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Antioxidant and Anti-inflammatory Effects of Vegetable Spices Fermentation Extracts in NAFLD

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ABSTRACT

Currently, the prevention strategy for Non-Alcoholic Fatty Liver Disease (NAFLD) has several limitations. Fermentation prolongs the shelf life of food products and enhances the bioavailability of bioactive compounds, essential vitamins, and minerals, thereby improving their nutritional efficacy. This study evaluated the antioxidant activity and the effects of Vegetable Spices Fermentation Extract (VSFE) on Peroxisome Proliferator-Activating Receptor (PPAR-γ) and Interleukin (IL)-6 mRNA expression in a NAFLD rabbit model. Using a posttest-only control group design, 24 rabbits were divided into four groups: standard diet group, high-fat diet (HFD) group, treatment group 1 (HFD+VSFE 100 mg/kg BW), and treatment group 2 (HFD + VSFE 200 mg/kg BW). PPAR-γ and IL-6 mRNA expression were measured using real-time polymerase chain reaction (RT-PCR). The fermented extract had a higher phenolic content (519.95 ± 61.9 mg GAE/g) than the non-fermented extract (357.45 ± 58.66 mg GAE/g) ($p<0.05$). The expression of PPAR-γ and IL-6 significantly decreased in groups 1 and 2 compared with the control group. In conclusion, VSFE could act as a source of antioxidants and anti-inflammatory effects to inhibit the establishment of NAFLD.
Keywords: Flavonoid; IL-6; NAFLD; Phenolic; PPAR-γ; Vegetable Spices Fermented Extract

INTRODUCTION

NAFLD is defined by the presence of over 5% fat accumulation in hepatocytes, not caused by alcohol consumption or other liver diseases. One of the primary causes of NAFLD is the consumption of high-fat foods, which leads to the accumulation of free fatty acids and liver triglycerides (TG) (Sufyan, 2019). The prevalence of NAFLD is increasing globally (Fachrureza & Pratomo, 2020). This trend is evidenced by the rise in NAFLD cases, from 27.94% during 2000–2010 to 31.63% during 2011–2021 (Liu et al., 2022). In Indonesia, the prevalence of NAFLD is around 30.6%, higher than that in China (20%) and India (24.6%) (Sufyan, 2019).

High-fat diets are major risk factors for NAFLD, characterized by hepatic steatosis (Fontes-Cal et al., 2021; Guo et al., 2022). In the development of NAFLD, PPAR-γ is one of the transcription factors that aggravate hepatic steatosis. PPAR-γ gene expression can be induced by Activating Transcription Factor 4 (ATF4), promoting the expression of pro-adipogenic mediators and triggering steatosis in the liver (Platko et al., 2019). Furthermore, the ATF4 transcription pathway regulates DNL through fatty acid synthesis in hepatic tissue (Zhang et al., 2014). Transcription of this gene induces the expression of Adipose Differentiation-Related Protein (FSP27), causing excess lipid uptake in the liver, which triggers endoplasmic reticulum stress in hepatocytes, leading to the development of NAFLD. Excessive fat accumulation is recognized as damage-associated molecular patterns (DAMPs), which serve as ligands for Toll-like Receptor (TLR) 4 (Martin et al., 2022). Activation of TLR4 activates the NLR family pyrin domain-containing 3 (NLRP3), which induces the maturation of caspase-1, breaking down pro-IL-1β into IL-1β. Nuclear Factor Kappa Beta (NF-κB) and interleukin-6 (IL-6) increase in response to stimulation by IL-1β as transcription factors through binding to the NF-κB and IL-6 gene promoters (Duan et al., 2022). Based on the description above, it can be concluded that IL-6 and PPAR-γ are the main markers of cell inflammation.

Before NAFLD is established, an appropriate strategy is essential. Current medications primarily address the underlying causes of NAFLD, such as using statin drugs to reduce cholesterol levels (Jeznach-Steinhagen et al., 2019). However, the long-term use of statins

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may result in side effects, particularly muscle-related issues, including myalgia, myopathy, and myositis (Ward et al., 2019). To minimize these side effects, safer, more accessible, and cost-effective non-pharmacological interventions, such as dietary modifications, are needed.

