



Lipid-Lowering and Antioxidant Activity of RF13 Peptide From Vacuolar Protein Sorting-Associated Protein 26B (VPS26B) by Modulating Lipid Metabolism and Oxidative Stress in HFD Induced Obesity in Zebrafish Larvae

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Accepted: 30 January 2022 / Published online: 3 March 2022
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Abstract

Obesity is one of the major contributing factors responsible for the onset of various metabolic syndrome. In obesity-related metabolic syndrome, increased oxidative stress in accumulated fat is a key pathogenic mechanism. The aim of this study is to investigate the lipid-lowering and antioxidant effects of RF13 peptide derived from vacuolar protein sorting associated protein 26B (VPS26B) in high fat diet (HFD) induced zebrafish larvae. Based on the arrangement, composition, and bioinformatics analysis of the VPS26B protein sequence, we predicted and synthesized a short peptide, ¹RRGKGRRVTMSF¹³ (RF13). *In vitro* analysis (DPPH, ABTS and nitric oxide assay) of RF13 on showed its potential antioxidant properties and radical scavenging capacity. Rat skeletal muscle cells (L6) and human erythrocyte cells are used for the evaluation of RF13 cytotoxicity at differing concentrations showed no cytotoxic effect. Zebrafish larvae feeding HFD, on day 4 post fertilization (dpf) to 6 dpf, there was an increase in production of reactive oxygen species (ROS) and lipid accumulation, which showed decreased expression of antioxidative enzymes such as glutathione S-transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPx). However, RF13 treatment has significantly reduced the total cholesterol and triglycerides levels, enhanced antioxidant enzyme levels, and decreased lipid peroxidation. In addition, RF13 treatment has significantly decreased lipid accumulation, intracellular ROS and apoptosis in zebrafish larvae as compared to the HFD group. Furthermore, RF13 significantly downregulated the lipid metabolizing genes such as CCAAT/enhancer-binding protein- α (C/EBP- α), sterol regulatory element-binding protein 1 (SREBP1) and fatty acid synthase (FAS), while they upregulated the antioxidant-related genes. These results suggests that RF13 peptides can be used as functional foods and natural drugs to enhance the antioxidant ability and also to alleviate lipid accumulation by regulating lipid metabolism.

Keywords Vacuolar protein sorting-associated protein · Lipid accumulation · Antioxidant · Zebrafish · High fat diet

Abbreviation

| | |
|-----------------|---|
| VPS26B | Vacuolar protein sorting associated protein 26B |
| HFD | High fat diet |
| FAS | Fatty acid synthase |
| LPL | Lipoprotein lipase |
| PPAR | Peroxisome proliferator-activated receptor |
| C/EBP- α | CCAAT/enhancer-binding protein- α |
| SREBP1 | Sterol regulatory element-binding protein 1 |
| ROS | Reactive oxygen species |
| GR | Glutathione reductase |
| GPx | Glutathione peroxidase |

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| | |
|-------|--|
| SOD | Superoxide dismutase |
| CAT | Catalase |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| ABTS | 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid |
| HFD | High fat diet |
| DCFDA | 2',7'-dichlorofluorescein diacetate |
| DPPP | Diphenyl-1-pyrenylphosphine |
| RNS | Reactive nitrogen species |

Introduction

Obesity has emerged as a major public health problem in developed countries. It has been linked to a number of metabolic disorders, including hypercholesterolemia, type 2 diabetes, fatty liver, atherosclerosis, high blood pressure and certain types of cancers (Sung et al. 2011). Obesity is defined as the excessive body fat deposition in the adipose tissue (adiposity) due to imbalance in food intake and energy expenditure (Friedman, 2009). The fatty acid synthase (FAS) and lipoprotein lipase (LPL) are the two important enzymes involved in the accumulation of fat in the cytoplasm of adipocytes (Guru et al. 2021a). Three major transcription factors have been found to regulate the transcriptional expression of these lipid metabolizing enzymes: peroxisome proliferator-activated receptor (PPAR), C/EBP- α , and SREBP1 (Chang et al. 2006).

Obesity and associated pathologies, such as insulin resistance and type 2 diabetes, are intricately linked to oxidative stress. Overconsumption of high fat and carbohydrate containing diet can stimulate specific signalling pathways in different cell types which can promote oxidative stress and inflammation (Savini et al., 2015). On the other hand, oxidative stress can affect food intake and stimulates adipocyte proliferation and differentiation, making it important for bodyweight management (Dandona et al. 2010). Overproduction of reactive oxygen species (ROS) can degrade proteins, nucleic acids and cause cell damage. Obesity also affects the antioxidant enzyme system by depleting the activities of GR, GPx, superoxide dismutase (SOD) and catalase (CAT) (Batista et al. 2014).

Many new pharmacological approaches for obesity management have recently been investigated. However, these pharmacological drugs to control appetite were found to be linked to various side effects like stomachache, constipation, vomiting, insomnia and heart attack (Bray 2005). The naturally occurring components have been shown to help people in losing weight and also shown to avoid diet-induced obesity. Antioxidants are the most important compounds used in modern nutrition and pharmacology (Moro and Basile 2000). They can lower body weight and adiposity

by lowering blood glucose, triglycerides and low density lipoprotein (LDL) cholesterol levels and also increase the energy expenditure and fat oxidation (Mashmoul et al. 2013). According to the literature, natural compounds are reported to inhibit various enzymes involved in fat metabolisms, such as pancreatic lipase, LPL and glycerophosphate dehydrogenase (Birari and Bhutani 2007; Issac et al. 2020; Petra et al. 2009).

Vacuolar protein sorting associated protein 26B (VPS26B) functions as a retromer complex subunit. VPS26B is to be predominantly present in the cytoplasm and is localized to the actin polymerization-rich regions of the plasma membrane (Kerr et al. 2005). VPS26B is a retromer complex-binding protein that does not associate with the endosome. This VPS26B found in lamellipodia contains VPS26A and VPS35 retromer components (Seaman 2004). In this study, we have derived a peptide, RF13 from VPS26B retromer component previously identified from our transcriptome study on freshwater teleost, striped murrel, *Channa striatus* (Kumaresan et al. 2018). The physical and chemical properties of this peptide has been determined by using various bioinformatics tools. The *in vitro* antioxidant activity of RF13 peptide was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and nitric oxide assays. Furthermore, the *in vivo* antioxidant and antiobesity effects of RF13 peptide was evaluated using a high fat diet induced obese zebrafish larvae model.

Materials and Methods

Chemicals and Reagents

Dulbecco's modified eagle's medium (DMEM) (containing 4.5 g glucose, 4.0 g glucose, 4.0 mM L-Glutamine, 1 mM sodium pyruvate and 1.5 g/L of sodium bicarbonate) were purchased from Himedia, Mumbai, India. Tricaine, Oil red O, Nile red, Triton X-100, Trolox, and 1–2-propylene glycol were purchased from Sigma-Aldrich. KAPA SYBR FAST one-step qRT-PCR master mix kit was obtained from Roche. Triglycerides and cholesterol kit was purchased from Robonik, India. All other chemicals and reagents purchased are molecular grade.

Prediction and Synthesis of Antioxidant Peptide

The full-length VPS26B cDNA was obtained from the earlier established cDNA library of *C. Striata* (Arockiaraj et al. 2013; Kumaresan et al. 2018). On the Expasy translate tool (<https://web.expasy.org/translate/>), the obtained cDNA was translated into protein sequence in silico. The

ProtParam tool (<https://web.expasy.org/protparam/>) was used to determine the physical and chemical parameters of the cDNA and protein sequences. Polyview-2D (<https://polyview.cchmc.org/>) was used to predict the secondary structure, and the I-TASSER (Iterative Threading Assembly Refinement) (<https://zhanggroup.org/I-TASSER/>) program was used to predict the 3D structure of VPS26B, which was then visualized using Pymol (version 2.5) (Velayutham et al. 2021a). The amino acid sequence, molecular weight, amino acid composition, hydrophobicity, and evolutionarily conservation were all characterized for RF13. ProtParam Tool-Expasy was used to examine the number, composition, theoretical value, molecular weight, and stability index of the amino acids in RF13. RF13 possess a net charge of +5, and the sequence composition is predicted based on the peptide property calculator (version 3.1). The peptide (version 2.0) program (<https://www.peptide2.com/>) was used to investigate the attribute and hydrophobic property of the RF13 peptide. Helical wheel projection analysis (<https://www.bioinformatics.nl/cgi-bin/emboss/pepwheel>) was used to identify the hydrophobicity and helical structure of RF13. Based on the confirmation from bioinformatics outputs, RF13 has been synthesized at Zhengzhou peptides pharmaceutical technology Co.Ltd., China. Peptide stock of 1 mM was dissolved in PBS was utilized for all the assays.

