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Pancreatic lipase inhibitory activity of butterfly pea flower (*Clitoria ternatea*) kombucha

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ABSTRACT. Preventing the action of pancreatic lipase is believed to be an effective method for treating obesity. Pancreatic lipase inhibitor acts by suppressing the activity of pancreatic lipase, leading to decreased lipid absorption. Kombucha is a traditional fermented drink believed to have numerous health benefits, including anti-obese. It can be produced using a range of substrates, such as butterfly pea flowers. The aim of this research was to investigate the inhibitory activity of kombucha made from butterfly pea flowers toward pancreatic lipase. The fermentation parameters monitored in this study included changes in cell density (OD600), the dry weight of the kombucha mushroom, pH, reducing sugar content, and the percentage of titratable acid. The total phenolic and flavonoid content were also analyzed before and after fermentation. The inhibitory effect of butterfly pea flower kombucha on pancreatic lipase was presented as IC_{50}. The findings indicated that as fermentation progressed, the pH level and amount of reducing sugar decreased while the % titratable acid, cell density (OD600), and dry weight of the kombucha mushroom increased. The phenol and flavonoid content of butterfly pea kombucha was greater than butterfly pea infusion, with respective levels of 0.040 mg GAE/g and 0.017 mg QE/g. This study confirms that butterfly pea flower kombucha, with an IC_{50} value of 162.83 µg/ml, has the ability to inhibit pancreatic lipase *in vitro* more effectively compared to butterfly pea infusion, which exhibits an IC_{50} value of 239.39 µg/ml. Thus, butterfly pea flower kombucha might be a promising candidate to combat obesity.

Keywords: butterfly pea flower; *Clitoria ternatea*; kombucha; pancreatic lipase; total flavonoid

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INTRODUCTION

Obesity is a health problem caused by an imbalance between energy intake and output, which is related to various diseases such as hypertension, cardiovascular, diabetes, and others (Lunagariya *et al.*, 2014). WHO states that 1.9 billion people are overweight and 650 million of them suffer from obesity (WHO, 2016). About 21.8% of adults in Indonesia suffer from obesity (KEMENKES, 2018). A change in lifestyle is the first approach in the treatment of obesity, but maintaining this effort, in the long run, can be a challenge for obesity sufferers (Wadden *et al.*, 2020). Alternatively, obesity treatment is done using anti-obesity agents. One anti-obesity agent that works by reducing calorie absorption is pancreatic lipase inhibitors. An important part of fat metabolism is played by the enzyme pancreatic lipase, which is responsible for 60% of fat absorption (Kim *et al.*, 2016). Orlistat is a common commercial drug used to combat obesity by inhibiting pancreatic lipase. However, some unpleasant side effects may occur such as oily stools, stained underwear, bloating, and inhibition of vitamin K absorption (Li *et al*., 2020; Liu *et al*., 2020). The use of this drug by pregnant women or those with malabsorption syndrome is not advised. It is meant only for adults and teenagers aged 12 to 18 and above. Some individuals may experience difficulty in achieving positive outcomes from medication if they do not adhere to a consistent dosage schedule and have apprehensions about potential long-term side effects (Behl & Misra, 2017). Therefore, it is important to explore other options like the use of functional foods for obesity treatment (Asgary *et al*., 2018; Payab *et al*., 2020).

An example of functional food that has become popular is the fermented drink known as kombucha. Made from fermenting black tea with a microbial culture in the form of a symbiotic

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relationship between yeast, fungus, and acetic acid bacteria, commonly referred to as symbiotic culture of bacteria and yeast (SCOBY), kombucha is widely consumed as a beverage believed to enhance overall health (de Miranda *et al.*, 2022). Research has indicated that kombucha can prevent cardiovascular disease (Doudi *et al*., 2020; Morales, 2020), has a hepatoprotective effect (Wang *et al*., 2014), improves digestive function (Tamer *et al*., 2021), stimulates the immune system (Haghmorad *et al*., 2021) and reduces cholesterol levels (Watawana *et al.*, 2015; Zubaidah *et al.*, 2019). *In vivo* studies on rat also suggest that kombucha has better activity in inhibiting pancreatic lipase activity compared to black tea (Aloulou *et al*., 2012).