One of the typical Indonesian vegetable-based preparations is fresh vegetables commonly consumed cucumber and vegetables, served with sambal. The chemical compounds present in vegetables can be reduced if processed incorrectly. Spontaneous fermentation is an old vegetable processing method that is still unfamiliar in Indonesia. Fermentation can extend the shelf life of food and increase the bioavailability of active compounds, vitamins, and minerals in a food product, including vegetables (Şanlier et al., 2019). Fermentation improves the absorption of bioactive secondary metabolites such as vitamins and minerals by converting complex substances into simpler, more readily assimilated forms. Additionally, it minimizes the presence of anti-nutritional components that could interfere with nutrient uptake, thus enhancing both the nutritional value and functional characteristics of the food (Knez et al., 2023). One study reported by Shahbazi (2021) demonstrated that fermented cabbage has anti-inflammatory properties by suppressing pro-inflammatory cytokines. Preliminary studies have shown that the number of secondary metabolite compounds in the fermented chili sauce was increased compared to the non-fermented version. However, the effect of fermented sambal lalapan on NAFLD remains elusive. Therefore, the study examined how fermented sambal lalapan may prevent NAFLD by controlling lipid signaling pathways and inflammatory activity.

Antioxidants inhibit and prevent oxidative damage caused by free radicals by donating electrons (Solichah et al., 2021). They are categorized into three main types based on their origin. The first type is endogenous antioxidants, which are naturally produced in the body, such as glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD). The second type is exogenous antioxidants, obtained from external sources, including synthetic compounds like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate, which are commonly used in food products. The third type is natural plant antioxidants, including vitamins A, C, E, phenolic, and flavonoid compounds (Neha et al., 2019). Phenolics, the largest group of natural antioxidants, consist of a benzene ring with a hydroxyl group (-OH) that allows them to neutralize free radicals by donating hydrogen atoms (Dhurhania & Novianto, 2018). Flavonoids, a subgroup of phenolics, have a more complex structure with multiple benzene rings and a core of 15 carbon atoms (Al Mamari, 2022). Flavonoids found abundantly in foods like chili peppers, tomatoes, and vegetables could exhibit stronger antioxidant effects than vitamins C and E (Cheng et al., 2019).

MATERIALS AND METHODS

Materials

This study utilized several plant-based ingredients, including Brassica oleracea (cabbage), Ocimum sanctum L. (basil), Cucumis sativus L. (cucumber), Lycopersicum esculentum Mill. (tomato), Capsicum frutescens (cayenne pepper), Allium cepa L. (shallot), and Allium sativum (garlic), all of which were procured from a local traditional market in Malang, 24 male New Zealand White (NZW) rabbits obtained from the Veterinary Laboratory of Brawijaya University, salt, sugar, 70% ethanol, aquadest, GENAzol (Genazzon bioscience), RNA Extraction Reagent (Cat. No. 15596026; Thermo Fisher, Waltham, MA, USA), GoScript™ Reverse Transcriptase kit (Cat. No. A5001; Promega, Madison, WI, USA), GoTaq Master Mix (GoTaq 2-Step RT-qPCR System, Cat. No. A6010; Promega), Galic Acid (SIGMA), Folin-Ciocalteu (MERCK), Sodium Carbonate, Quercetin (SIGMA), Sodium Nitrate, Aluminum Chloride, and 1 M Sodium Hydroxide.