***In vitro* ROS scavenging activity of RF13**

To evaluate the antioxidant potential of RF13 peptide, the following antioxidant assays, including DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-di-(3-ethylbenzothiazoline sulfonic acid) and nitric oxide radical scavenging assay were performed using a UV-Vis spectrophotometer, as reported in our earlier study (Velayutham et al. 2021b). Trolox was used as the positive control for these cell-free assays.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

The effect of RF13 on the viability of L6 cells was assessed by MTT assay (Guru et al. 2021b; Issac et al. 2021b). Briefly, 3×10^5 cells were seeded in a flat bottomed 96 well microtitre plate containing fresh DMEM medium and treated with different concentrations of RF13 peptide (5 μ M, 25 μ M, 50 μ M, and 100 μ M). After 24 h incubation, 5 mg/mL of MTT solution was added to each well and incubated for 4 h at 37 °C in CO₂ incubator (Heal force, China). Further, the MTT was removed, and 100 μ L of DMSO was added to solubilize the formazan crystals. The absorbance was measured at 570 nm using a microplate reader (Multiskan GO, Thermo scientific, Finland).

Hemolytic Assay

The hemolytic activity was evaluated against human erythrocytes to determine the toxicity of the RF13 peptide. The assay was performed as reported in our previous study (Gopinath et al. 2021; Guru et al. 2021c). The human erythrocytes were treated with different concentrations of RF13 peptide; Triton X-100 was used as a positive control. The absorbance was measured at 560 nm using a UV-Vis spectrophotometer (UV1800, SHIMADZU, Kyoto, Japan).

Zebrafish Larvae Maintenance and Treatment

Wild type (AB strain) zebrafish were obtained from S.B Aquarium, Guduvanchery, Tamilnadu, India). Zebrafish embryos were obtained through spawning of adult zebrafish, and the embryos were collected immediately after spawning. The embryo and larvae are reared in the embryonic medium at 28 °C. For the HFD experiments, the zebrafish larvae were subjected to cotreatment as described previously (Lee et al. 2015; Misselbeck et al. 2019). The 4 dpf larvae were divided into three groups (n = 100 larvae/group): (1) a control group, in which larvae are given normal diet and kept untreated in E3 medium; (2) HFD group, in which larvae were fed with HFD from 4 to 6 dpf, and (3) the RF13 treatment groups (5 μ M, 25 μ M, and 50 μ M) of zebrafish larvae were exposed to co-treatment of HFD. The HFD was stopped at the end of 6 dpf, and the sample was collected for further analysis.

Zebrafish Toxicity Test

To determine whether RF13 peptide is toxic to zebrafish larvae (n = 20), 4dpf larvae were placed in a 6 well plate containing embryonic medium and exposed to various concentrations of RF13 peptide while untreated larvae were served as a control. During the 24 h of RF13 peptide treatment, parameters such as mortality rate and heart rate were measured (Ramachandran et al. 2017).

Measurement of Larval Weight

The weight of the larvae was measured using the microbalance (Wensar, India) (Hachicho et al. 2015). The larvae fed with HFD and treatment with the RF13 group were transferred to tin capsules and water. The sample was dried at 60 °C for 30 min, and dry weight was calculated.

Oil red O Staining

Oil red O stain was performed as described previously (Lin et al. 2017) with slight modification. The whole larvae were

incubated for 1 h at 65 °C in the dark in 0.5% Oil red O diluted in 100% 1,2-propylene glycol. The stained larvae were washed serially with volume/volume % of 100%, 80%, 40%, and 20% 1,2-propylene glycol for about 25 min to fade the background color. The stained larvae were washed three times in PBS and stored in 70% glycerol. Using a bright-field dissecting microscope, images of lipid droplets were observed.

Nile Red Staining

Nile red staining was performed as described previously (Ahmad et al. 2019). To prepare a final concentration of 0.5 µg/mL, Nile red was dissolved in acetone. The larvae were stained using Nile red for 30 min in the dark. After staining, the zebrafish larvae were washed in PBS and anesthetized with 0.05% tricaine. Finally, using fluorescent microscope Cool SNAP-Pro colour digital camera (Olympus, Tokyo, Japan), the stained larvae were observed and photographed.

Table 1 Primers used in the gene expression study

| Gene | Primer | Reference |
|---------|---|----------------------------|
| β-actin | Forward: 5'- ATGGATGAGGAAATCGCT-GCC -3' | (Liang et al. 2015) |
| | Reverse: 5'- CTCCCTGATGTCTGGGTC-GTC -3' | |
| C/EBP-α | Forward: 5'- AACGGAGCGAGCTT-GACTT -3' | (Shieh et al. 2010) |
| | Reverse: 5'- AAATCAT-GCCATTAGCTGC -3' | |
| SREBP1 | Forward: 5'- CATCCACATGGCTCT-GAGTG -3' | (Shieh et al. 2010) |
| | Reverse: 5'- CTCATCCACAAAGAAGC-GGT -3' | |
| FAS | Forward: 5'-GCACCGGTAATAAGGTTGGA-3' | (Cha et al. 2020) |
| | Reverse: 5'-CAGACGCCATGTTCAAGAGA-3' | |
| GPx | Forward: 5'-AGATGTTCATCTGCACACG-3' | (Timme-Laragy et al. 2009) |
| | Reverse: 5'-AAGGAGAAGCTTCTCAGCC-3' | |
| GST | Forward: 5'-TCTGGACTCTTCCCGTCTCTCAA-3' | (Timme-Laragy et al. 2009) |
| | Reverse: 5'-ATTCACTGTTGCCGTTGCCGT-3' | |
| GSR | Forward: 5'-TAAACAAAACCGAAGATGGG-3' | (Gopinath et al. 2021) |
| | Reverse: 5'-TTAAACCTGTAGCCGAAAGC-3' | |

Estimation of Triglycerides and Total Cholesterol Level

Each of the treatment group were homogenized in 200 µL of pre-cooled PBS and centrifuged for 4 min at 8000 xg. The supernatant was then collected and used for the analysis. According to the test kit instructions, the total cholesterol and triglyceride levels in the supernatant were measured in a microplate reader at a wavelength of 510 nm (Robonik, India).

Antioxidant Enzyme Assay

The enzyme assay was performed in HFD induced zebrafish larvae (6 dpf). Briefly, zebrafish larvae were homogenized using 0.1 M ice-cold phosphate buffer (pH 7.4). The homogenate was then centrifuged at 5000 x g for 15 min. The supernatant was collected and used for protein, SOD, and CAT estimation (Bradford, 1976; Marklund and Marklund 1974; Sinha 1972). For performing the biochemical assays, 30 embryos/group was taken, all experiment was done in triplicates.

Quantification of ROS, Apoptosis, and Lipid Peroxidation in HFD Zebrafish Larvae

The levels of intracellular ROS were measured using the 2'-7'-dichlorofluorescein diacetate (DCFDA) staining dye, while lipid peroxidation was measured using the Diphenyl-1-pyrenylphosphine (DPPP) oxidation-sensitive fluorescent probe dye. Furthermore, oxidative-stress-induced cell death was measured using the acridine orange nucleic acid selective dye (Issac et al. 2021a, c; Wang et al. 2020). In brief, HFD and RF13 peptide exposed larvae were washed and incubated separately for 1 h at 28.5 °C in the dark with DCFDA solution (20 µg/mL), DPPP (25 µg/mL) and acridine orange (7 µg/mL). The larvae were washed to remove excess dye and anesthetized with ice-cold PBS for visualization after incubation with fluorescence dye. The fluorescence image was captured using fluorescent microscope equipped with Cool SNAP-Pro colour digital camera (Olympus, Tokyo, Japan). Image J software (Version 1.49, NIH, USA) was used to quantify the fluorescence intensity (Gopinath et al. 2021).

