

RESEARCH ARTICLE

Vegetables Spices Fermentation Extract Inhibit NAFLD Development by Attenuation of Steatosis through Suppression of *C/EBPα* and *TNFα* mRNA Expression

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Abstract

BACKGROUND: Non-alcoholic fatty liver disease (NAFLD), characterized by inflammation and steatosis, is one of the metabolic problems that trigger serious complications. Statin is usually used for NAFLD therapy, however, statin intolerance and resistance reduce the effectiveness of NAFLD treatment. Therefore, this study was conducted to develop the phytopharmaca against NAFLD by elucidating the anti-inflammatory and steatosis suppression of vegetable spices fermentation extract (VSFE) in rabbits induced with high-fat diet (HFD).

METHODS: Twenty New Zealand White rabbits were determined into 4 groups; which were groups receiving standard diet (SD), HFD, HFD and 100 mg/kgBW VSFE, as well as HFD and 200 mg/kgBW VSFE. mRNA expressions of tumor necrosis factor alpha (*TNFα*) and CCAAT/enhancer-binding protein alpha (*C/EBPα*) were evaluated. The *TNFα* concentration was quantified after protein and RNA isolation. Inflammation and steatosis were

observed in liver hematoxylin-eosin-stained prepartate using microscope.

RESULTS: Macrophage cells and hepatic steatosis showed significant decrease in a drug dependent manner. An effective dose of 200 mg/kgBW decreased *C/EBPα* and *TNFα* expression to 0.45 ± 0.32 and 0.72 ± 0.29 ($p=0.013$ and $p=0.002$, compared to the HFD group), respectively. *TNFα* inflammatory cytokine concentrations also responded to the administration of 100 mg/kgBW and 200 mg/kgBW doses, with the value of 31.72 ± 10.40 mg/dL and 48.35 ± 7.15 mg/dL ($p=0.009$ and $p=0.002$, compared to the HFD group), respectively.

CONCLUSION: VSFE might prevent NAFLD by inhibit steatosis and inflammation through suppression of *C/EBPα* expression and *TNFα* expression.

KEYWORDS: *C/EBPα*, *TNFα*, fermentation, steatosis, NAFLD

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by macrovesicular steatosis in 5% of hepatocytes without

additional factors like drug or alcohol abuse.(1) Globally, NAFLD is the primary cause of chronic liver disease, with a prevalence of 25% (2), 5-30% of which is in Asian regions such as Sri Lanka, Malaysia and Indonesia (3). The United States recorded 8% of deaths caused by liver disease and

diabetes.(4) Consequently, NAFLD's high incidence is a concerning disease burden that needs further attention.

An important strategy in preventing NAFLD is dietary improvement (5), based on the fact that a high-fat diet causes abnormal accumulation of free fatty acids in the blood circulation and liver cells, which will induce endoplasmic reticulum (ER) stress (6). One consequence of ER stress is tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2), which will attach to the cytosolic domain of inositol-requiring enzyme type 1 (IRE1) α . Then, as an initiator of the inflammatory process, the IRE1-TRAF2 complex activates the inhibitor of nuclear factor- κ B kinase (IKK) and induces phosphorylation of the inhibitor of nuclear factor- κ B (I κ B). This causes nuclear factor- κ B (NF- κ B) to translocate into the nucleus towards the target gene and undergo transcription of antiapoptotic and inflammatory genes. In addition, the IRE1-TRAF2 complex can also activate c-Jun N-terminal kinases (JNK), which phosphorylate and activate activator protein 1 (AP-1). It therefore causes the synthesis of pro-inflammatory mediators like TNF, interleukin (IL)-6, and IL-1 β .(7) Another consequence is that ER stress will activate IRE1, which leads to the splicing of X-box binding protein 1 (XBP1) and produces the potent transcription factor XBP1s, which encodes expression of unfolded protein response (UPR) gene target, namely the adipogenic factor *C/EBP*, which further cause droplet lipid accumulation.(7)

Currently, NAFLD therapy in the form of statins and lifestyle changes has not provided optimal results. There were reports regarding the incidence of hepatotoxicity, myopathy, neuropathy, cognitive decline, pancreatic malfunction, and sexual dysfunction when using statins for a long time.(8) In addition, statin resistance and intolerance are reasons for the discontinuation of statin therapy, thus reducing the effectiveness of NAFLD therapy. Statin intolerance can occur partially or totally. A total of 322/1500 patients (22.1%) discontinued statin therapy due to side effects and intolerance.(9,10) Therefore, efforts are urgently required to complete prevention strategies based on diet modification, in addition to the recommended therapies.