Methods

Preparation of Vegetables Spices Fermentation Extract (VSFE)

The fermentation extract was prepared following established protocols. Cabbage was mixed with 3% salt and fermented at 4°C overnight. The following day, peeled cucumbers and slices of basil were added to the pickled cabbage, along with a mixture of shallots, garlic, chili, tomatoes, sugar, and salt. The entire blend was sealed in a jar and incubated at 20°C for 14 days (Rachmawati et al., 2023). The fermented product was then oven dried at 60°C for 5 days and milled into powder (Vatansever et al., 2017). Extraction was carried out using the Ultrasound-Assisted Extraction (UAE) method at 27°C for 30 minutes. The extract was concentrated via rotary evaporation at 50°C for 60 minutes and dried again at the same temperature until a solid extract was obtained (Wahyu et al., 2020). For in vivo studies, dosage variations of 100 mg/kg body weight (BW) and 200 mg/kg BW were determined based on previous research (Kieczykowska & Musik, 2020).

Preparation of Non-Fermented Vegetables Spices Extract (NFVSE)

The non-fermented extract was prepared through a thermal frying process. In the initial preparation stage, 30 grams of basil leaves were washed and air-dried. Subsequently, garlic, shallots, small red chili and peppers and tomatoes are sautéed in 100 mL of coconut oil at a controlled temperature of 32°C, followed by draining to remove excess oil (Sudarmadji~ & Markakis, 2018). The sautéed mixture is then homogenized into a chili paste. Separately, the cabbage and cucumber are thinly sliced. These vegetable components are then combined with the basil and chili paste, with the addition of salt and sugar to enhance flavor and preservation (Rachmawati et al., 2023). For the preparation of *simplicia*, the unfermented *sambal lalapan* undergoes a drying process in an oven at 40°C until a stable, dry consistency is achieved (Chao et al., 2017).

Animal Experiment Design

This study used 24 male NZW rabbits, 5 months old. The rabbits were housed in cages measuring 50 x 70 x 70 cm^3 at 20°C temperature with a 12:12-hour light-dark cycle. The Ethics Committee of Brawijaya University approved the research protocol, with number 174-KEPUB-2024.

The rabbits were fed regular Nova feed and allowed unlimited access to water. Following two weeks of acclimation, the rabbits were split into four groups at random: the standard diet (SD) group (n=6), which was fed a standard diet (protein 16%, fat 4%, fiber 13%, starch 52%, phosphorus 657 mg/kg BW, calcium 787 mg/kg BW) (Lozano et al., 2019); the high-fat diet (HFD) group (n=6), which was standard diet feed supplemented with 1% cholesterol from cow brain (Rachmawati & Muhammad, 2021) ; the treatment group 1 (n=6), which was fed an HFD and 100 mg/kg BW VSFE; and treatment group 2 (n=6), which was fed an HFD and 200 mg/kg BW VSFE. After receiving care for eight weeks, every rabbit was euthanized under anesthesia, and surgical procedures were performed to collect organ samples.

Organ Sampling

The rabbits were terminated after a 24-h fast. A subcutaneous injection of 0.1 mg/kgBW of xylazine and ketamine in a 1:1 ratio was used to induce anesthesia (Baumgartner et al., 2010). Liver samples were obtained after a particular process: the liver was surgically dissected after the rabbits were put down. To remove any remaining blood, the livers were cleansed using cold 0.9% NaCl. The livers were then separated longitudinally into the left and right lobes and allowed to air dry on filter paper. Immediately after, the liver was incubated for 48 hours in 10% neutral-buffered formalin (NBF). The liver tissue is kept clean and preserved for additional examination thanks to this meticulous procedure. In order to preserve the integrity of the liver specimens, liver samples are meticulously processed by integrating the processes of dissection, cleaning, and incubation (Figen, 2020).

Total Phenolic Content

The total phenolic content test in this study was carried out using the Folin–Ciocalteu method with gallic acid as the standard compound, involving the mixing of 400 µL of Folin–Ciocalteu and 200 µL of the sample in a 1.5 mL microtube, followed by vortexing, incubation for 5 minutes, the addition of 300 µL sodium carbonate, further vortexing, and incubation in the dark for 2 hours, with four repetitions using Vegetables Spices Fermentation Extracts at the same concentration to confirm the total phenolic content in the sample (Budiono et al., 2023).