Real-Time PCR

The expression of lipogenic specific genes (C/EBP-α, SREBP1 and FAS) and antioxidant gene (GPx, GST and GR) were analyzed to assess the effect of RF13 peptide on HFD induced zebrafish larvae to investigate the fundamental mechanisms of action. Total RNA was isolated using

Trizol method from zebrafish larvae ($n = 30$) and quantified using a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, USA) (Velayutham et al. 2021b). The primers listed in Table 1 were used to perform the quantitative real-time PCR (qPCR) assay. According to the manufacturer's protocol, KAPA SYBR FAST one-step qRT-PCR master mix kit was used to perform qPCR in a Light Cycler 96 (Roche Diagnostics GmbH, Germany). The $2^{-\Delta\Delta C_t}$ method was used to determine relative gene expression (Livak and Schmittgen 2001).

Statistical Analysis

All experiments were performed in triplicates, and the data were provided in the study is the mean of triplicates \pm standard deviation (SD). One-way and two-way ANOVA followed by Dunnett's multiple comparisons was performed for all data sets using GraphPad prism (Version 5.03).

Results

Prediction and Characterization of RF13 Peptide

VPS26B cDNA sequence was identified from the previously developed *C. striatus* transcriptome dataset. The VPS26B protein sequence consists of 382 amino acid residues with a molecular weight of 43443.31 g/mol, and the theoretical isoelectric point (pI) is 7.21. The protein sequence has negatively charged residues (Asp + Glu) and positively charged residues (Arg + Lys). The aliphatic index of protein is 79.35, and the instability index is computed to be 43.40. The amino acid arrangement of the RF13 peptide identified from the VPS26B protein is $^1\text{RRGKGGRRVTMSF}^{13}$. The sequence consists of basic (38.46%) and neutral (38.46%) residues which were identified through the helical wheel prediction program; in addition, the program has also predicted polar and non-polar regions of the peptides as well (E-Suppl. Figure 1A and 1B).

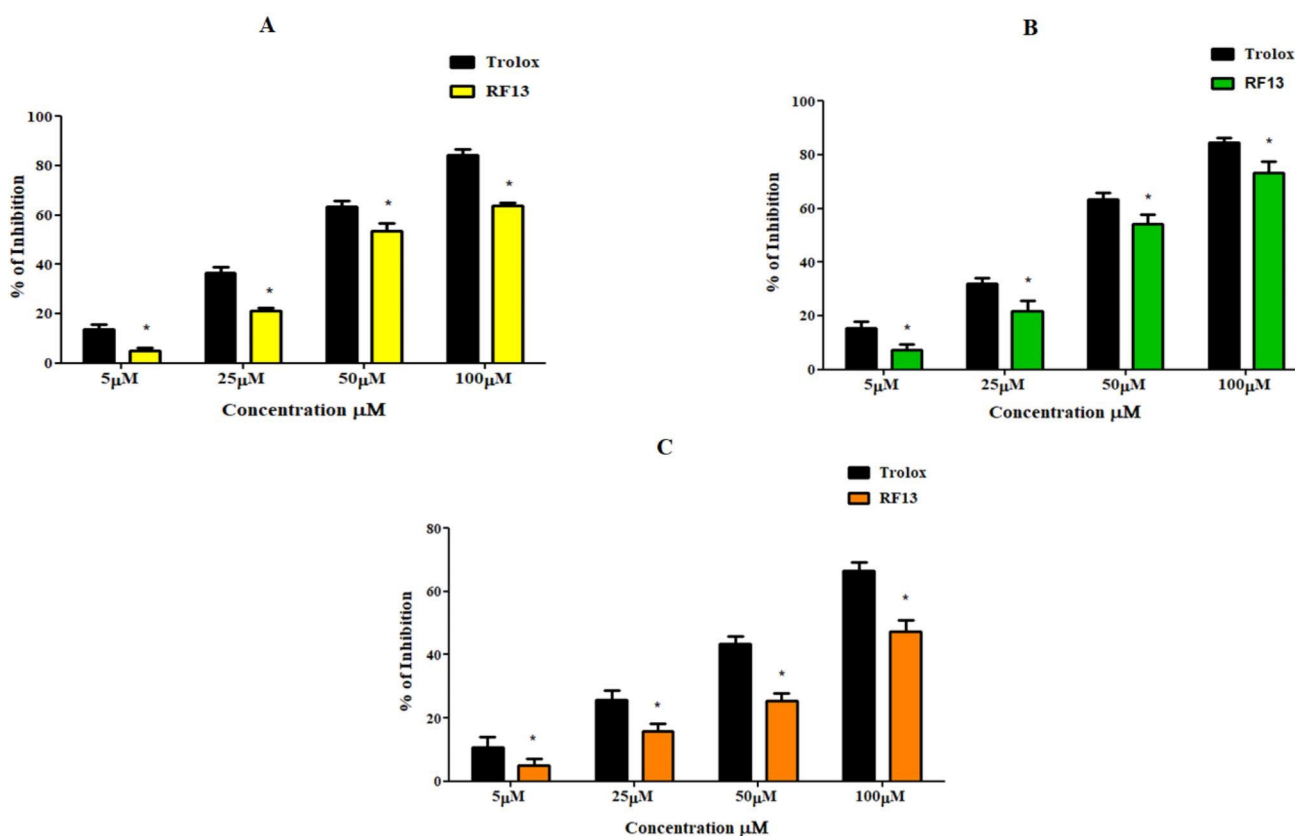


Fig. 1 Free radical scavenging activity of RF13 peptide was compared with control Trolox. The data represent the percentage of (A) DPPH radical scavenging activity, (B) ABTS radical cation decolorization and (C) Nitric oxide scavenging activity. The single asterisk (*) denotes the significant difference between control and peptide treatment (5 μM to 100 μM) at $p < 0.05$ level by one-way ANOVA followed by Dunnett's multiple comparisons test. All the experiments are done in triplicates and the values are represented in mean \pm SD, $n = 3$

In vitro antioxidant activity of RF13

The DPPH radical scavenging activity of RF13 showed significantly higher radical scavenging activity (64%) at 100 μM and lower activity (5%) at 5 μM compared to positive control (Trolox) at the same concentrations (83% at 100 μM and 14% at 10 μM , respectively). The radical scavenging activity of RF13 peptide was also found to be dose-dependent (Fig. 1 A).

The ABTS radical scavenging activity of RF13 was found to be dose-dependent. Significantly higher ($p < 0.05$) antioxidant activity was observed at 100 μM (73%) while the least activity was observed at the lowest concentration of the RF13 peptide (7.3% at 5 μM) and Trolox (15% at 5 μM and 85% at 100 μM) (Fig. 1 B).

The RF13 peptide exhibited nitric oxide scavenging activity, leading to reducing the nitrite concentration compared to Trolox. The nitric oxide scavenging activity of RF13 was concentration-dependent, with 100 μM concentration showing the most efficient scavenging activity. At the same time, Trolox exhibited significantly ($p < 0.05$) higher activity (67% at 100 μM), as mentioned in Fig. 1 C.

Cytotoxicity Assay

The cytotoxic effect of RF13 was determined in L6 and human erythrocytes (Fig. 2A and 2B). RF13 treatment at 100 μM showed a significant toxicity effect on cell viability after 24 h. In both, the cells treated with RF13 at 100 μM showed a cytotoxic effect. The results indicate that RF13 had no cytotoxic activity at concentrations between 5 μM and 50 μM .

Toxicity assay in zebrafish larvae

At 4 dpf, zebrafish larvae were treated for 24 h with RF13 (5 μM to 50 μM) and showed no significant mortality compared to untreated larvae. A higher concentration of RF13 treatment (100 μM) resulted in a moderate mortality rate (E-Suppl. Figure 2A). The heart rate of 4 dpf zebrafish larvae treated with RF13 for 24 h was measured to determine cardiotoxicity. The larval arterial and ventricular blood flow were microscopically recorded for 1 min, and the heart rate per min was recorded. Even at a higher concentration (100 μM), only a slight reduction in heart rate was observed (E-Suppl. Figure 2B).

Effect of RF13 on the Weight of Zebrafish Larvae

The weight of zebrafish larvae was measured after the HFD induction, and it was found to be significantly higher ($p < 0.05$) than the control group (E-Suppl. Figure 3). The weight of the larvae decreased dose-dependently after RF13 was co-treated with the HFD induction. This demonstrates that RF13 can induce weight loss in HFD zebrafish larvae.