Several reports showed that consumption of vegetables such as cucumber (*Cucumis sativus*) and white cabbage (*Brassica oleracea*) had a hypolipidemic effect. However, based on previous studies, the serving of raw vegetables is easily oxidized, and boiling with improper methods could reduce the nutritional content of vegetables. Previous studies reported that fermentation of cucumber as pickles, and white cabbage as sauerkraut could enhanced the bioavailability of secondary metabolites and antioxidant

contents.(11) Previous study demonstrated that 14th day spontaneous fermentation of combination cucumber, white cabbage with additional condiment basil leaves (*Ocimum sanctum*) and spicy sauces from garlic (*Allium sativum*), shallot (*Allium cepa*), tomatoes (*Lycopersicon esculentum*), chili (*Capsicum frutescens*) had hypolipidemic effect.(12) Therefore, this study was conducted to investigate the benefits of fermented vegetables in preventing NAFLD through its work in modulating inflammatory and lipid signaling pathways which has not been done before.

Methods

Preparation of Vegetables Spices Fermentation Extract (VSFE)

Cabbage (*Brassica oleracea*), basil (*Ocimum sanctum* L.), cucumber (*Cucumis sativus* L.), tomatoes (*Lycopersicon esculentum* Mill), cayenne pepper (*Capsicum frutescens*), shallots (*Allium cepa* L.), and garlic (*Allium sativum*) obtained from traditional market in Malang, and determined by UPT Materia Medica Batu, Indonesia with reference No. 067/2250-55/102.20/2023, respectively.

The preparation of extract fermentation was stated in the previous study.(11) The cabbage was added with 3% salt overnight at 4°C. The next day, peeled cucumber and sliced basil leaves were mixed with the salted cabbage. The prepared chili sauce consisted of shallots, garlic, chilies, tomatoes, sugar and salt were added to the vegetables mixture and then placed in a closed sealed jar at 20°C for 14 days.(13) The fermented products were further ground into simplicia powder after being dried for five days at 60°C in a hot oven, and then extracted using the ultra-assisted extraction (UAE) method.(14) Following extraction, the plant extract was treated at 50°C in a rotary evaporator to produce extract concentrate. Then the extract was dried at 50°C in a hot oven until the mass settled. For the *in vivo* investigation, the determination of dose variation used with concentrations of 100 mg/kgBW and 200 mg/kgBW was made based on previous study.(15)

Animal Experiment Design

Twenty male New Zealand White rabbits aged 5-month-old from Animal Laboratory of Universitas Brawijaya, Malang, was used in this study. The rabbits were housed in 50 x 70 x 70 cm³ individual cages at 20°C with a light-dark cycle of 12:12 hours. The protocol of this study was approved by Ethical Committee of Universitas Brawijaya (No. 104-KEP-UB-2022).

The rabbits were fed with standard food Giant Feed (Wijaya Inti Nutrisi, Karanganyar, Indonesia). and water *ad libitum*. Following 2 weeks of acclimatization, rabbits were randomly carried out into 4 groups; standard diet (SD) group (n=5) that received a regular diet; high fat diet (HFD) group (n=5) that was fed 1% cholesterol without given extract; HFD+100VSFE group (n=5) that was feed with HFD and 100 mg/kgBW VSFE; as well as HFD+200VSFE group (n=5) that was feed with HFD and 200 mg/kgBW VSFE. (10) After 8 weeks of treatment, all rabbits were terminated with anesthesia, and a surgical procedure was done to obtain the organs (Figure 1).

Animal Termination and Blood Sampling

Rabbits were subjected to a HFD and VSFE for 8 weeks. The rabbits underwent fasting for 24 hours and followed by termination, anesthesia was conducted with a ketamine and xylazine injection at a 1:1 ratio by subcutaneous injection of 0.1 mg/kgBW. Blood was collected through heart puncture, and anesthesia continued with inhalation of 15 mg/kgBW chloroform.(16) The rabbits were then affixed to a dissecting board. The surgical process involved shaving the abdominal fur, cleansing with water, and making an incision from the abdomen to the chest using curved scissors. After exposing internal organs, the liver was extracted for further examination. This experimental protocol was performed to assess the impact of the high fat diet and VSFE extract on rabbit health, particularly focusing on hepatic observations.

Organ Sampling

Hepatic samples were collected following specific procedures: after terminating the rabbits, the liver was

resected surgically.(17) The liver was cleansed with cold 0.9% NaCl to remove blood residues. Subsequently, the liver was air-dried on filter paper and longitudinally sectioned into right and left lobes. Immediate incubation of the liver followed in 10% neutral buffered formalin (NBF) for 48 hours. This meticulous process aimed to ensure the cleanliness and preservation of hepatic tissues for subsequent analysis. The liver samples were prepared with precision, incorporating surgical, cleansing, and incubation steps, which are critical for maintaining the integrity of the hepatic specimens.(18)

Histopathological Examination

The immersed hepatic tissue was dehydrated and embedded into paraffin block, then trimmed to a thickness of 5 μm. After that, deparaffinized using xylol and hydrated the tissue with graded alcohol concentrations.(18) Tissues were stained with hematoxylin eosin (HE). Total number of 10 different fields of the preparation were examined under a Nikon Eclipse E200 binocular microscope (Nikon Instruments Inc., Melville, NY, USA) at 400x magnification. The results of the examination inflammatory cells are characterized by accumulation of aggregated macrophage cells. Observation of hepatic macrophages by counting the number of dots that were purple-black in color and hepatic steatosis by counting hepatocyte cells. Images were quantified using ImageJ (National Institutes of Health, Bethesda, MY, USA).