In order to achieve a diverse range of flavors, kombucha is commonly created using a variety of substrates, for example, mint leaves, salak, ginger, grapes, and others (de Miranda *et al*., 2022). An ingredient that has gained popularity as a healthful beverage is the butterfly pea flower (*Clitoria ternatea*). Based on some research results on animals, butterfly pea flower extract has properties as an anti-asthma, anti-inflammatory, analgesic, antipyretic, anti-diabetic, anti-lipidemic, anti-rheumatic, and antioxidant (Oguis *et al*., 2019). Like other natural ingredients, butterfly pea flower also has the potential to be used as a substrate for making kombucha. However, at present, information on its potential as a lipase inhibitor for obesity therapy is still unknown. Therefore, the purpose of this research is to determine the lipase inhibitor activity of butterfly pea flower (*C. ternatea*) kombucha. It is anticipated that the findings of this study will clarify the key ingredients that support kombucha's lipase inhibitory action and anti-obesity characteristics, facilitating safe and efficient ingestion.

MATERIALS AND METHODS

Samples and kombucha culture collection. Butterfly pea (*Clitoria ternatea*) collected from a market in D.I.Yogyakarta, Indonesia. Kombucha culture used as a starter of the fermentation process was obtained from the Laboratory of Microbiology, Faculty of Applied Science and Technology, Universitas Ahmad Dahlan.

Kombucha preparation and fermentation process. The production of butterfly pea flower kombucha refers to Ahmed *et al*. (2020), with modifications. About 1 gr of butterfly pea flowers was brewed with 200 ml of boiling water for 15 min, then poured and filtered into sterilized glass bottles or containers. The SCOBY used is one week old, it has formed a dense, solid nata with a firm texture and a thickness of 0.3 mm. Butterfly pea flowers 1 gr was soaked with 200 ml of boiling water for 15 min, then poured and filtered into five sterilized glass bottles, each containing 40 ml. Twenty gr of sugar (10% w/v) was added to the bottles containing the solution of butterfly pea flower tea. The bottles are sealed and left to cool to around 30℃. Then, 12 ml of liquid culture and 8 gr of solid culture (SCOBY) were added to each bottle. The bottles are then sealed with sterilized cheesecloth, tied with rubber bands, and incubated for 12 days at room temperature (±28-30℃).

Biological determination. To measure the optical density (OD) of the fermented culture, a spectrophotometer was used to read the value at 600 nm. The weight of the kombucha mushroom was determined by separating it from the culture, washing it thrice with distilled water, and drying it at 80°C until it reached a constant weight.

Chemical determination. The pH levels were determined by utilizing an electronic pH meter that had been calibrated at pH 4.0 and 7.0. To determine the titratable acidity (TA), a 10 ml sample of the fermentation broth was taken and heated in a water bath at 100°C for 10 min to remove the CO_2. The sample was then diluted to 250 ml and 25 ml of the solution was added to a 100 ml Erlenmeyer flask. About 3-5 drops of phenolphthalein (PP) indicator were added and the solution was titrated with a 0.1 N standard NaOH solution until it turns light pink. The total acidity was expressed as acetic acid (BM=60). To measure the amount of reducing sugars in the kombucha sample, the Nelson-Somogyi method was employed (Somogyi, 1952). A 1 ml sample was diluted to 100 ml and subsequently mixed with 1 ml of Nelson reagent. After being heated for 20 min in a boiling water bath, it was cooled to 25℃ in running water and combined with 1 ml of

