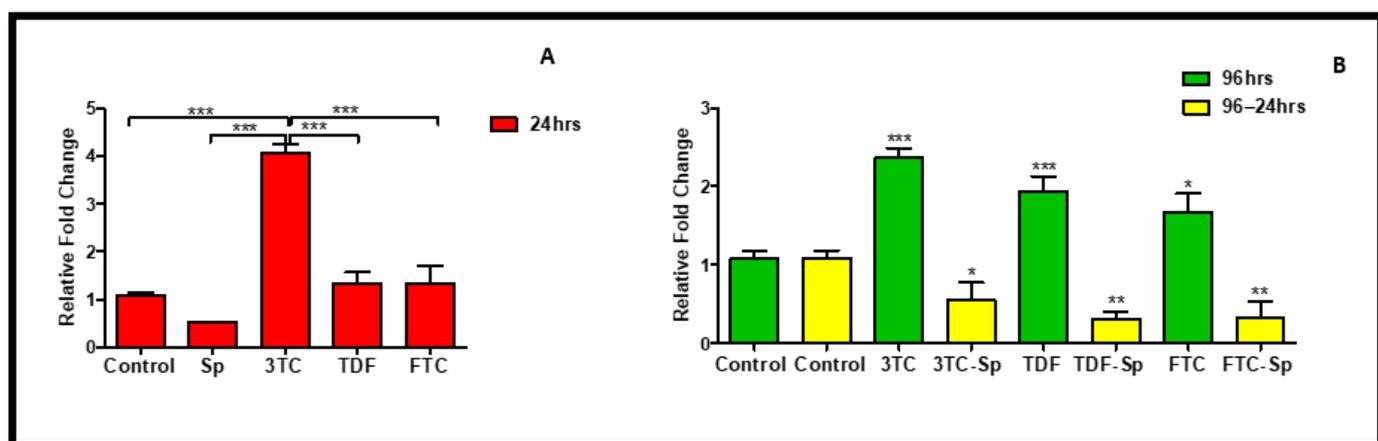


Figure 2. Effects of SP and HAART (3TC, TDF, and FTC) on *eNOS* mRNA levels. *eNOS* mRNA levels after exposure of HepG2 cells to (A): SP and HAART for 24 h, (B): HAART (96 h), and HAART (96 h) followed by SP (24 h); * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

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 ($OH\cdot$) and nitric oxide ($NO\cdot$); 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).

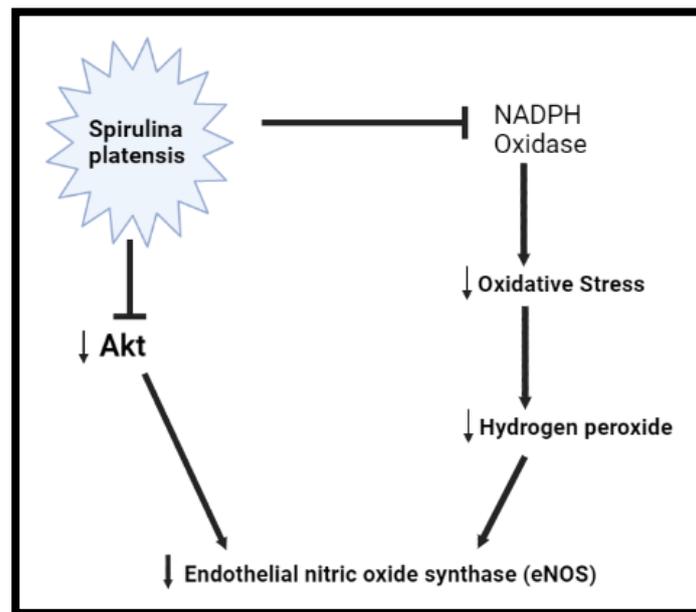


Figure 3. Diagrammatic representation of *Spirulina platensis* reduction of oxidative stress via inhibition of NADPH oxidase, leading up to *eNOS* mRNA downregulation. *Spirulina platensis* inhibits NADPH oxidase, reduces ROS, and blocks free radical species including H_2O_2 , leading to the reduction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