Total Flavonoid Content

The total flavonoid content test in this study was conducted using the aluminum chloride colorimetric assay method with quercetin as the standard compound, involving the addition of 200 µL of the sample, 280 µL of distilled water, and 60 µL of sodium nitrate into a 1.5 mL microtube, followed by vortexing and incubation in the dark for 5 minutes, then the addition of 60 µL of aluminum chloride, vortexing, and further incubation in the dark for 6 minutes, followed by the addition of 400 µL of 1 M sodium hydroxide and vortexing, with four repetitions using vegetables Spices Fermentation extracts at the same concentration to confirm the total flavonoid content in the sample (Acero et al., 2024).

Gene Expression Quantification

To maintain RNA integrity until gene expression analysis, a 20-mg liver sample was promptly surgically excised and stored in GENAzol solution at -80°C. Total RNA was extracted using the Total RNA Extraction Reagent (Cat. No. 15596026; Thermo Fisher, Waltham, MA, USA). The GoScriptTM Reverse Transcriptase kit (Cat. No. A5001; Promega, Madison, WI, USA) was used to synthesize cDNA. A MyGo PCR kit was used to carry out the polymerase chain reaction (PCR). Each tube was filled with cDNA, certain primers, and GoTaq Master Mix (GoTaq 2-Step RT-qPCR System, Cat. No. A6010; Promega). The primers listed below were taken from earlier research and validated by NCBI.

PPAR-γ:
Forward 5'-TGGGGATGTCTCATAATGCCA-3'
Reverse 5'-TTCCTGTCAAGATCGCCCTCG-3'
IL-6:
Forward 5'-GTCTTCCTCTCTCACGCACC-3'
Reverse 5'-TGGGCTAGAGGCTTGTCACT-3'
GAPDH:
Forward 5'-GGAGAAAGCTGCTAA-3'
Reverse 5′- ACGACCTGGTCCTCGGTGTA-3′

PCR was started with denaturation at 95°C temperature for 15 seconds. With a total run period of 60 minutes, annealing temperatures were set to the Tm of each gene: PPAR-γ at 53°C and IL-6 at 60°C. The analytical program was used to determine each gene's Ct values. The relative expression levels were calculated using the Livak formula ($2^{-\Delta\Delta Ct}$) (Livak & Schmittgen, 2001).

Statistical Analysis

The comparison of the flavonoid and phenol contents was analyzed using an independent t-test. Moreover, the effects of VSFE on gene expression in the rabbit NAFLD model were tested using a one-way ANOVA. LSD was used in a post hoc test to conduct additional analysis. SPSS version 29.0 (IBM Corporation, Armonk, NY, USA) was the software used, and a value of $p<0.05$ was deemed statistically significant.

RESULTS

Vegetable Spices Fermented Extract Has Been Proven to Have a Higher Phenolic but Lower Flavonoid Content Compared to Non-fermented Extract

The results of the gallic acid and quercetin measurements were used to create standard curves showing the relationship between the absorbance and varying concentrations of each compound to derive their respective regression equations. The regression equation for gallic acid was y = 0.0011x 0.0092 with a coefficient of determination $R^2 = 0.9293$, where y represents the absorbance value and x represents the total phenolic content. Similarly, for quercetin, the regression equation obtained was y = 0.0007x + 0.0094 with a coefficient of determination $R^2 = 0.9459$, where y represents the absorbance value and x represents the total flavonoid content.

VSFE Suppressed PPAR-γ Expression in NAFLD Rabbits Model

The administration of VSFE significantly reduced PPAR-γ expression across all experimental groups (p = 0.003). As shown in Figure 1, the relative mRNA expression level of PPAR-γ in the HFD group was 2.15 [0.65–1.55], which differed significantly from that in the SD group (1.00 [0.66–1.52]). This research revealed that VSFE at dosages of 100 and 200 mg/kgBW reduced PPAR-γ expression, with corresponding expression values of 1.10 [0.76–1.32] and 1.29 [0.57–1.76], respectively. However, there were no statistically significant differences found between the treatment groups and the SD group. (p = 0.984 and p = 0.900).