arsenomolybdate reagent. The mixture was thoroughly agitated until the cuprous oxide sediment dissolved and then 7 ml of distilled water was added. Based on a glucose standard curve, the spectrophotometer was used to measure the absorbance at a 540 nm wavelength to determine the amount of reducing sugar in kombucha. The total phenol content in kombucha fermented solutions was determined using the Folin-Ciocalteu method (Singleton *et al*., 1999). A 0.1 ml sample was transferred into a 100 ml Erlenmeyer flask and diluted with distilled water to a volume of 46 ml. Next, 1.0 ml of Folin-Ciocalteu reactive solution was added and the mixture was allowed to incubate at room temperature for 3 min. After that, 3 ml of 2% w/v sodium carbonate solution were added and the mixture was left for 30 min. Finally, the absorbance was measured at 760 nm. The total phenol content was reported as gallic acid equivalents using a calibration curve. A colorimetric method was utilized to determine the flavonoid content of the sample. The process began by adding 100 µl of the extract to 4 ml of distilled water. Afterward, 0.3 ml of 5% sodium nitrite was added and allowed to sit for 5 min. Next, 0.3 ml of 10% aluminum chloride was added and left for another 6 min. Then, 2 ml of 1M sodium hydroxide was added to the mixture, which was immediately diluted by adding 3.3 ml of distilled water and mixed thoroughly. The absorbance was then measured at 510 nm against a blank. To establish a calibration curve, a standardized measurement of quercetin was utilized and the gathered data was recorded in mg/g of the sample.

***In vitro* assay for lipase inhibitory activity.** The *in vitro* lipase inhibitory activity test was conducted according to our previous method (Aji *et al*., 2020) with modifications. Lipase activity was determined by evaluating the conversion of p-nitrophenyl palmitate (pNPP) to p-nitrophenol. Five different concentrations, 50, 100, 150, 200, and 250 ug/ml, were made. Then, a 20 mg/ml enzyme stock was made in a 50 mM Tris HCl pH 8 buffer solution, and a 50 mM substrate stock (pNPP) was made in acetone. The activity test was conducted by mixing 0.1 ml of the 20 mg/ml lipase, 0.2 ml of the extract (at different concentrations), and 0.7 ml of 50 mM Tris HCl pH 8. After mixing, the solution was incubated for 15 min at 37℃, and then 0.1 ml of 50 mM pNPP was added and incubated for another 30 min at 37℃. The absorbance of the test results was measured using a spectrophotometer at a wavelength of 410 nm. In this experiment, the activity of infuse of butterfly pea flower (without fermentation) and the kombucha of butterfly pea flower was measured. Orlistat was used as a positive control, while aquadest was used as a negative control. The activity of the enzyme was determined by measuring the rate of reaction that produced 1 mmol of p-nitrophenol per minute at 37℃. The test was conducted three times. The inhibitory activity of the lipase enzyme was calculated as lipase inhibition (I %) = 100 – (A/B x 100), where A is the activity without inhibitor and B is the activity with inhibitor (Vangoori *et al*., 2019). The lipase inhibitory activity was presented as the IC_{50} value, which is the concentration required to reduce enzyme activity by 50%. This value is determined by plotting the substrate concentration and percent inhibition results on a regression equation.

Data analysis. All the analyses were conducted in triplicate. Data were represented as mean ± standard deviation and statistically analyzed using SPSS Statistics ver. 26. Identification of any significant differences in the mean comparison between the two samples, an independent t-test was performed on the data for total phenolic and total flavonoids. Meanwhile, the lipase inhibitory results were analyzed using a one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test (DMRT).

RESULTS AND DISCUSSION

Biological activity of butterfly pea flower kombucha. In this study, kombucha was produced using butterfly pea flowers as the substrate. Mushrooms exhibited exponential growth during the initial 2 or 4 days of fermentation, as illustrated in Fig. 1. The data demonstrates that as the fermentation time increased, the growth of the kombucha cultures, as measured by OD600, also increased and reached its peak after 9 days. During the fermentation process, the OD600 of kombucha increased in the initial four days and continued to rise until the end of the fermentation period (Abou-

Taleb *et al*., 2017). The total count of yeast and acetic acid bacteria in kombucha liquor increases over time and reaches its peak after 10 days of fermentation (Neffe-Skocińska *et al*., 2017).