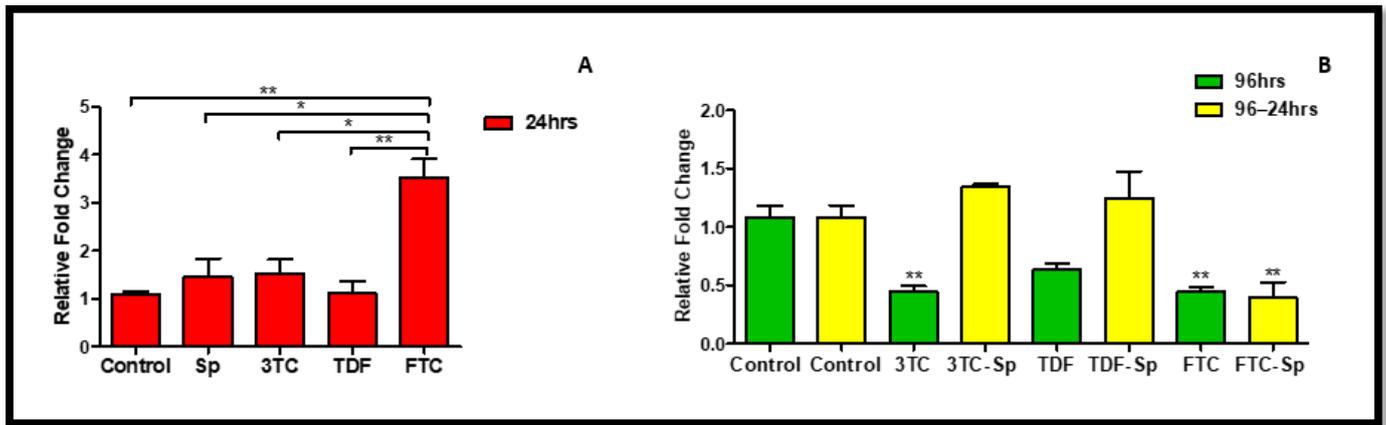


Figure 4. Effects of SP and HAART (3TC, TDF, and FTC) on *NRF-2* mRNA levels. *NRF-2* mRNA levels after exposure of HepG2 cells to (A): SP and HAART for 24 h, (B): HAART (96 h), and HAART (96 h) followed by SP (24 h); * $p < 0.05$, ** $p < 0.005$.

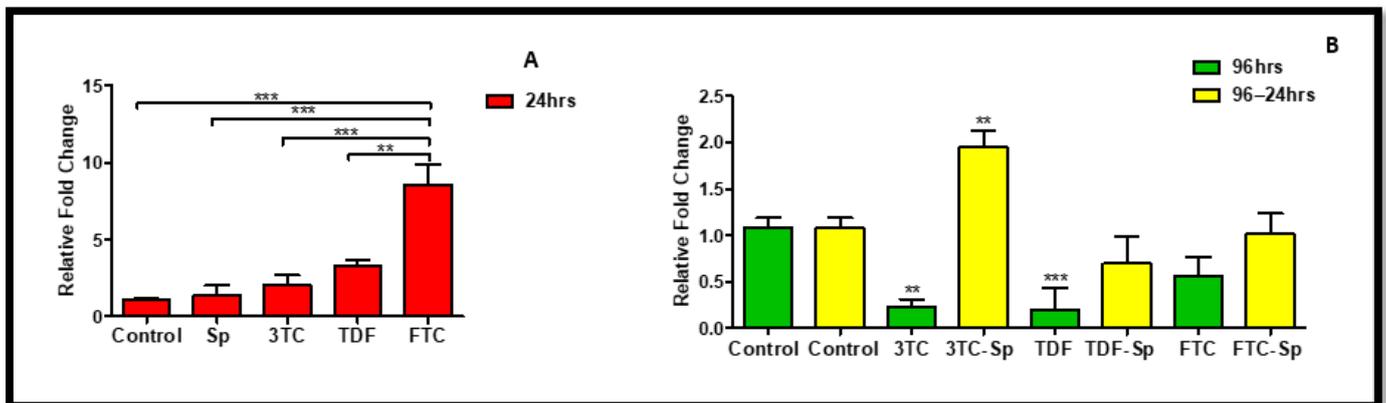


Figure 5. Effects of SP and HAART (3TC, TDF, and FTC) on *Keap1* mRNA levels. *Keap1* mRNA levels after exposure of HepG2 cells to (A): SP and HAART for 24 h, (B): HAART (96 h), and HAART (96 h) followed by SP (24 h); ** $p < 0.005$, *** $p < 0.0001$.