Lipid Modulating Effect of RF13 on HFD Zebrafish Larvae

Using O red O staining, the effect of the RF13 peptide on HFD group lipid accumulation in zebrafish larvae was calculated. When comparing the HFD group to the control group (Fig. 3 A and 3B), appearance of red color in the abdomen of the larvae showed an increase in lipid accumulation (83%). RF13 has significantly ($p < 0.05$) reduced lipid accumulation in a dose-dependent manner. The lipid modulating activity

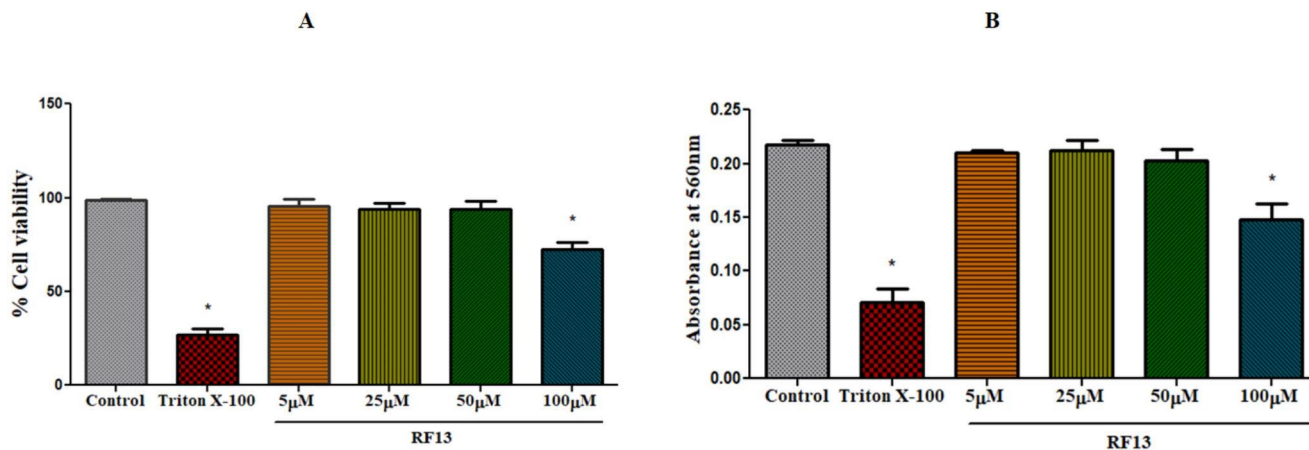


Fig. 2 (A) Effect of RF13 peptide on the viability of L6 cells, evaluated by MTT assay. Triton X-100 (0.01%) and untreated cells are used as positive control and control for the experiment, respectively. (B) Hemolytic activity of the RF13 peptide against human erythrocytes. Data were expressed as mean \pm SD. * denotes $p < 0.05$ as compared to the control

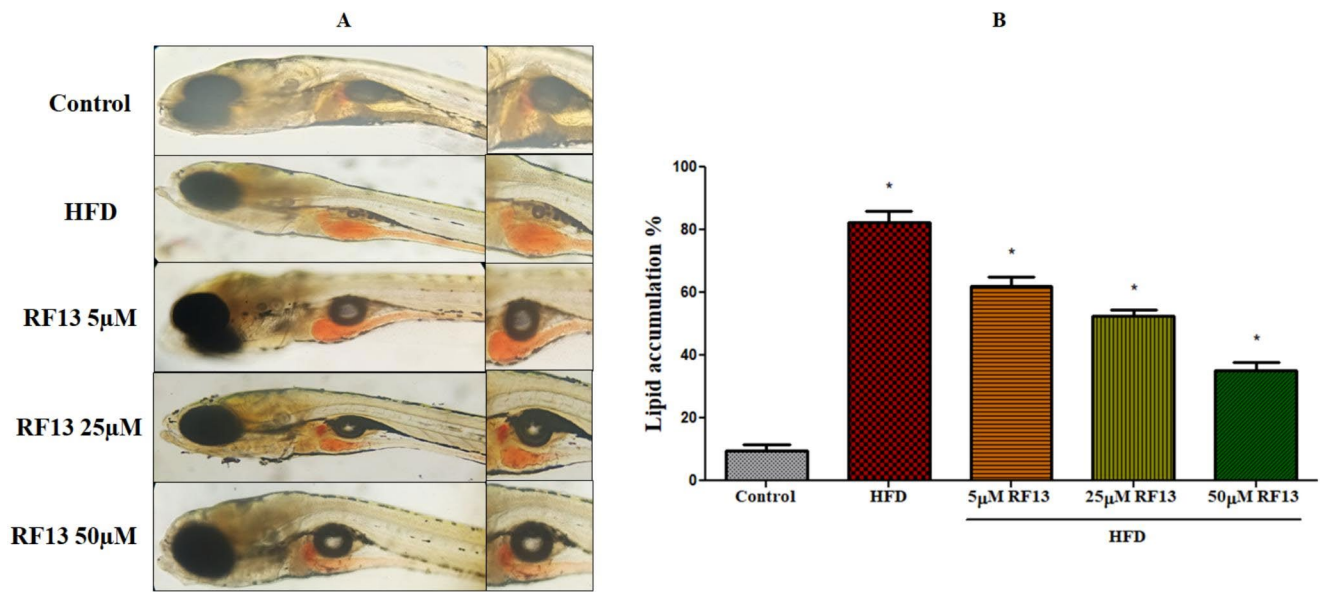


Fig. 3 (A) Oil red O staining of zebrafish larvae (the red area indicates the lipid accumulation). (B) Quantitative analysis of lipid accumulation in the zebrafish larvae (statistical analysis by ImageJ software). Experiments were performed in triplicate, and the data were expressed as mean ± SD (n = 30/group). * represents the statistical significance at $p < 0.05$