Assessment of TNFα, Serum Glutamic Oxaloacetic Transaminase (SGOT), and Serum Glutamate Pyruvate Transaminase (SGPT) Concentration

Samples for measuring TNFα concentration were collected

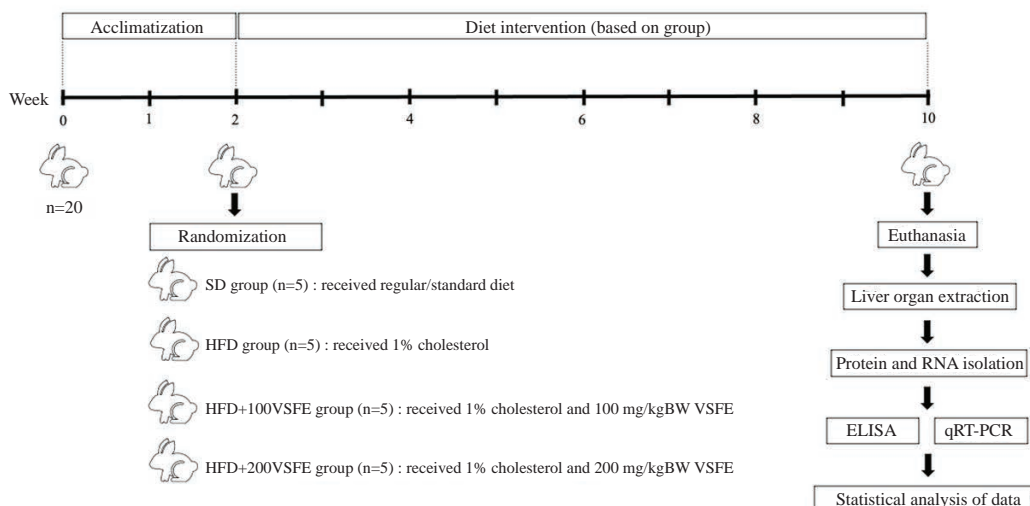


Figure 1. Study timeline.

from protein isolation of liver tissue using commercially available sensitive Rabbit Tumor Necrosis Factor A, TNF-A ELISA kit (Cat. No. MDE0065Rb; MedikBio, Malang, Indonesia). Twenty serum samples were also collected for SGOT and SGPT analysis. Using a specific substrate and the enzyme activities of aspartate transaminase and alanine transaminase, the colorimetric approach was used to determine the quantities of SGOT and SGPT. Additionally, an absorbance of 340 nm was used to read the nicotinamide adenine dinucleotide (NADH) quantification. The EnzyChrom™ Aspartate Transaminase Assay Kit (Cat. No. MDASTR-100; MedikBio) was the commercial kit used for the SGOT assay. EnzyChrom™ Alanine Transaminase Assay Kit (Cat. No. MDALT-100; MedikBio) was utilized for the SGPT assay in the meantime. Previously, the protein isolate and serum stored at -80°C before examination to preserved protein structure and activity.(19)

Gene Expression Quantification

The 20 mg liver was surgically removed right away, and it was stored at -80°C in TRIzol solution to protect the integrity of the RNA until gene expression analysis was performed. The total RNA Extraction Reagent (Cat. No. 15596026; Thermo Fisher, Waltham, MA, USA) was used to extract the total RNA. The cDNA synthesis was done by using a GoScript™ Reverse Transcriptase kit (Cat. No. A5001; Promega, Madison, WI, USA). Polymerase chain reaction (PCR) was conducted using MyGo PCR equipment. cDNA, particular primers, and GoTaq Master Mix (GoTaq 2-Step RT-qPCR System, Cat. No. A6010; Promega) were all included in each tube. The following primers were obtained from previous studies and then confirmed by NCBI.