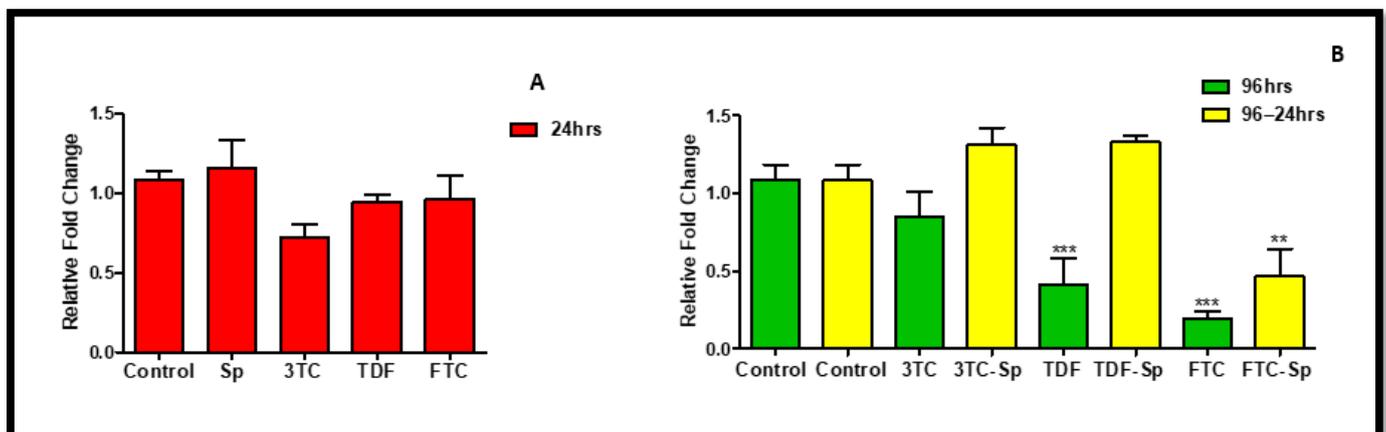


Figure 6. Effects of SP and HAART (3TC, TDF, and FTC) on *NQO-1* mRNA levels. *NQO-1* mRNA levels after exposure of HepG2 cells to (A): SP and HAART for 24 h, (B): HAART (96 h), and HAART (96 h) followed by SP (24 h); ** $p < 0.005$, *** $p < 0.0001$.

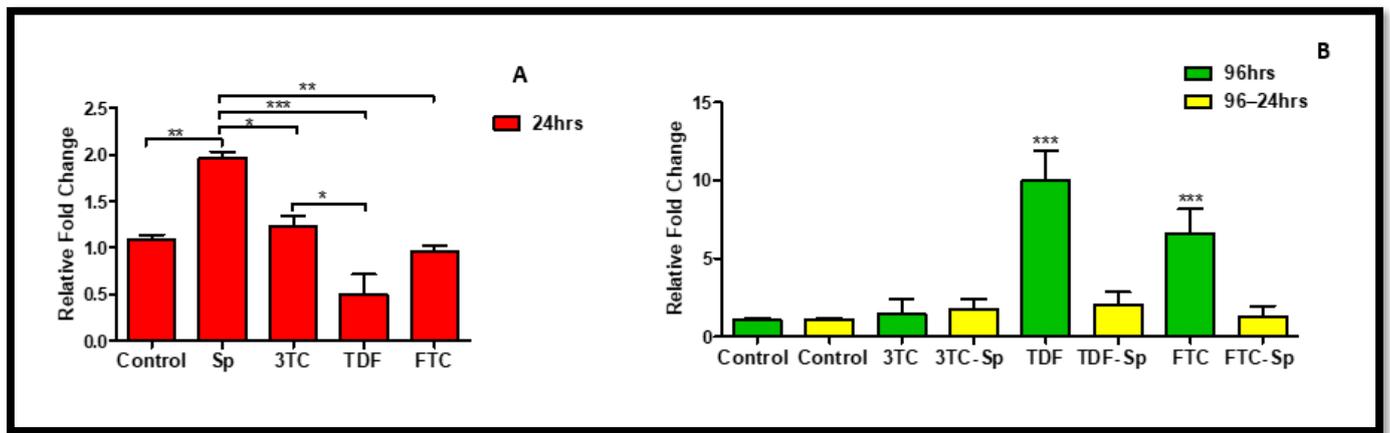


Figure 7. Effects of SP and HAART (3TC, TDF, and FTC) on *CAT* mRNA levels. *CAT* mRNA levels after exposure of HepG2 cells to (A): SP and HAART for 24 h, (B): HAART (96 h), and HAART (96 h) followed by SP (24 h); * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

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).