The dry weight of the kombucha mushroom showed a similar pattern, with a peak output of 4.74 g after 9 days of fermentation. The pattern of the dry weight of kombucha mushrooms increasing as the fermentation period progresses is linked to the expansion of a cellulosic structure made up of acetic acid bacteria (Ahmed *et al*., 2020). The kombucha mushroom, also known as the SCOBY or mother, is a cellulose biofilm composed of a symbiotic colony of bacteria and yeast. The composition of microorganisms in kombucha can differ from one culture to another, but certain types of microorganisms are commonly present in all SCOBYs, including acetic acid bacteria (AAB) and yeast (Bishop *et al*., 2022). The number of bacteria present in the liquid portion of kombucha was higher than in the solid portion (SCOBY) (Watawana *et al*., 2015). The partnership between the acetic acid bacteria and yeast in kombucha effectively prevents the contamination of spoilage and pathogenic microorganisms (Kapp & Sumner, 2019).

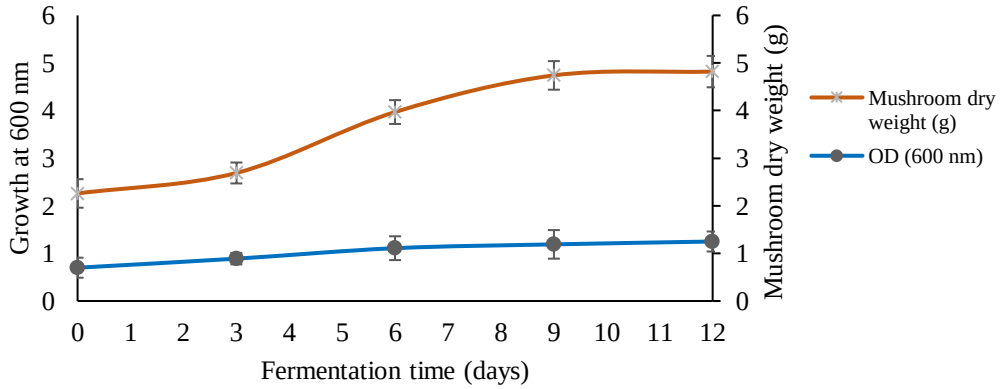

Fig. 1. Optical density (OD 600 nm) and mushrooms dry weight patterns during butterfly pea flower kombucha fermentation

Chemical composition of butterfly pea flower kombucha. Data shown in Fig. 2 indicates that the pH values decreased as the fermentation period prolonged and reached its lowest point at 2.04 on day 12. In contrast, the total acidity, represented as acetic acid concentration, increased throughout fermentation and reached 0.34% on the 12th day.

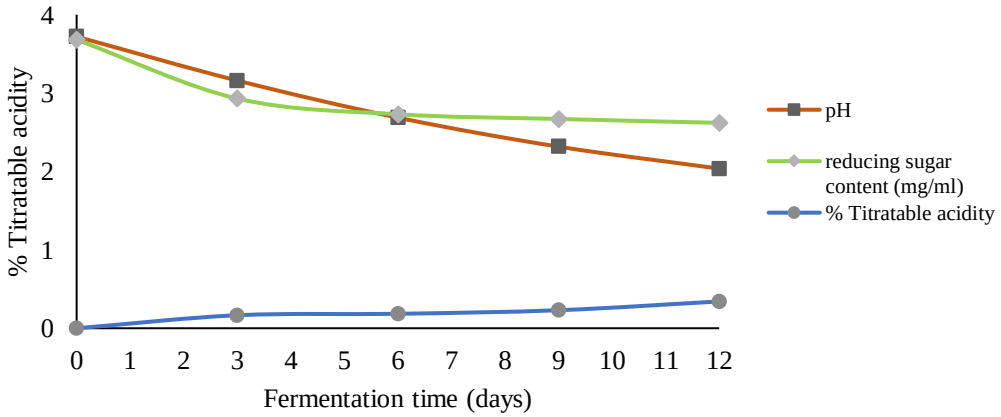

Fig. 2. Titratable acidity and pH during butterfly pea flower kombucha fermentation

These results align with Ahmed *et al.* (2020), who observed that kombucha's pH steadily declined as the fermentation progressed. This decline is due to the presence of organic acid, which is produced as a result of sugar metabolism by bacteria and yeast, leading to an increase in the acidity of the kombucha. The drop in pH results from the microorganisms in kombucha producing more organic acids as the fermentation period extends, reducing pH (Amarasinghe *et al.*, 2018).