C/EBP α : Forward 5'-GCGGGAACGAACAACAT-3'
Reverse 5'-GGCGGTCATTGTCACTGGTC-3'
TNF α : Forward 5'-GTCTTCTCTCTCACGCACC-3'
Reverse 5'-TGGGCTAGAGGCTTGTCCT-3'
GAPDH : Forward 5'-GGAGAAAGCTGCTAA-3'
Reverse 5'-ACGACCTGGTCCTCGGTGTA-3'

Following the manufacture guidelines, PCR was performed with denaturation at 95°C for 15 seconds, Annealing adjusted to T_m of each gene for *C/EBP α* and *TNF α* at 53°C and 60°C , respectively (60 minutes). Ct values for each gene were calculated on the analysis software. The Livak formula $2^{-\Delta\Delta\text{Ct}}$ was utilized to compute the relative expression.(20)

Statistical Analysis

The effect of VSFE on steatosis, macrophage infiltration, and gene expressions in rabbit model NAFLD was performed in SPSS version 29.0 (IBM Corporation, Armonk, NY, USA) by one-way ANOVA test. A $p < 0.05$ was regarded as statistically significant (*), and $p < 0.01$ as highly statistically significant (**). Further analysis was carried out by *post hoc* test using LSD.

Results

VSFE Attenuated Macrophage Infiltration and Steatosis in Rabbits Supplemented with HFD

Pathological manifestation of NAFLD shown in this study were macrophage infiltration and steatosis, as shown in Figure 2 and Figure 3. The SD group showed the lowest number of macrophages at 2.90 ± 0.308 . Whereas HFD group demonstrated the highest number of macrophages at 13.72 ± 2.446 . The two treatment groups, both HFD+100VSFE and HFD+200VSFE, significantly decreased the number of macrophages with 8.18 ± 0.664 and 4.62 ± 0.704 ($p < 0.001$, compared to HFD group), respectively.

The development of steatosis in SD group obtained was 2.26 ± 1.103 , HFD group was 13.65 ± 0.961 , HFD+100VSFE group was 6.61 ± 1.668 , and HFD+200VSFE group was 4.94 ± 1.277 . It could be concluded that group SD had the least amount of steatosis, followed by groups HFD+200VSFE, HFD+100VSFE and HFD. According to the result, compared to the HFD group, VSFE significantly decreased the amount of steatosis with 100 mg/kgBW VSFE ($p < 0.001$) and 200 mg/kgBW VSFE ($p < 0.001$).

VSFE Decreased SGOT and SGPT Concentration in Rabbits Supplemented with HFD

SGOT and SGPT are liver enzymes that determine hepatic damage in NAFLD. One-way ANOVA test shows significant differences in SGOT and SGPT levels across all groups ($p = 0.014$ and $p < 0.001$) (Table 1). The highest value of SGOT was found in the HFD group (62.32 ± 26.07 mg/dL). After administration of 100 mg/kgBW VSFE, SGOT level showed no significant difference with the HFD group ($p = 0.270$). Meanwhile, in the HFD+200VSFE group, the SGOT level decreased significantly to 21.41 ± 7.83 mg/dL compared to the HFD group ($p = 0.013$). Conversely, the SGOT level of HFD+100VSFE group showed a significant difference compared to the SD group ($p = 0.038$), but not with HFD+200VSFE group ($p = 0.553$).

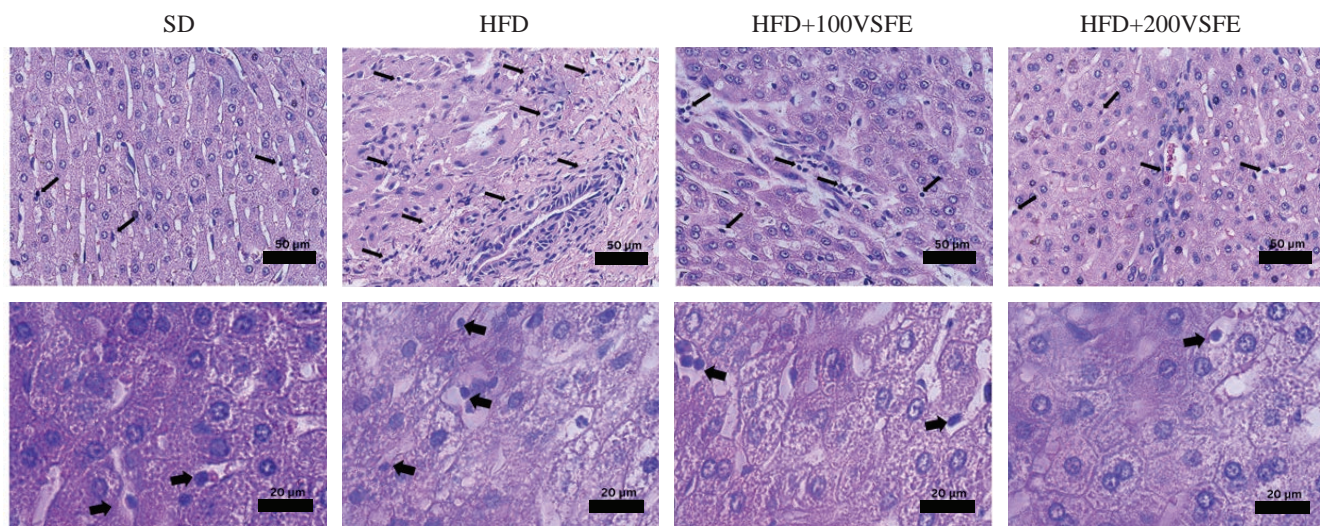


Figure 2. Liver histopathology showed that VSFE reduced macrophage infiltration. The 200 mg/kgBW VSFE considered more optimal than 100 mg/kgBW VSFE. Black arrows indicate presence of macrophages that appear as small circles in the black hepatic sinusoids. SD group showing no lobular inflammation; HFD group showing >4 foci lobular inflammation; HFD+100VSFE group showing 2-4 foci lobular inflammation; HFD+200VSFE group showing <2 foci lobular inflammation.