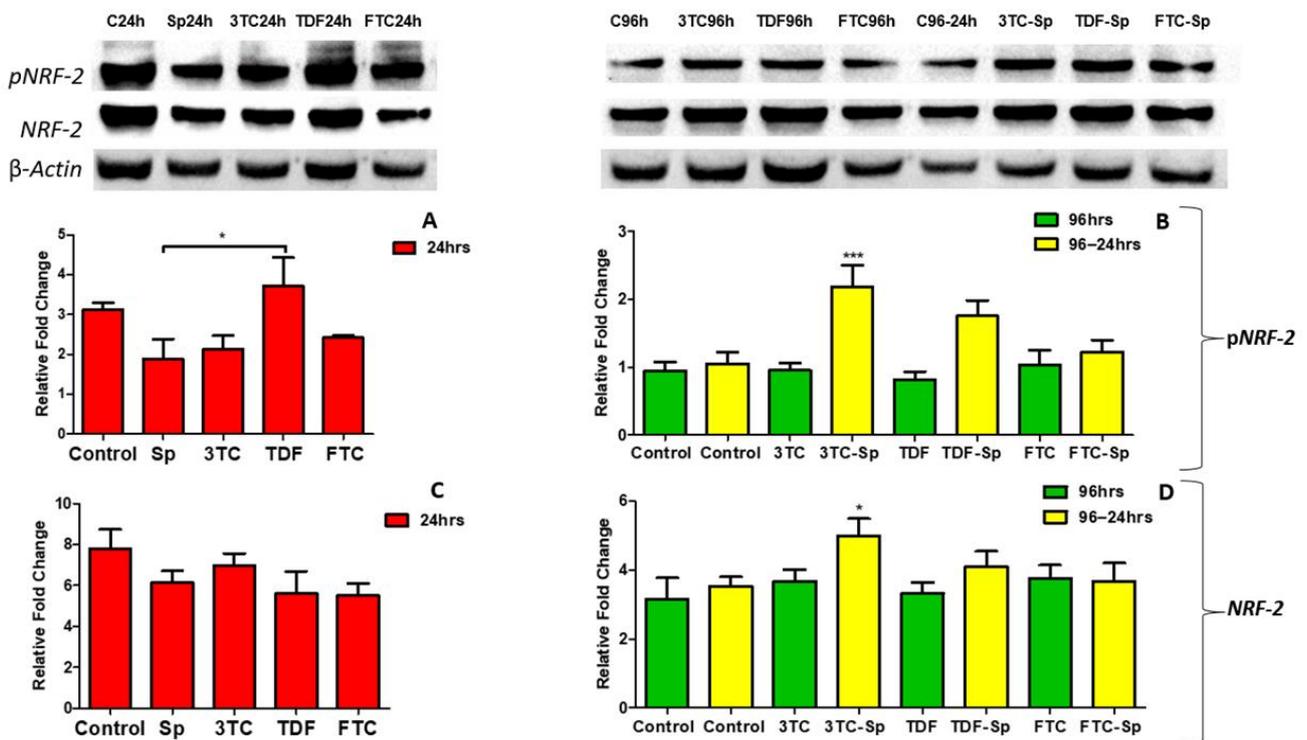


Figure 8. Effects of SP and HAART (3TC, TDF, and FTC) on pNRF-2 and NRF-2 protein expression. pNRF-2 protein expression after exposure of HepG2 cells to (A): SP and HAART for 24 h, (B): HAART (96 h), and HAART (96 h) followed by SP (24 h); NRF-2 protein expression after exposure of HepG2 cells to (C):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

