Development of fermented vegetables in their ability to inhibit pancreatic lipase as antidyslipidemic agent

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Abstract. Improvement of public awareness of healthy living encourages the increase of demand for natural food products and provides health benefits for the body. Cardiovascular disease caused by atherosclerosis of blood vessel walls and thrombosis is the leading cause of death in the world. Dyslipidemia and coagulation disorders are one of the most significant risk factors for the development of atherosclerotic conditions. The purpose of this study is to develop one of the healthy food products from fermented green vegetables (spinach, broccoli and katuk leaves) fermented by kombucha culture during fermentation as a functional beverage in its ability inhibits pancreatic lipase as anti-dyslipidemia. Results showed a correlation between the ability to defend against free radicals and the ability to inhibit pancreatic lipase enzyme activity. The optimum conditions obtained in the fermentation of katuk leaf vegetables based on the ability to inhibit pancreatic lipase activity (% inhibition) and the ability to inhibit free radicals (DPPH), which is for 6 days fermentation of 91.44%, and 88.62%, with a pH value 3.98.

Keywords: fermentation, green vegetables, kombucha, antidyslipidemic, lipase pancreatic inhibition.

1. Introduction

Recently research developments in the cardiovascular field are very rapid. This is indicated by the increasing number of studies in this field. Atherosclerosis, especially regarding the role of lipids also undergoes rapid development in accordance with the results of the research conducted (1). Cardiovascular disease due to atherosclerosis of blood vessel walls and thrombosis is the leading cause of death in the world. Epidemiological studies have shown that dyslipidemia and coagulation disorders are one of the most significant risk factors for the development of atherosclerotic conditions (2). Dyslipidemia is an abnormality in blood lipid levels characterized by elevated triglyceride levels, LDL cholesterol, total cholesterol and decreased HDL cholesterol in the blood. Without immediate treatment, dyslipidemia can lead to many diseases, which one of them is coronary heart disease (CHD)(3).

Conventional treatment of dyslipidemia is done by regulating the correct diet and taking blood lipid-lowering drugs, such as statins (4). Adjuvant therapy is performed by giving additional treatment to primary therapy by using an adjuvant form, such as functional food.

Katuk (Sauropus androgynous), spinach (Amaranthus) and broccoli (Brassica oleracea var. Italic) are a vegetable plant, which is widely available in Southeast Asia. Utilization is currently limited to direct consumption as a daily vegetable menu. Fermentation of vegetables is considered to be one of the processes for developing products that are beneficial to health. One of the most important sources of
potential pancreatic lipase inhibitors represents the class of polyphenols and flavonoids as natural bioactive compounds their potential effect on metabolic diseases as a health food. The functional foods have physiological properties for the body by developing products from green vegetables, and one of them is fermented food by kombucha.

The functional foods have physiological properties for the body by developing products from green vegetables, and one of them is fermented food by kombucha. Kombucha is a fermented drink that is popular among many other traditional fermented foods. The presence of bacteria and yeast in the medium plays a role in creating a strong symbiosis that can inhibit the growth of microorganism contamination (5). The dominant bacteria that grows in kombucha culture is acetate acid bacteria, which is an aerobic bacterium that can use alcohol as a substrate to form acetic acid. These bacteria, in contrast to yeast, require large amounts of oxygen for their growth and activity (6).

The physiological effects of these bioactive compounds from high antioxidant beverages can help to reduce the risk of cardiovascular disease. Green vegetables contain high levels of polyphenols and flavonoids. Polyphenols work by slowing up the absorption of triacylglycerol through inhibition of pancreatic lipases, increasing excretion of cholesterol through feces, increasing excretion of LDL receptors in the liver, and inhibiting secretion of apolipoprotein B100 (7)(8).

Alkaloid compounds can inhibit pancreatic lipase enzyme activity that increases fat secretion through feces so that it cannot be converted into cholesterol (9). Flavonoids act as antioxidants that can reduce LDL cholesterol and triglyceride levels, by increasing the activity of the lipoprotein lipase enzyme that plays a role in the hydrolysis of triglycerides into fatty acids, the mechanism of action is reducing LDL sensitivity to free radicals. Flavonoids also inhibit HMG-CoA reductase in reducing cholesterol levels in the blood (10).

The purpose of this study is to develop one of the healthy food products from fermented green vegetables (spinach, broccoli and katuk leaves) fermented by kombucha culture during fermentation as a functional beverage in its ability inhibits pancreatic lipase as anti-dyslipidemia.