The data on reducing sugar concentration, as presented in Fig. 3, demonstrates a decrease in the concentration at the start of the fermentation process after two days, and it slightly decreased until day 12. The decline in reducing sugar in kombucha can be attributed to the ability of microorganisms to break down sugar molecules through hydrolysis (Kitwetcharoen *et al*., 2023). As the fermentation process progresses, the decreasing level of reducing sugar results from the sugar consumed by microorganisms as a source of carbon, which in turn leads to the production of ethanol and organic acids. Therefore, the acid level in kombucha progressively increases with the prolongation of the fermentation period (Amarasinghe *et al.*, 2018). The acidic environment in kombucha favors acid-tolerant microbes and inhibits the growth of potential competitors or invaders. Certain yeast species, like Dekkera/Brettanomyces, can survive and flourish in the acidic conditions of kombucha, which would be harmful to other yeast genera. Similarly, acid-tolerant bacteria, such as Komagataeibacter, thrive in kombucha, while other bacteria are less tolerant and unable to survive in highly acidic conditions (May *et al*., 2019).

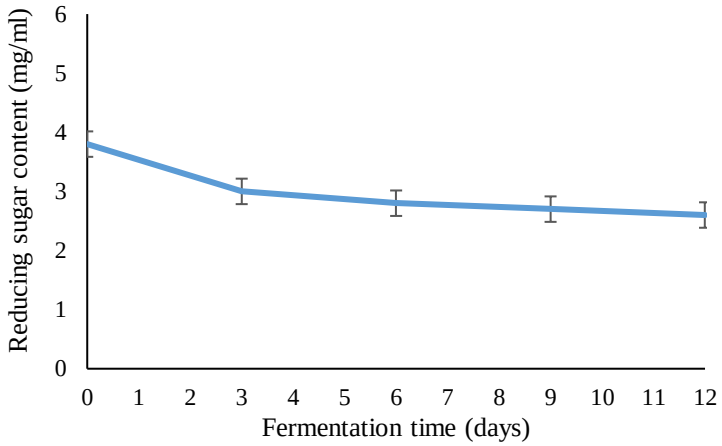

Fig. 3. Reducing sugar content during butterfly pea flower kombucha fermentation

Total phenolic and total flavonoid compound of butterfly pea flower kombucha. The total phenolic content was greater after 12 days of fermentation, as indicated in Table 1, than before fermentation. The findings of this study are consistent with prior research that found kombucha fermented drinks to contain a higher amount of total phenolics than the initial fermentation process (Vitas *et al*., 2018). Another investigation demonstrated a rise in total phenolic content as the duration of fermentation progressed (Ayed & Hamdi, 2015). The enhancement of phenolic compounds in kombucha was observed after three days due to the increased kinetics growth of microorganisms (Antolak *et al.*, 2021). The variety of tea utilized in kombucha production affects the amount of polyphenols present (Bishop *et al*., 2022).

Table 1. Total phenolic and flavonoids before and after butterfly pea flower kombucha fermentation

Fermentation time (days)	Total phenolic (mg GAE/g sample)	Total flavonoids (mg QE/g sample)
0	0.015 ± 0.002^{a}	0.012 ± 0.002^{a}
12	0.040 ± 0.001^{b}	0.017 ± 0.001^{a}

Note: Mean values are significantly different at $p < 0.05$ (Independent t-test). Values are given in means ± standard deviation

The total flavonoid content also shows a similar trend, as visualized in Fig. 3. The increase of phenols and flavonoids in the final stages of kombucha fermentation is likely due to the growth and metabolic activity of the yeast and bacteria in the mixture. These microorganisms produce various bioactive compounds, including phenols and flavonoids, which contribute to the kombucha's characteristic flavor and health-promoting properties. The fermentation of kombucha involves several chemical reactions, one of which is the oxidation of polyphenolic compounds by some enzymes, resulting in the formation of flavonoids and other healthy compounds through microbial hydrolysis

(Antolak *et al.*, 2021). The Kombucha samples exhibit significant antioxidant capacity due to a high concentration of total phenolic and flavonoid substances (Ivanišová *et al.*, 2019).