The results of the current study showed that the HFD group had the highest SGPT value (213.72±110.77 mg/dL). Following the administration of 100 mg and 200 mg/kgBW VSFE, the SGPT enzyme considerably decreased in comparison to the HFD group ($p < 0.001$, for both). On the other hand, there was no significant difference in the SGPT levels between HFD+100VSFE and HFD+200VSFE when compared to the SD group ($p = 0.720$ and $p = 0.964$, respectively).

VSFE Alleviated mRNA Expression of *C/EBPα* in Rabbits Supplemented with HFD

VSFE administration significantly decreased *C/EBPα* expression compared to the whole group ($p = 0.009$). Based on the data collected there was a significant difference in the relative expression of *C/EBPα* between the HFD group (2.28±0.55) and the SD group (0.68±0.22), with $p = 0.026$. In the HFD+200VSFE group, the relative expression decreased to 0.45±0.32, which was significantly lower

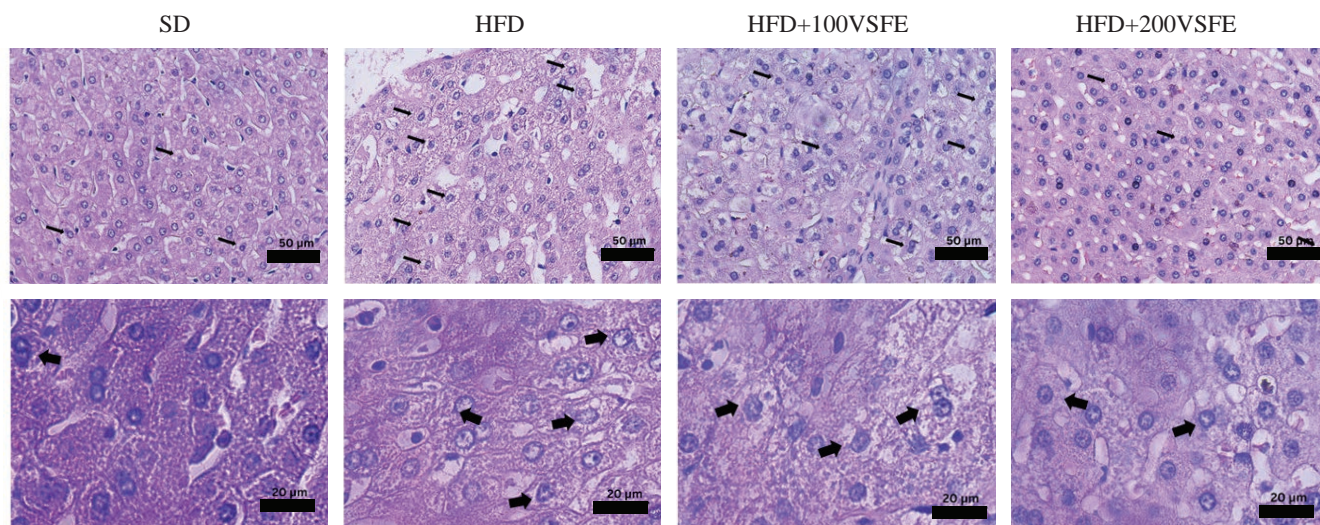


Figure 3. Liver histopathology demonstrated that VSFE could lessen steatosis in hepatocytes. Black arrows indicate the presence of lipid droplets in hepatocyte cells characterized by clear cells with nuclei in the center/edge. SD group showing little microsteatosis; HFD group showing many microsteatosis and macrosteatosis; HFD+100VSFE group showing many microsteatosis and little macrosteatosis; HFD+200VSFE group showing little microsteatosis and macrosteatosis.

Table 1. Liver enzyme profile animal model of NAFLD.