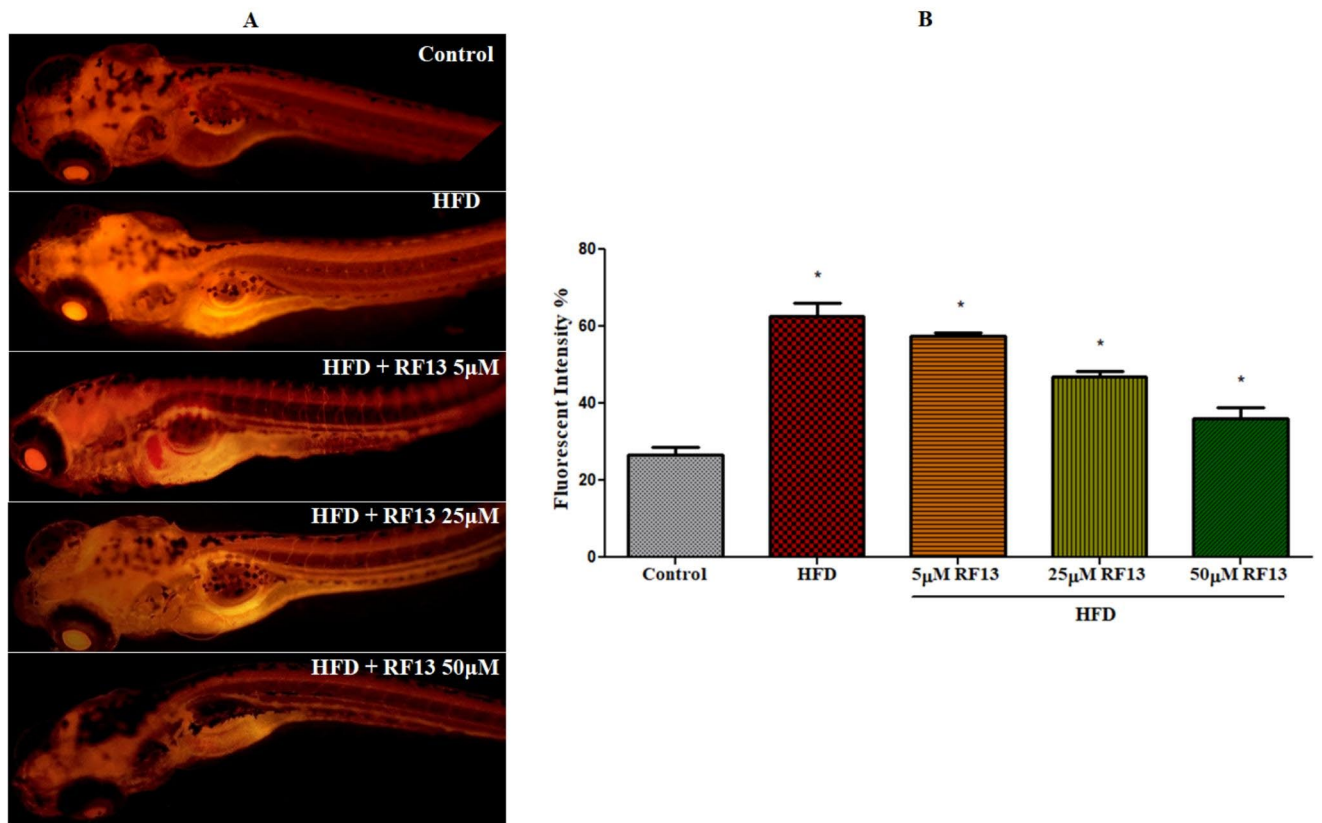


Fig. 4 (A) Representative photomicrographs of zebrafish larvae stained with Nile red staining. (B) Quantitative analysis of results of Nile red staining in zebrafish larvae measured by image J. The results were expressed as a percentage (%) of Nile red staining and were compared with respect to untreated control. Data were expressed as mean ± SD (n = 30/group) of three independent experiments. * $p < 0.05$ as compared to the control

of RF13 was observed to be high at 50 μM concentration (35%) which can lead to inhibition of lipid accumulation.

The Nile red assay was used to investigate the RF13 lipid-lowering activity. There was a significant increase in orange fluorescence in the abdomen of the HFD zebrafish larvae group, indicating lipid accumulation (63%) (Fig. 4 A and 4B). On the other hand, the orange fluorescence of larvae in the 50 μM RF13 (36%) treatment group was found to be significantly reduced compared to the HFD larvae group. These results showed that RF13 peptide has lipid modulating activity on HFD induced lipid accumulated zebrafish larvae.

Triglycerides and Cholesterol Level in Zebrafish Larvae

The triglyceride and total cholesterol were further tested in the HFD zebrafish larvae. As shown in Fig. 5 A and 5B,

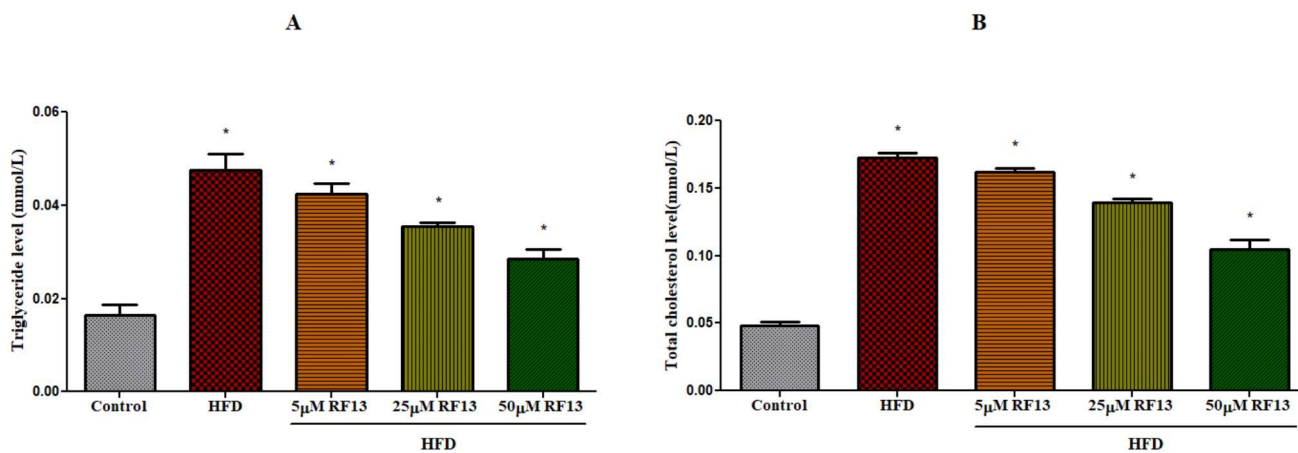


Fig. 5 (A) Triglyceride levels and (B) Total cholesterol levels in zebrafish larvae of each group ($n = 30/\text{group}$). Experiments were performed in triplicate, and the data were expressed as mean \pm SD. * represents the statistical significance at $p < 0.05$

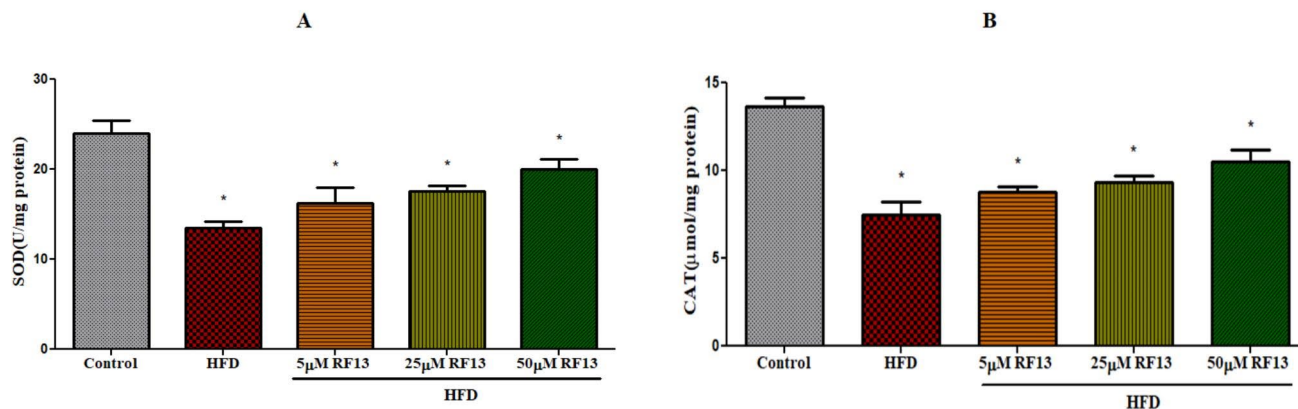


Fig. 6 (A) Total SOD and (B) CAT activity in HFD induced oxidative stressed zebrafish larvae co-treated with RF13 peptide. Data expressed as mean \pm standard deviation ($n = 30/\text{group}$). Values are statistically significant at * $p < 0.05$ compared to the control

the HFD larvae group showed an increase in triglycerides (0.0475 mmol/L) and total cholesterol level (0.1725 mmol/L) compared to the control. Compared to the HFD larvae, the RF13 peptide co-treatment showed a dose-dependent reduction, and the higher concentration of 50 μM showed a maximum reduction in the triglycerides (0.0285 mmol/L) and total cholesterol (0.105 mmol/L).

Antioxidant Enzyme Activity in Zebrafish Larvae

In HFD zebrafish larvae, antioxidant enzyme activities (SOD and CAT) of RF13 were measured. The HFD larvae group had significantly lower SOD (14 U/mg of protein) and CAT (8 $\mu\text{mol}/\text{mg}$ of protein) activity (Fig. 6 A and 6B). Meanwhile, SOD (20 U/mg of protein) and CAT (11 $\mu\text{mol}/\text{mg}$ of protein) activity were higher in the RF13 peptide treatment at 50 μM .

Protective Effect of RF13 Against ROS, Apoptosis, and Lipid Peroxidation in Zebrafish

To assess the protective effect of RF13 against HFD induced oxidative stress in zebrafish larvae, the larvae were stained with DCFDA, acridine orange, and DPPP. In comparison to the control group, fluorescence was more evenly distributed in the abdomen of HFD zebrafish larvae. The HFD larval group showed an increase in fluorescence intensity in DCFDA, acridine orange, and lipid peroxidation, indicating an increase in ROS level (82%), apoptotic cells (66%), and lipid peroxidation (51%) in the abdomen of zebrafish larvae (Figs. 7, 8 and 9). At the same time, when treated with the differing concentration of RF13 peptide, a decrease in fluorescence intensity of ROS level, apoptosis, and lipid peroxidation was observed. The maximum activity of reduction in ROS level (22%), apoptosis (31%), and lipid peroxidation (16%) were observed in the 50 μ M of RF13.