Data are presented as mean±SD. Relative mRNA expression levels of PPAR-γ were normalized to GAPDH levels. [a]significantly different from the SD group; [b]significantly different from the HFD group; [c]significantly different from treatment group 1; [d]significantly different from treatment group 2. The [a,b,c,d] symbols indicate a significant difference with a $p<0.05$. SD: standard diet; HFD: high-fat diet; T1: treatment 1 (HFD+VSFE 100 mg/kg BW); T2: treatment 2 (HFD+VSFE 200 mg/kg BW).

VSFE Suppressed IL-6 Expression in NAFLD Rabbits Model

Suppression of IL-6 expression by VSFE showed significant differences in all treatment groups (p = 0.000). Figure 2 displays the relative expression of IL-6 mRNA in the HFD group (2.10 [1.60-5.21]), and a significant difference was observed when compared to the SD group (1.00 [0.58-2.43]). This study demonstrated that VSFE suppressed the expression of IL-6 at treatment doses of 100 and 200 mg/kgBW, with relative expression values of 0.42 (0.40-0.62) and 0.18 (0.04-1.38), respectively. A marked reduction was observed in both treatment groups relative to the HFD control, with p-values indicating high statistical significance ($p < 0.001$).

Data are presented as mean±SD. Relative mRNA expression levels of IL-6 were normalized to GAPDH levels. [a]significantly different from the SD group; [b]significantly different from the HFD group; [c]significantly different from treatment group 1; [d]significantly different from treatment group 2. The [a,b,c,d] symbols indicate a significant difference with a $p<0.05$. SD: standard diet; HFD: high-fat diet; T1: treatment 1 (HFD+VSFE 100 mg/kg BW); T2: treatment 2 (HFD+VSFE 200 mg/kg BW).

DISCUSSION

The absence of Food and Drug Administration (FDA) approved pharmacological interventions for the prevention and treatment of this condition underscores the pressing need for the development of targeted therapies. Existing