Group	SGOT (mg/dL)		SGPT (mg/dL)	
	Mean±SD	p- value	Mean±SD	p- value
SD	12.57±1.26 ^{b,c}		27.16±1.81 ^b	
HFD	62.32±26.07 ^{a,d}	0.014*	213.72±110.77 ^{a,c,d}	0.001**
HFD+100VSFE	45.63±37.26 ^a		39.94±4.21 ^b	
HFD+200VSFE	21.41±7.83 ^b		28.79±2.69 ^b	

* $p < 0.05$, ** $p < 0.01$. ^{a,b,c,d} represent significant difference ($p < 0.05$) compared to SD, HFD, HFD+100VSFE, HFD+200VSFE groups, respectively.

compared to the HFD group ($p=0.013$). Similar with the HFD+100VSFE (2.55 ± 1.94 , $p=0.005$, compared to the HFD group) (Figure 4). In this study, we demonstrated that the combined fermentation extract was able to suppress the relative expression of the *C/EBP α* .

VSFE Suppressed *TNF α* Expression in Rabbits Supplemented with HFD

The suppression of *TNF α* expression by VSFE exhibited statistical significance across all groups ($p=0.003$). Figure 5 displayed the relative expression of *TNF α* mRNA in the HFD group (2.01 ± 0.95) and showed a significant difference when compared to the SD group (0.57 ± 0.32). This study showed that VSFE was able to suppress the relative expression of *TNF α* at the 100 mg/kgBW and 200 mg/kgBW dose treatments, with relative expression values of 0.84 ± 0.34 and 0.72 ± 0.29 , respectively. Both doses were significant compared to the HFD group ($p=0.004$ and $p=0.002$, respectively). On the other hand, treatment groups

did not exhibit any significant difference in comparison to the SD group ($p=0.452$ and $p=0.672$).

VSFE Attenuated *TNF α* Concentration in Rabbits Supplemented with HFD

The reduction of *TNF α* by VSFE demonstrated statistically significant differences across all groups with $p < 0.001$ (Figure 6). The test results showed that the HFD group had a final *TNF α* concentration of 66.87 ± 5.79 mg/dL. Whereas the concentration of the SD group showed 28.22 ± 6.37 mg/dL. The dose variance of 100 and 200 mg/kg BW VSFE treatment groups was able to suppress *TNF α* concentration to 31.72 ± 10.40 mg/dL ($p < 0.009$) and 31.72 ± 10.40 mg/dL ($p < 0.002$), respectively. The results were significant compared to the HFD group. HFD group and HFD+100VSFE group demonstrated a significant difference compared to the SD group ($p < 0.001$, for both). Meanwhile, there was no significant difference for the HFD+200VSFE group ($p=0.479$).

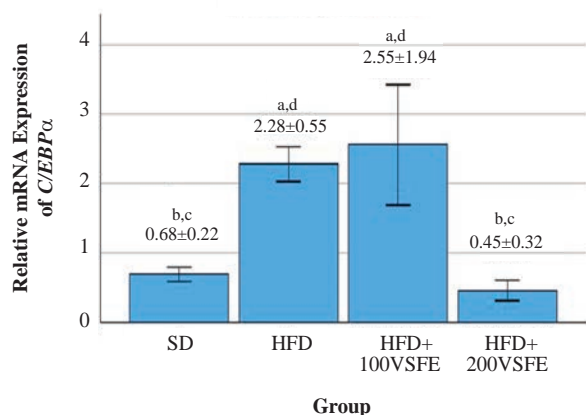


Figure 4. Relative mRNA expression of *C/EBP α* . Data was presented as mean±SD. Relative mRNA expression levels of *C/EBP α* was normalized to *GAPDH* levels. ^asignificantly different from the SD group; ^bsignificantly different from the HFD group; ^csignificantly different from HFD+100VSFE; ^dsignificantly different from HFD+200VSFE. The ^{a,b,c,d} symbol indicates a significant difference with a $p < 0.05$.

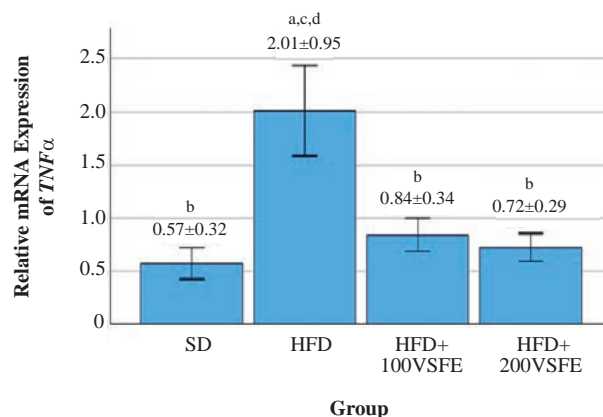


Figure 5. Relative mRNA expression of *TNF α* . Data was presented as mean±SD. Relative mRNA expression levels of *TNF α* was normalized to *GAPDH* levels. ^asignificantly different from the SD group; ^bsignificantly different from the HFD group; ^csignificantly different from HFD+100VSFE; ^dsignificantly different from HFD+200VSFE. The ^{a,b,c,d} symbol indicates a significant difference with a $p < 0.05$.