Effect of RF13 Peptide on Lipogenic and Antioxidant Gene Expression

The expression levels of lipogenesis-related genes such as C/EBP- α , SREBP1, FAS, and antioxidant genes like GPx, GST, and GR in HFD zebrafish larvae were measured using qPCR. The expression of C/EBP- α (3.5 fold), SREBP1 (2.9 fold), and FAS (3.3 fold) was significantly upregulated ($p < 0.05$) in the HFD larvae group, while GPx, GST, and GR expression were downregulated (Fig. 10 A and 10B). The RF13 peptide treatment at 50 μ M resulted in a decrease in C/EBP- α (2.7 fold), SREBP1 (1.9 fold), and FAS (2.2 fold) expression. Furthermore, when compared to control, RF13 treatment at 50 μ M showed the highest level of expression of antioxidant enzyme genes GPx (2.3 fold), GST (1.8 fold), and GR (2.1 fold).

Discussion

Consumption of high fat containing diet linked to lipid accumulation or high levels of total cholesterol and triglycerides (Liu et al. 2018). In epidemiological and clinical studies,

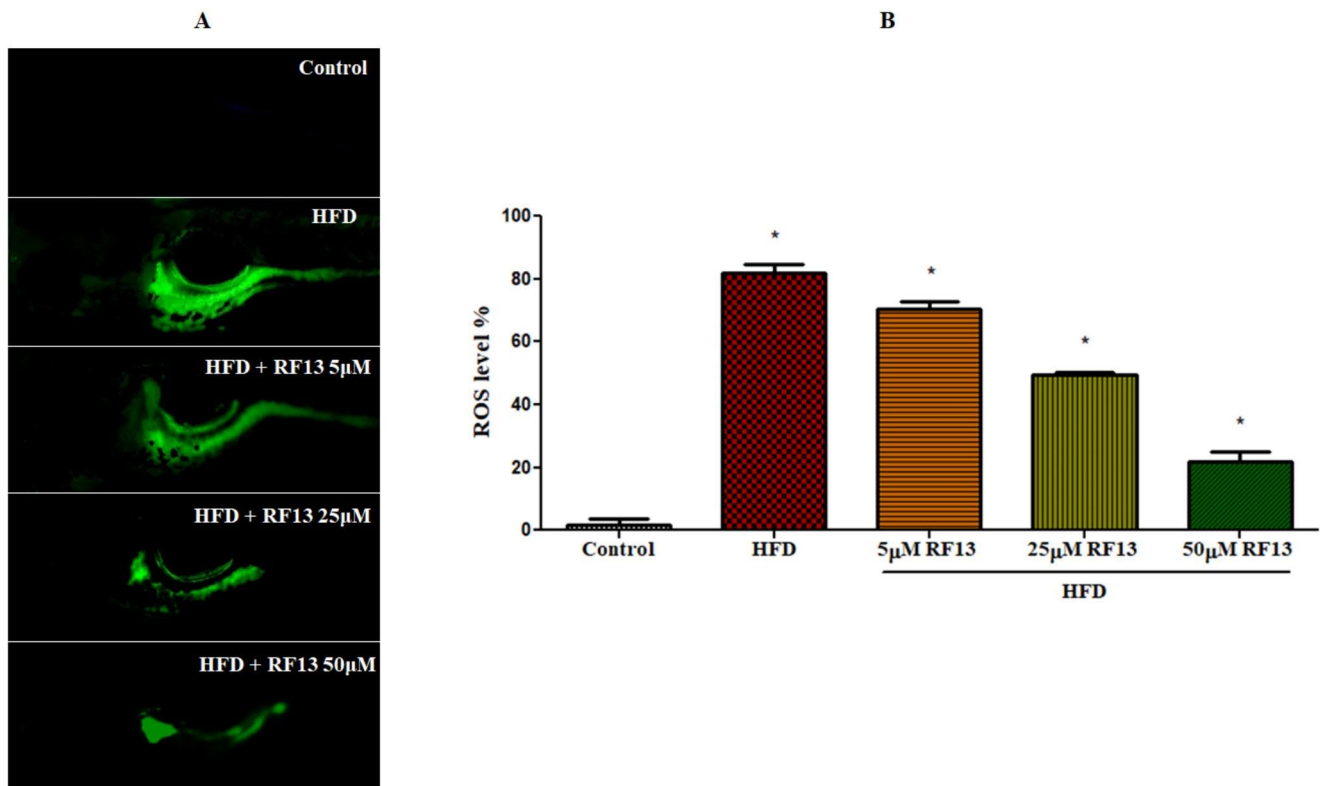


Fig. 7 (A) Quantitative analysis of *in vivo* ROS generation in HFD induced zebrafish larvae. The fluorescence intensity was quantified using ImageJ. (B) Representative photomicrographs of HFD zebrafish larvae by an oxidation-sensitive DCFDA fluorescent probe. The fluorescence image was captured using a fluorescence microscope. Experiments were performed in triplicate, and the data were expressed as mean \pm SD. * represents the statistical significance at $p < 0.05$

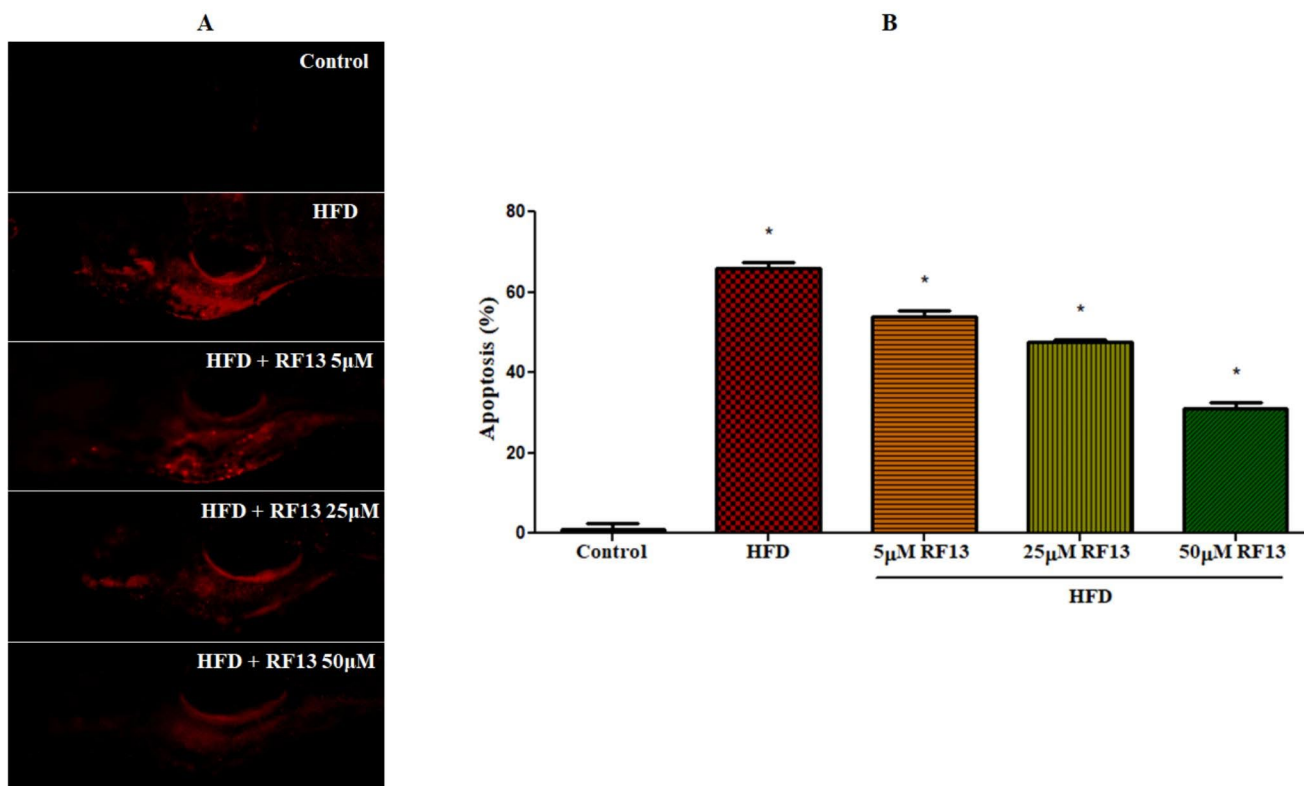


Fig. 8 (A) Representative photomicrographs of zebrafish larvae stained with acridine orange. (B) Quantitative analysis of apoptosis activity in the HFD induced zebrafish larvae. The fluorescence intensity was quantified using ImageJ. The fluorescence image was captured using a fluorescence microscope. Experiments were performed in triplicate, and the data were expressed as mean \pm SD ($n = 30$ /group). * represents the statistical significance at $p < 0.05$

high lipid levels in the blood have been linked to cardiovascular disease, type 2 diabetes, and hypertension (Koyama et al. 2009). Moreover, because of the interaction between superoxides and nitric oxide, this HFD causes excessive ROS production, which leads to an increase in reactive nitrogen species (RNS). These interconnected events lead to the onset of multiple risk factors and chronic diseases (Kesh et al. 2016). Therefore, pharmacological drug therapies for lipid accumulation that are effective and safe are required.