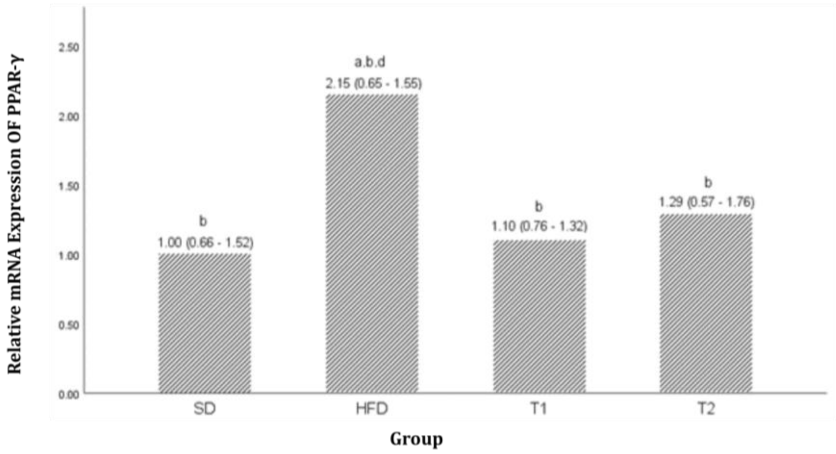

Figure 1. Relative mRNA expression of PPAR-γ

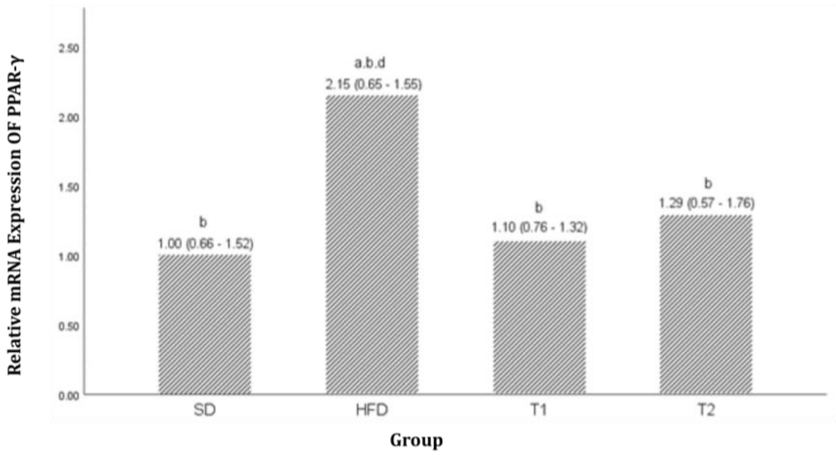

Figure 2. Relative mRNA expression of IL-6

Table 1. Total Phenolic and Flavonoid Content in Fermented and Non-Fermented Vegetable Spices Extracts

Sample	Phenolic Mean ± SD (mg GAE/g)	Flavonoid Mean ± SD (mg QE/g)
Fermented	519.95 ± 61.9*	34.42 ± 8.92*
Non-fermented	357.45 ± 58.66*	83 ± 25.56*

*p < 0.05

approaches for NAFLD prevention involve the use of statins and lifestyle changes. However, these interventions did not achieve optimal results. Reports have highlighted the risks associated with long-term statin use, including hepatotoxicity and myopathy (Karahalil et al., 2017). Additionally, statins can increase the risk of muscle-related complaints such as myalgia and rhabdomyolysis, nervous system issues, and type 2 diabetes mellitus (T2DM) (Thompson et al., 2016). Thus, additional preventive strategies based on dietary modifications are needed to complement existing therapies. Numerous studies suggest that antioxidants are a potential therapeutic option for preventing NAFLD (Perumpail et al., 2018). Antioxidants are abundant in vegetables and herbs, making them a promising source for overcoming NAFLD. A processing method that can increase the antioxidant content of a food ingredient is fermentation; this occurs because during the fermentation process, a hydrolysis reaction occurs, which is catalyzed by microorganisms, causing an increase in its antioxidant content (Hur et al., 2014). Therefore, VSFE can be used as a candidate for a functional food.

According to BPOM (Badan Pengawas Obat dan Makanan), quoted by Suter (2013), a functional food is defined as a natural or processed food containing one or more ingredients that, according to scientific research, have certain physiological functions that can benefit health. In addition, functional food is consumed, such as food with sensory characteristics in terms of color, texture, and flavor profiles that meet consumer preferences. VSFE can be used as a candidate for functional food products. The research conducted by Rachmawati et al. (2023) showed that VSFE contains phenolic and flavonoid compounds. After analyzing the possible interactions between the active compounds (phenolic and flavonoids) and the incidence of dyslipidemia, endothelial dysfunction, and atherosclerosis, the results showed that VSFE has the potential to improve lipid profiles, reduce the occurrence of endothelial dysfunction, inhibit the formation of atherosclerotic plaque, and inhibit the occurrence of coronary heart disease.

One key contributing factor is the antioxidant properties of phenolics and flavonoids, which were the focus of this study. The phenolic content in non-fermented foods is often low because most active compounds remain trapped in the food matrix and are bound in large molecular complexes linked to glycosides. The increase in the phenolic content in the fermented vegetable spices can be attributed to the activity of lactic acid bacteria (LAB), which thrive during fermentation. LAB can release enzymes, such as tannase, esterases, phenolic acid decarboxylase, and glycosidases, which liberate the bound phenolics in the food matrix. This biotransformation increases the quantity and variety of free phenolics in fermented foods (Yang et al., 2023).