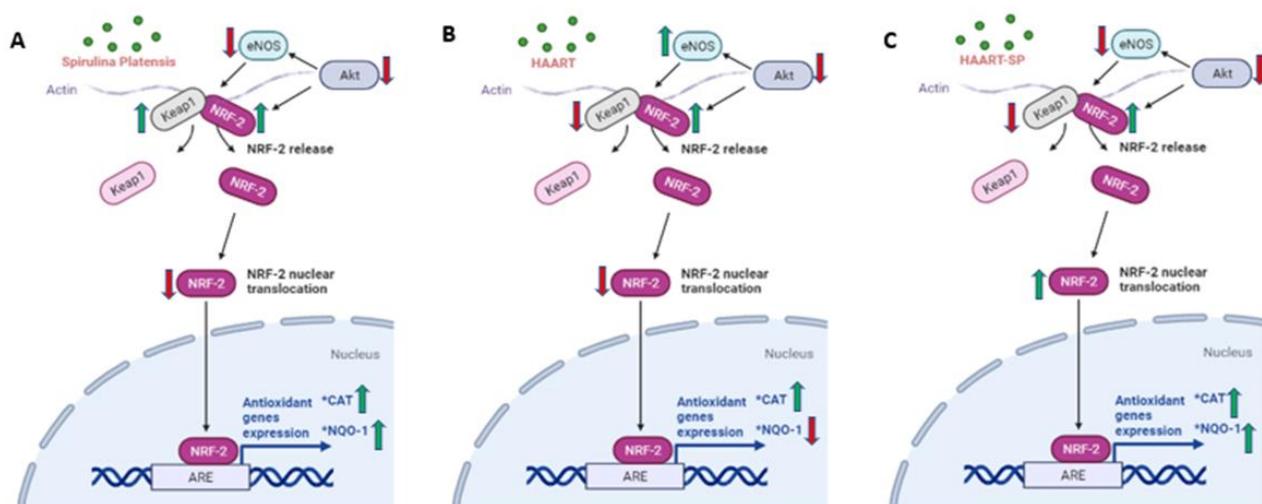


Figure 9. Effects of *Spirulina platensis* and HAART (3TC, TDF, and FTC) on antioxidant induction pathway in HepG2 cells; (A): the effect of *Spirulina platensis*; (B): the effect of HAART; (C): the effect of HAART-SP (created with BioRender.com).

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 (dH₂O)), 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 (10⁶ cells per 25 cm³ 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 cm³ 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 Laemmli buffer [dH₂O, 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 μ L) 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 dH₂O, 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% H₂O₂ 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/ μ L. 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 μ L), forward primer (1 μ L), reverse primer (1 μ L), nuclease-free water (2 μ L), and the cDNA template (1 μ L). 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 $2^{-\Delta\Delta C_t}$ 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 \pm 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
<i>Nrf-2</i>	60 °C	Forward Reverse	5'-CACATCCAGTCAGAAACCAGTGG-3' 5'-GGAATGTCTGCGCCAAAAGCTG-3'	2446	NM_006164.5	772 bp
<i>Keap1</i>	59.3 °C	Forward Reverse	5'-CTGGAGGATCATACCAAGCAGG-3' 5'-GGATACCCTCAATGGACACCAC-3'	2524	NM_012289.4	1599 bp
<i>CAT</i>	53 °C	Forward Reverse	5'-GTGCGGAGATTCAACACTGCCA-3' 5'-CGGCAATGTTCTCACACAGACG-3'	2291	NM_001752.4	1043 bp
<i>NQO-1</i>	59.7 °C	Forward Reverse	5'-GAAGAGCACTGATCGTACTGGC-3' 5'-GGATACTGAAAGTTCGCAGGG-3'	2407	NM_001025434.2	1907 bp
<i>eNOS</i>	59.7 °C	Forward Reverse	5'-GTGGCTGTCTGCATGGACCT-3' 5'-CCACGATGGTGATTGGCT-3'	2058	NM_001160109.2	1788 bp
<i>Akt</i>	59.7 °C	Forward Reverse	5'-TGGACTACCTGCACTCGGAGAA-3' 5'-GTGCCGCAAAGGTCTTCATGG-3'	2922	NM_001382431.1	1468 bp
<i>GAPDH</i>	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 ₂ O

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 ₂ O 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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