The present study reported the lipid-lowering effect and antioxidant activity of RF13 on HFD induced zebrafish larvae. The synthesized RF13 was found to contain positively charged amino acids like arginine and lysine and hydrophobic residues like glycine, valine and phenylalanine. The peptide contained amino acids such as methionine, lysine, arginine, serine and phenylalanine, which are all reported to have specific antioxidant activities. The hydrophobic amino acids have a potential free radical scavenging and lipid peroxidation activity (Rajakpse et al. 2005). Glycine (23.1%) and arginine (30.8%) was found to dominate the amino acid composition in RF13. Glycine and arginine are essential amino acids for good health and disease prevention,

particularly in the cardiovascular system. Arginine and glycine supplementation can improve liver function, glucose tolerance and oxidative stress (Lee and Kim 2019). By reducing inflammation and oxidative stress associated with HFD, oral supplementation with glycine and arginine can attenuate obesity-related changes in the heart and liver (Alam et al. 2013; Muthu et al. 2021; McKnight et al. 2010; Razak et al. 2017). The above-mentioned amino acids indicate that RF13 has a beneficial effect on exhibiting lipid lowering and antioxidant activity.

In vitro antioxidant activity of RF13 shows dose-dependent free radical scavenging activity. A previous study reported that a peptide chain made up of hydrophobic amino acids can easily scavenge the DPPH radical (Sonklin et al. 2018). RF13, which had the strongest DPPH scavenging activity, contained hydrophobic amino acids that are important for donating a hydrogen atom to the DPPH radical. When comparing RF13 to trolox in the ABTS assay for evaluating radical scavenging activity, the antioxidant activity of RF13 increased steadily. The ABTS radical can react with aromatic amino acids, making it more sensitive than DPPH (Dash and Ghosh 2017). As aromatic amino acids react with ABTS free radicals to form stable compounds,

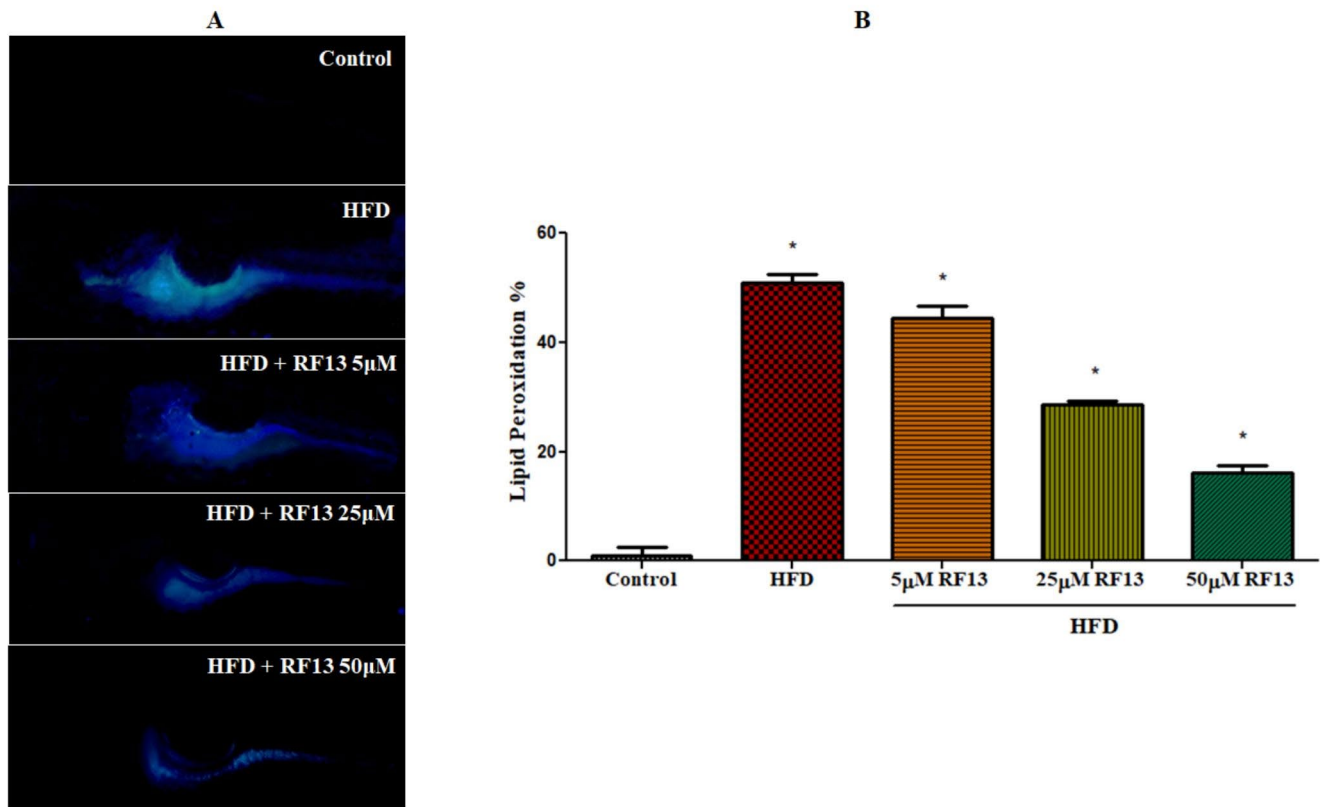


Fig. 9 (A) Representative photomicrographs of HFD induced zebrafish larvae stained with DPPP. (B) Quantitative analysis of lipid peroxidation in the zebrafish larvae. The relative fluorescence intensities of zebrafish larvae were determined using Image J software. The experiments are conducted in triplicate, and the data are expressed as mean ± SD (n = 30/group). * represents the statistical significance at $p < 0.05$

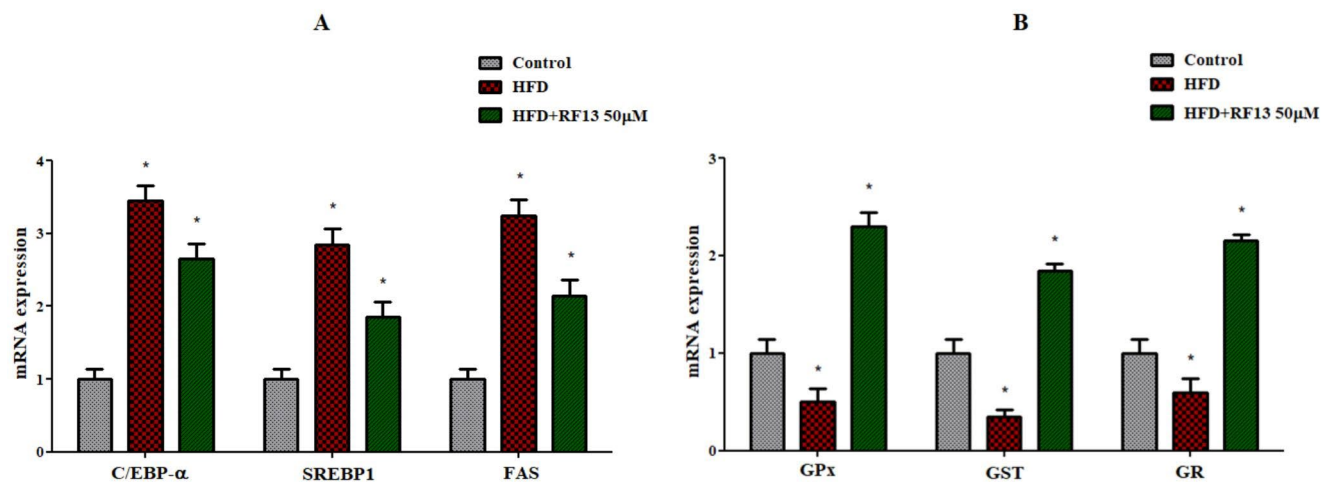


Fig. 10 Effect of RF13 on the mRNA expression of a lipogenic specific genes and antioxidant-related genes. (A) Effect of RF13 on mRNA expression of C/EBP-α, SREBP1, and FAS. (B) Effect of RF13 on mRNA expression of GPx, GST, and GR. Data were expressed as mean ± SD of three independent experiments. * $p < 0.05$ as compared to the control

phenylalanine in the RF13 sequence may be responsible for enhancing antioxidant activity. Nitric oxide may react with a superoxide anion to generate highly reactive peroxynitrite,

which can damage DNA. These extremely reactive intermediates have the potential to cause cellular degeneration (Pacher et al. 2007). The scavenging activity of nitric oxide

by RF13 peptide was enhanced with increasing concentration from 5 μM to 100 μM . The previous study reported that peptides derived from flaxseed showed an increase in nitric oxide scavenging activity (Udenigwe et al. 2009). The sequence of RF13 peptides contributes to the potential nitric oxide scavenging.