2. Material and Methods

2.1. Kombucha starter culture

Kombucha starter culture, also known as 'SCOBY', (generally consists of Acetobacter xylinum, Glukonobacter dan S.cerevisiae ) is obtained from the commercial collection of Indo Kombucha, Indonesia. The starter culture used in this study was stored in a refrigerator (4°C) and consisted of sour broth and cellulosic layer ("tea fungus" floating on the liquid surface).

2.2. Kombucha beverage from green vegetables preparing and sampling procedures

The ingredients consisted of spinach vegetables (Amaranthus spp.), (Brassica oleracea L.) and katuk leaves (Sauropus androgynous), each of which was fermented in a Kombucha starter culture. The following ingredients were used to make the drink consisting of sterile water, starter culture of kombucha tea, filtrate spinach leaves (Amaranthus spp.), Broccoli (Brassica oleracea L.) and katuk leaves (Sauropus androgynous) and sucrose. Each drink was made from the ratio of spinach leaves, broccoli and katuk leaves (1 part each with 4 parts of water then 80 mesh escaped filtering and 100 g/L of sucrose. The filtrate of each vegetable was pasteurized at 90–100 °C for 5–10 minutes, and sucrose was added to each juice filtrate and then stirred until dissolved, then placed in a sterile jar until it cools to room temperature 27–30 °C. After that, the juice filtrate was inoculated with a starter culture of kombucha in 60 ml acidic liquid (sour broth) and 40 g of cellulose layer per 1 L juice (total 10% wt/vol). Jars were covered with sterile gauze to ensure aerobic conditions for fermentation. The fermentation process was carried out in a range of 0 to 12 days with 3 days interval in temperature dark room (± 28–30 °C).

2.3. pH measurement Acidity

pH measurement Acidity of the tested product was determined with the use of a calibrated pH meter (pH700, Eutech Instrument). Three replicate measurements were performed.
2.4. Determination of polyphenol content
Total phenolic content was estimated using the Folin-Ciocalteu method (11). Samples of 0.1 mL were added with 700 µL of aquadest, and 500 µL of Folin Ciocalteu reagent and 1 mL of saturated Na₂CO₃ solution. Afterwards, the mixture was added with another 1.4 mL of aquadest, then vortexed. The result was analyzed by UV-Vis Spectrometer at 760 nm against a blank, after 2 h at dark room temperature. Gallic acid was used as a standard and the concentration was expressed as micromoles of gallic acid equivalents (GAE) per g of wet weight (mL).

2.5. DPPH• (2,2-Diphenyl-1-picrylhydrazyl radical) scavenging activity assay
The free radical scavenging activity of kombucha was evaluated using the DPPH• scavenging activity assay, as described by (12). In brief, aliquots of beverage samples from each fermentation result (500 µL) was added to 500 µL of a daily prepared solution of 0.1 mM DPPH (0.4 mg/mL) in methanol. Next, the mixture was added with 1.5 mL of methanol. The solution was stored at room temperature in a dark room for 30 minutes and DPPH free radical reduction was measured by reading absorbance at 515 nm using a spectrophotometer (Agilent Technologies, Cary 60 UV-Vis). The following equation was used to measure inhibitory activity:

\[
\text{Scavenging capacity} (\%) = \frac{(\text{Control Absorbance} - \text{Sample Absorbance})}{\text{Control Absorbance}} \times 100
\]

2.6. Measurement of Porcine Pancreatic Lipase Inhibitory Activity
The ability of the kombucha green vegetables to inhibit pancreatic lipase was measured using the method previously reported by Kim et al.(13) Briefly, an enzyme buffer was prepared by the addition of 25 mg/mL porcine pancreatic lipase solution (Sigma-Aldrich) in buffer containing 10 mM MOPS (morpholinepropane sulphonic acid) and 1 mM EDTA, pH 6.8, to 155 µL Tris buffer (100 mM Tris-HCl and 5 mM CaCl₂, pH 7.0). Then, 20 µL of either the vegetables extracts at the test concentration (10 µg/mL) or orlistat (Roche, Basel, Switzerland) were mixed with 175 µL enzyme buffer and incubated for 15 min at 37°C with 5 µL substrate solution (10 mM p-NPB (p-nitrophenylbutyrate) in dimethyl formamide); the enzymatic reactions were allowed to proceed for 15 min at 37°C. Lipase activity was determined by measuring the hydrolysis of p-NPB to p-nitrophenol at 405 nm using an ELISA reader (Varioskan Flash, Thermo Scientific). Inhibition of lipase activity was expressed as the percentage decrease in OD when porcine pancreatic lipase was incubated with the test materials. Lipase inhibition (%) was calculated according the following formula:

\[
\text{Inhibition} (\%) = 100 - \left( \frac{B - b}{A - a} \times 100 \right)
\]

where A is the activity without inhibitor, a is the negative control without inhibitor, B is the activity with inhibitor, and b is the negative control with inhibitor.