Spontaneous fermentation in vegetable spices can lead to the breakdown of flavonoids into phenolics, a process facilitated by microorganisms (LAB) through the production of the enzyme β-glucosidase (Zhu et al., 2022). The β-glucosidase enzyme breaks down the sugar moiety in the flavonoid compounds, resulting in aglycones (the free forms of flavonoids). In addition to β-glucosidase, tannase is another enzyme identified during fermentation. Tannase hydrolyzes tannins into gallic acid and pyrogallol, phenolic compounds (Rahmi et al., 2020). Phenolics have high bioavailability, which allows maximum absorption in the body. According to research by Adebo & Medina-Meza (2020), fermentation alters flavonoid compounds, transforming them into other compounds with unidentifiable structures. These changes occur due to the decarboxylation, hydrolysis, and esterification reactions during fermentation.

Increased PPARγ expression can intensify hepatic steatosis by inducing the expression of adipose differentiation-related protein (ADRP), which leads to excessive lipid uptake (Zhang et al., 2014). This finding is supported by the present study, where PPARγ expression significantly decreased in treatment groups 1 (100 mg/kgBW VSFE) and 2 (200 mg/kgBW VSFE) compared with the HFD group. The mechanism by which this fermentation product reduces PPARγ levels is likely attributed to the presence of active compounds in VSFE. Previous studies have shown that VSFE contains secondary metabolite compounds, such as flavonoids and fisetin, which reduce PPARγ expression. Flavonoids, acting as antioxidants, have been shown to decrease PPARγ expression by reducing ROS levels, thereby inhibiting hepatic steatosis (Ammazzalorso & Amoroso, 2019). Similarly, fisetin can reduce the mRNA expression of PPARγ in the liver of mice fed a high-fat diet. This reduction occurs through decreased lipid accumulation in the liver tissue, as fisetin can block lipid synthesis in hepatocytes by inhibiting the PPARγ transcription factor (Liou et al., 2018). Based on these findings, it can be concluded that the fermentation compounds present in VSFE may act as antioxidant agents.

Another key pathway contributing to hepatic steatosis involves increased IL-6 levels, which intensify lipid accumulation by inducing the expression of lipogenic enzymes regulated under the control of STAT3. This finding is supported by the present study, where IL-6 expression significantly decreased in treatment groups 1 (100 mg/kgBW VSFE) and 2 (200 mg/kgBW VSFE) compared with the HFD group. The mechanism by which this fermentation product reduces IL-6 levels is likely attributed to the active compounds in VSFE. Previous studies have shown that VSFE contains secondary metabolite compounds like capsaicin, which reduce IL-6 expression. Capsaicin has been shown to reduce TLR4 expression, thereby inhibiting NF-κB activation (Bian et al., 2019; Zhao et al., 2021). Based on these findings, it can be concluded that the fermentation compounds present in VSFE may act as anti-inflammatory agents. This study primarily focused on evaluating the biological effects of VSFE. Although the non-fermented extracts were analyzed for phenolic and flavonoid content, in vivo testing was not conducted. Future studies are encouraged to directly compare both fermented and non-fermented extracts in biological models to provide a more comprehensive understanding of their effects.

CONCLUSION

The results of this study indicate that in rabbits receiving a high-fat diet (HFD), the fermented VSFE exhibited a higher total phenolic content than the non-fermented extract, indicating enhanced antioxidant potential. The breakdown of the total flavonoid content into phenolics during fermentation increases the bioavailability, enabling maximum absorption in the body. This transformation leads to the formation of simpler phenolic acids, such as gallic acid or ferulic acid, which are more readily absorbed and exhibit stronger antioxidant properties compared to their original flavonoid forms. These antioxidant properties are likely responsible for suppressing the relative expression of PPAR-γ and IL-6, key markers of inflammation in the NAFLD rabbit model, observed at doses of 100 mg/kg BW and 200 mg/kg BW of VSFE. The differences in the bioactive compound content between fermented and non-fermented VSFE further highlight the therapeutic potential of fermented herbal preparations. These findings support further investigations into the development of therapeutic agents by utilizing the bioactive components of herbal medicines—such as those present in Sambal Lalapan—for the prevention and treatment of liver-related disorders, including NAFLD.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in the conduct and publication of this research.

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