The cytotoxicity of a drug is an important parameter in determining its toxicity. The peptide must be non-toxic to function as a therapeutic agent. The hemolytic and cytotoxic effects of the RF13 peptide were investigated to confirm the peptide's non-toxic nature towards L6 cells and human blood cells. The findings showed that RF13 has no cytotoxic effect on HDF cells and is non-hemolytic to human blood cells at concentrations up to 50 μM . The zebrafish larvae model is increasingly being used to examine drug toxicity and safety, and multiple studies demonstrate that mammals and zebrafish toxicity profiles are highly comparable (McGrath and Li 2008). The toxic effect of RF13 at various concentrations was studied using zebrafish larvae. The results showed that RF13 peptide treated zebrafish larvae at various concentrations (5 μM to 50 μM) had no adverse effect on the larvae. However, RF13 treatment at 100 μM caused mortality and lowered heart rate. L6 cells, human erythrocytes, and zebrafish larvae were shown to be toxic at concentrations of 100 μM . Hence, the doses from 5 μM to 50 μM concentration were selected for the study.

Zebrafish are a well-known model organism in lipid studies because they have similar genetic backgrounds to mammals. The lipid distribution in the body can be visualized using zebrafish. Furthermore, new genes linked to diet-induced obesity and energy balance have been discovered in recent studies, implying that zebrafish could be employed as a model for diet-induced obesity (Flynn et al. 2009; Lee et al. 2015). The results of this study revealed that larvae fed with HFD has gained more weight than larvae feeding the usual diet. The observation described here is similar to previous reports (Vargas and Vásquez 2017) showing that overfeeding or HFD favours the development of overweight and fat accumulation in larvae. These alterations can be considered to contribute to obesity. Additionally, the whole body lipid accumulation could be observed from Oil red O staining. The results revealed that lipid accumulation was diminished after the RF13 peptide treatment, especially under the concentration of 50 μM . Nile Red is a heterotetracyclic organic compound that interacts with lipid droplets in the cell (Türkoğlu et al. 2021). During this experiment, enhanced fluorescence was seen in the abdomen of HFD zebrafish larvae compared to control, indicating that the abdomen of larvae had an increase in triglycerides and cholesterol. In the abdomen of the zebrafish larvae, however, when RF13 was used in co-treatment with the HFD, there was a decrease in fluorescence intensity, indicating that it

reduced the intracellular lipids in HFD larvae. RF13 treatment at various concentrations also enhanced HFD zebrafish larvae lipid metabolism by lowering triglycerides and total cholesterol levels. These results suggest that RF13 can inhibit lipid accumulation by regulating lipid metabolism.

Changes in lipid metabolism have been reported as contributory factors to obesity-related oxidative stress. In both human and animal models of obesity, increased generation of ROS and diminished antioxidant defense systems have been shown to play a major role (Noeman et al. 2011). The effective mechanism to avoid free radical-induced cell damage is accomplished by a set of endogenous antioxidant enzymes like SOD and CAT (Blokchina et al. 2003). Indeed, studies have found that hypercholesterolemia impairs the antioxidant defence mechanism by lowering SOD and CAT activity (Belguith-Hadriche et al. 2016). The HFD has considerably reduced the levels of SOD and CAT enzymes in this study, which could be due to oxidative and tissue damage. However, in zebrafish larvae, RF13 treatment caused a significant increase in SOD and CAT levels in a dose-dependent manner. Obesity can enhance lipid peroxidation by causing progressive and cumulative cell injury due to the pressure of a large body mass, which generates ROS (Lechleitner et al. 2000). An increase in ROS and lipid peroxidation may aggravate mitochondrial dysfunction by causing oxidative damage to lipids. Apoptosis can be triggered by mitochondrial dysfunction (Carmiel-Haggai et al. 2005; Noeman et al. 2011). This study observed increased ROS level, apoptosis, and lipid peroxidation through the DCFDA, acridine orange, DPPH staining in the HFD zebrafish larvae. This may be due to the decrease of antioxidant enzymes and exhaustion of storage of this enzyme in fighting free radicals generated during the development of obesity. Supporting this data, alteration in antioxidant enzymes and their oxidative stressed condition in HFD induced rats have been reported (Noeman et al. 2011). The findings show that RF13 reduces the deleterious effects of free radicals by enhancing antioxidant enzymes. Furthermore, when the RF13 was treated with the HFD in zebrafish larvae, the level of ROS, apoptosis, and lipid peroxidation in the zebrafish larvae was significantly reduced. RF13 showed increased activity with no side effects at a dose of 50 μM . As a result, the optimum concentration (50 μM) was chosen for further research.

To explore the molecular mechanism of RF13 against HFD zebrafish larvae, the lipogenic specific gene expression of C/EBP- α , SREBP1, and FAS was investigated. C/EBP- α is a potential key regulator of adipocyte development that regulates lipogenic gene expression (Jiang et al. 2006). SREBP1 is another important factor in fatty acid metabolism, as it increases the activity of other lipogenic factors, including FAS. FAS activates fatty acid synthesis

enzymes directly, and it is a basic metabolic enzyme that produces palmitic acid, a triglyceride that is utilized to store energy (Ming et al. 2014; Moon et al. 2012). The current study found that RF13 treatment (50 μ M) has reduced the expression of lipogenic specific genes so that fatty acid and triglycerides were inhibited and lipid accumulation reduced. Further while investigating the antioxidant activity of the RF13 peptide, we have evaluated its effect on antioxidant-related genes (GTPx, GST and GR). HFD has suppressed the expression of antioxidant genes compared to control. These findings show that increased antioxidant expression via transcriptional regulation may contribute to the improved antioxidant status noticed in HFD zebrafish larvae treated with RF13 (50 μ M). Overall, the potential of RF13 to prevent HFD-induced lipid accumulation and free radicals suggests that it could be used as a supplement to reduce oxidative stress and other metabolic disorders associated with obesity.

Conclusions

We concluded that the RF13 reduced fat accumulation and HFD-induced oxidative stress via transcriptional regulation of lipogenic and antioxidant genes. RF13 has consistently lowered triglycerides and total cholesterol levels while increasing antioxidant enzyme levels. The exact molecular mechanism of fat metabolism remains unknown and complicated. Further findings will pave the way for the benefits of RF13 to be applied to humans.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10989-022-10376-3>.

Acknowledgements The authors acknowledge Dr. Kanchana K. Mala for providing necessary help in performing haemolytic assay and associated ethical clearance (885/IEC/2015).

Author Contributions Conceptualization, Methodology, Formal analysis and investigation Writing-original draft preparation: AJ, MV; Conceptualization, Formal analysis, Funding acquisition, Supervision: JA.

Data Availability Statement Data will be made available on reasonable request.

Declarations

Conflict of Interest All the authors declare that they have no conflict of interest.

Research Involving Human and Animal Participants This research does not involve any human or animal objects; however, we have collected human blood from healthy volunteers for the hemolytic assay performed in this study. The blood from the healthy individuals was

collected as per the guidelines of the Institutional Ethical Committee regulation (Ethical Clearance No. 885/IEC/2015). For blood collection, informed consent was obtained from all the participated adults in written form. All authors are aware of the details of their research work that are presented in the current manuscript and gave their consent to publication.

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