3. Result and Discussion
3.1. Chemical characterization of raw material (green vegetables) before fermentation process
The compositions of raw material for healthy food products from fermented green vegetables (spinach, broccoli and katuk leaves) before being fermented. The results were shown below (Table 1).

| Composition                  | Material (Green vegetables)* |
|------------------------------|------------------------------|
| Lipase pancreatic inhibitory activity (%) | Spinach | Broccoli | Katuk leaf |
| DPPH Inhibitory activity (%) | 53,05 | 36,74 | 90,85 |
| Polyphenol (mg GAE/mL)       | 0,09 | 0,04 | 0,23 |
| pH                           | 6,32 | 4,99 | 6,07 |
| Dissolved Protein (mg/mL)    | 25,06 | 20,00 | 57,03 |
| Reducing Sugar (mg/mL)       | 3,54 | 0,44 | 0,71 |

*) Ratio extract of green vegetables and destilation water (1:4)
The choice of broccoli, spinach, and katuk leaf were based on the composition, especially higher polyphenols compared to other types of vegetables. The difference in composition of the types of green vegetables varies as shown in Table 1. Spinach vegetable extracts have a high lipase inhibitory activity compared to other vegetables, but from the antioxidant activity and the highest total polyphenol content in katuk leaf vegetable extract each of 90, 85% and 0.23 mg GAE/mL.

3.2. Lipase pancreatic inhibitory activity and pH
Pancreatic lipase is a key enzyme for the absorption of triglycerides in the small intestine. It is removed from the pancreas and hydrolyzes triglycerides to glycerol and free fatty acids\cite{14,15}. Plants have a variety of natural chemical compounds that have diverse structural features, so many of them are potentially compatible with pancreatic lipase modulation mechanisms. Inhibition of pancreatic lipase is a viable strategy to combat obesity and lower blood lipid levels (16). Some vegetables have a good ability to inhibit the action of pancreatic lipases. The effect of the fermentation process on the vegetable lipase inhibitory activity can be seen in Figure 1, where the length of fermentation time tends to affect lipase inhibitory activity in vegetables, during the fermentation process the lipase inhibitory activity tends to increase during the fermentation time in green vegetables.

![Figure 1](image)

**Figure 1.** Effects of fermentation time and variation green vegetables with culture of *kombucha* to lipase pancreatic inhibitory activity and pH of fermented vegetables.

This is due to some active compounds in the fermentation process of vegetables were degraded due to this process and produce other bioactive compounds such as flavonoids. The results of this fermentation obtained the highest lipase inhibition activity in katuk vegetables with a 6 day fermentation process of 91.44% and the lowest in spinach vegetables at 12 days fermentation at 28.41%.

The pH value in this study was significantly influenced by the duration of fermentation. Kombucha products contain yeast and bacteria that metabolize sucrose to produce organic acids such as acetic acid and gluconate acid. According to Jayabalan (17) Yeasts and bacteria in vegetable kombucha are involved in such metabolic activities that utilize substrates in different and complementary ways. Yeasts hydrolyze sucrose into glucose and fructose by invertase and produce ethanol via glycolysis, with a preference for fructose as a substrate. Acetic acid bacteria make use of glucose to produce gluconic acid and ethanol to produce acetic acid. As a result, the pH value of kombucha beverage decreases due to the production of organic acids during fermentation.

3.3. DPPH inhibitory activity and polyphenol
Polyphenols are a group of powerful antioxidants that can protect the body from cell damage due to free radicals. Polyphenols can be found in green vegetables with a high enough level. It also functions as a color giver of vegetables. The effect of the fermentation process on total levels of vegetable polyphenols can be seen in Figure 2, where the length of fermentation time tends to affect the total levels of polyphenols in vegetables. During the fermentation process, the total value of polyphenols tends to fluctuate in green vegetables. This can happen because phenolic compounds in the fermentation process of vegetables are degraded due to this process. In addition, they produce other
bioactive compounds and forms organic acids, acetaldehyde, alcohol and carbon dioxide which play a role in the formation of specific kombucha scents.