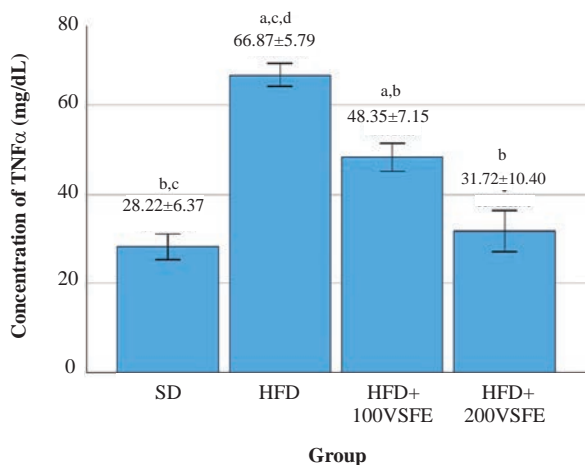


Figure 6. TNF α concentration after treatment of VSFE. Data was presented as mean \pm SD. ^asignificantly different from the SD group; ^bsignificantly different from the HFD group; ^csignificantly different from HFD+100VSFE; ^dsignificantly different from HFD+200VSFE. The ^{a,b,c,d} symbol indicates a significant difference with a $p < 0.05$.

Discussion

The result showed that the administration of 1% cholesterol for 8 weeks in New Zealand White rabbit could induce steatosis, macrophage infiltration, and elevation of liver enzyme which is the hallmark of NAFLD. This findings were consistent with previous studies.(21,22) Two liver indicators that can indicate liver disease are SGOT and SGPT. This was discussed through a previous study that detected elevated liver enzymes ($p < 0.01$) in HFD-induced rabbits.(23) As demonstrated by our research, liver enzyme levels considerably increased following HFD administration and markedly dropped in the VSFE-treated group.

In accordance with this study, VSFE decreased the amount of hepatic macrophages in rabbits with HFD. Several active compounds in the form of flavonoids and phenols act as antioxidants that could inhibit macrophage infiltration through the Toll-like receptor (TLR)-4 pathway so that the inflammatory response does not occur continuously.(22) Previous report described that one secondary metabolite in fermented extract was capsaicin, which could polarized macrophage liver to M2 more than M1.(24) Moreover, capsaicin worked through the transient receptor potential channel vanilloid type-1 (TRPV1) to enhance fat oxidation by inhibiting reactive oxygen species (ROS) production and modulating TNF α , thereby reducing triglyceride production in the liver. Antioxidants function by protecting against the damaging effects of ROS.(25)

Accumulated fat consumption from HFD exposure indicates an increase in lipid deposits in hepatocytes. The findings in this study are supported by previous research using fermented garlic extract in HFD-induced rats that was able to reduce hepatic steatosis and improve insulin resistance, accompanied by a decrease in liver triglyceride levels ($p < 0.05$).⁽²⁶⁾ Additionally, fermented cabbage extract was equally successful in reducing lipid build-up in red-oil staining in another work involving NAFLD model rats with HFD induction ($p < 0.05$).⁽²⁷⁾

The molecular mechanism by which VSFE reduces lipid droplets and inhibits macrophage infiltration was also investigated in this study, presumably through the role of transcription factors *C/EBP α* . Recent study reported that *C/EBP α* is a transcription factor that is highly expressed in hepatocytes.⁽²⁸⁾ A short 5-UTR (5 untranslated region), a distinct protein coding sequence (CDS-coding sequence), and a long 3-UTR make up the molecular basis of *C/EBP α* regulation.⁽²⁹⁾ When the *C/EBP α* gene's proximal promoter region was first identified, it revealed possible binding sites for NF- κ B, nuclear factor (NF), upstream stimulating factor (USF), basic transcription element-binding protein (BTEB), and additional *C/EBP* genes.⁽³⁰⁾ Further down the signaling transduction, *C/EBP α* interaction with transcription factors NF- κ B can controls cytokine production and cell survival.⁽³¹⁾

Moreover, active NF- κ B translocates into the nucleus where it binds to targets of inflammatory genes to cause the release of inflammatory cytokines such TNF α , interleukin (IL)-1 β , and IL-6, which aggravates steatohepatitis further.⁽⁷⁾ Administration of naringenin extract in previous study, which is rich in flavonoids, can provide protective effects against steatosis and inflammation in hepatocytes. In addition, the mRNA expression levels of inflammatory genes such as *TNF α* , nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (*NLRP3*), and *IL-18* increased in the negative control group and decreased significantly in the treatment group with naringenin.⁽³²⁾