![Figure 2](a)  (b)

**Figure 2.** Effects of fermentation time and variation green vegetables with culture of kombucha to DPPH inhibitory activity and polyphenol of fermented vegetables

In this extraction, the highest total polyphenols obtained in katuk vegetables with a 6 day fermentation process of 0.24 mg GAE/mL followed by spinach in 3 days fermentation (0.21 mg GAE/mL) and the lowest in broccoli vegetables in 12 days fermentation amounting to 0.07 mgGAE/mL. According to Sun, Jin Hur et al (18). Fermentation can increase antioxidant activity mainly due to an increase in the amount of phenolic compounds and flavonoids during fermentation, which is the result of microbial hydrolysis reactions. Furthermore, fermentation induces the structural breakdown of plant cell walls, leading to the separation or synthesis of various antioxidant compounds. These antioxidant compounds can act as free radical terminators, metal chelators, singlet oxygen quenchers, or hydrogen donors to radicals. The production enzymes such as protease and α-amylase can be influenced by fermentation that may have metal ion chelation activity.

The free radical (DPPH) inhibition activity of each vegetable has a different pattern during fermentation. In broccoli fermentation, it was seen that the longer the fermentation time increased the antioxidant value of katuk leaf kombucha juice by 93.48%. Increased antioxidant activity in kombucha tea was caused by the results of microorganism metabolism in kombucha during the fermentation process (19). However, in katuk leaf fermentation with increasing fermentation time, it will reduce antioxidant activity. This was caused by degradation of phenolic compounds by enzymes produced by kombucha culture, so that the compounds that have antioxidant activity lose their stability. Kombucha exhibited increased free radical scavenging activities during fermentation. The extent of the activity depended upon the fermentation time, type of material, and the normal microbiota of the kombucha culture, which in turn determined the nature of their metabolites. Although free radical scavenging properties of kombucha showed time-dependent profiles, prolonged fermentation is not recommended because of accumulation of organic acids, which might reach harmful levels for direct consumption (17).

### 3.4. Reducing sugar and Dissolved protein

The ability of SCOBY cultures to reducing sugar in each sample of various types of kombucha vegetables varies. During the fermentation process, sucrose is transformed into glucose and fructose by bacteria and yeast. Glucose is used to form alcohol and organic acids, so that the concentration of organic acids increases. During the fermentation process yeast kombucha breaks down sucrose into glucose and fructose (20). Glucose will be used for cell metabolism to produce ethanol and carbon dioxide.
Figure 3. Effects of fermentation time and variation green vegetables with culture of kombucha to reducing sugar and dissolved protein of fermented vegetables

Reducing sugar varied during the fermentation time for each different type of vegetable. There was a decrease in sugar content from day 0 to day 9 and it increases again after fermentation on the day 12 for fermented spinach and katuk leaves. The lowest reduction in reducing sugars at day 9 of spinach and katuk leaf fermentation was 0.97 mg/mL and 1.31 mg/mL, respectively, while for the broccoli was at 3 days fermentation at 2.06 mg/mL.

The dissolved protein produced in vegetable fermentation is a breakdown parameter of large molecular weight proteins into amino acids and peptides to be lower molecular weight proteins by microbial proteolytic activity in kombucha culture, resulting in higher water solubility as a dissolved protein. The tendency of dissolved protein content by proteolytic activity shows a different pattern of each vegetable during the fermentation process, as seen from the varied pattern of changes. Since the amount of dissolved protein is also influenced by protease enzymes from microbes, it can be assumed that proteolytic microbes do not control the kombucha culture, as shown in Figure 3.

4. Conclusions

Development of processed vegetables as functional beverages through the fermentation process yields inhibition of pancreatic lipases that vary during fermentation time. The results showed that katuk leaves exhibits the optimum lipase inhibition activity as anti-dyslipidemia compared to spinach or broccoli vegetable fermentation. The optimum katuk leaf fermentation was at 6 days of fermentation time with inhibition of pancreatic lipase by 91.44% with antioxidant activity of 88.62% at pH 3.98, total polyphenol at 0.24 mg GAE/mL, reducing sugar by 1.97mg /mL and dissolved protein at 50.90 mg/mL.

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