Increased transcriptional activity of *C/EBP α* , directly regulates leptin expression and increases transforming growth factor (TGF)- β production and promotes fibrosis in mice kidney cells.⁽²⁹⁾ *C/EBP α* triggers adipogenesis by inducing peroxisome proliferator-activated receptor (PPAR) γ transcription and the two have a close relationship in influencing each other for adipogenesis and normal adipocyte function.⁽³³⁾ The mechanism by which *C/EBP α* induces *PPAR γ 2* expression is through direct binding to a specific location in its promoter.^(33,34) *Cornus officinalis*

extract at a dose of 2 µg/mL was able to suppress *C/EBPα* expression ($p<0.05$) but increased *PPARγ* expression in insulin-induced 3T3-L1 preadipocyte cells.(35) In line with this study, the increase in *C/EBPα* expression at the 100 mg/kgBW dose was higher than the HFD group and significantly decreased at the 200 mg/kgBW dose. It is hypothesized that the cross-interaction between *C/EBPα* and *PPARγ* indicates that the 100 mg/kgBW dose may increase *PPARγ* expression, which then modulates *C/EBPα* expression. Physiologically, the role of *C/EBPα* in responding to toxic lipid levels is by regulating lipogenesis through the unfolded protein response (UPR) pathway. (7) As another hypothesis proposed, VSFE 100 mg/kgBW treatment increased the biological response of cells in the form of *C/EBPα* upregulation, so then that excessive lipid regulation in cells is responded as a stress stimulation that activates the UPR signaling cascade, thus increasing the *C/EBPα*. Previous studies used MTL-CEBPα therapy in an lipopolysaccharide (LPS)-challenged humanized mouse model. Upregulation of *C/EBPα* led to increased neutrophils and attenuated the production of several critical pro-inflammatory cytokines, including *TNFα*, IL-6, IL-1β, and interferon (IFN)-γ.(36) Where these results are consistent with our findings, namely a decrease in *TNFα* and a decrease in macrophage infiltration.

Meanwhile, a higher dose of *C. officinalis* extract was able to suppress *C/EBPα*.(35) This confirms the findings in this study, where the relative expression of *C/EBPα* decreased significantly in the HFD+200VSFE group compared to the HFD group, which was only given a high-fat diet. A dose of 100 mg/kg BW VSFE has not been able to suppress the relative expression of *C/EBPα*. A HFD also triggers the upregulation of *C/EBP*, as a component of the UPR gene to maintain homeostasis of lipid metabolism in hepatocytes.(7)

VSFE has several phytoconstituent such as peptides, derivatives of flavonoid, capsaicin, flavanols, or phenols. (12) With such action, the VSFE, contains flavonoid, can provide protective effects against steatosis and inflammation in hepatocytes and lower the concentration of *TNFα* in the rabbit liver given HFD. Our results indicate that VSFE has some potential to prevent NAFLD, as shown in the decreased *C/EBPα*, *TNFα* expression and *TNFα* protein concentrations as well as a reduction of SGOT and SGPT enzyme levels, macrophage number and degree of steatosis. Rosuvastatin and propylthiouracil medications were used to treat NAFLD rabbits that were given a HFD for 8 weeks. The improvement of the hyperlipidemia condition was demonstrated by a significant drop in the levels of total cholesterol, low-density

lipoprotein (LDL), and triglycerides ($p<0.0001$) as well as a significant decrease in the inflammatory marker *TNFα* ($p<0.0001$). (37) Another study regarding the use of 0.6 mg/kg/day ezetimibe as hepatoprotectan agent for rabbit with NAFLD induced by HFD for 2 month. There were markedly reduced total cholesterol serum ($p<0.01$), attenuation of area lipid droplet ($p<0.05$), and triglyceride content in liver ($p<0.05$) compared to the control group fed with HFD without ezetimibe.(38) This study also demonstrated similar results, with a marked decrease in the relative expression of *C/EBPα*, *TNFα*, and *TNFα* protein concentrations and a reduction of SGOT and SGPT enzymes. These findings indicate that fermented vegetable extracts have the potential to be developed as nutraceutical to prevent NAFLD.

In current study, the positive control as a standard marker in this study has not been implemented. In the future, *C/EBPα* and other transcription factors must be explored regarding their specific role in NAFLD disease. Knockdown or overexpression methods should be used to validate the target of the pathways involved in NAFLD at the transcriptional, translational, post-translational modification, and activation levels.

Conclusion

This study demonstrates that in rabbits fed with HFD and 200 mg/kgBW VSFE was able to attenuate SGOT and SGPT levels, which are a hallmark of liver steatosis, and lower the number of macrophages, suppress the relative expression of *C/EBPα* and *TNFα*. These findings provide a groundwork for further research in developing drug candidates as a therapies or agent preventing liver-related pathological conditions by harnessing the bioactive potential of herbal medicine into drug candidates.

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Authors Contribution

ER were involved in conceptualization, supervision, and writing the original draft, formal analysis, methodology, investigation, MM performed data curation, formal analysis, conceptualization, writing the original draft, LSK done the supervision, conceptualization, formal analysis, methodology, BPS and FCR performed methodology and data curation, DJ and SS involved in concept supervision and funding. All the authors have been read, reviewed, and approve the entire manuscript.

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