***In vitro* assay for lipase inhibitory activity of butterfly pea flower kombucha.** The results of the *in vitro* lipase inhibition study of butterfly pea flower Kombucha are displayed in Fig. 4. The study compared the lipase inhibition activity of the butterfly pea flower infusion (without fermentation), butterfly pea flower kombucha drink, and orlistat. The butterfly pea flower kombucha drink exhibited lower lipase inhibition activity compared to orlistat, with an IC_{50} of 239.39 ug/ml, 162.83 ug/ml, and 102.00 ug/ml for the butterfly pea flower infusion (without fermentation), butterfly pea flower kombucha drink, and orlistat, respectively. However, compared to the butterfly pea flower infusion (without fermentation), the butterfly pea flower kombucha exhibits a superior level of inhibition against pancreatic lipase. The results of this study align with the outcomes of previous research on the fermentation of green vegetables (such as spinach, broccoli, and sweet leaf) using kombucha culture as a functional beverage that possible to inhibit pancreatic lipase (Maryati *et al.*, 2020). Additionally, sea grape kombucha (*Caulerpa racemosa*) has been shown to inhibit pancreatic lipase activity and result in weight loss in experimental mice (Permatasari *et al.*, 2022).

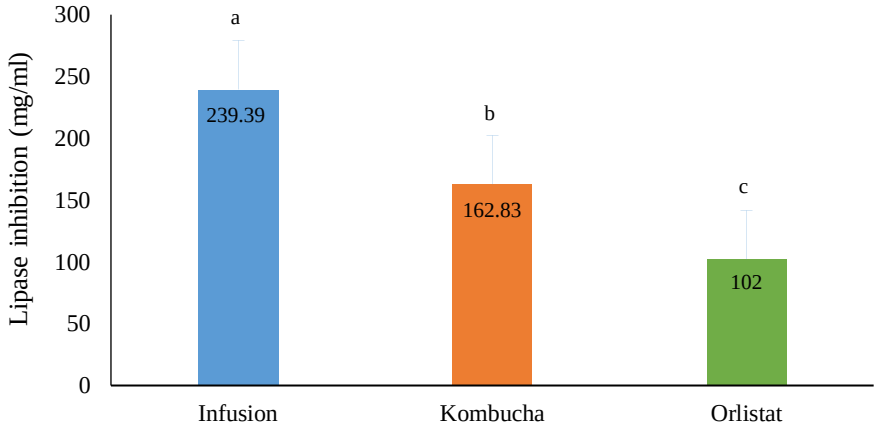

Fig. 4. Inhibitory activities (IC_{50}) of butterfly pea flower infusion, kombucha, and orlistat on pancreatic lipase. The data represented by distinct letters exhibit a significant difference ($P < 0.05$). The values are presented as means ± standard deviation

During kombucha fermentation, yeast and bacteria produce various bioactive compounds, including organic acids and polyphenols, which are believed to contribute to the inhibition of lipase activity. Several studies have shown that subclasses of polyphenols, including flavonoids, and phenolic acids, can effectively inhibit pancreatic lipase (Buchholz & Melzig, 2015). Kombucha contains phenol compounds, the main bioactive compound group in kombucha and responsible for the drink's health benefits (Cardoso *et al.*, 2020). These compounds play an important role in inhibiting pancreatic lipase activity (Buchholz & Melzig, 2015). Moreover, the optimal duration of fermentation produces organic acids, such as glucuronic acid, which serve as secondary metabolites and inhibit pancreatic lipase activity, while simultaneously increasing the polyphenol activity during the process (Maryati *et al.*, 2020). The bioavailability of polyphenols is increased by glucuronic acid (GlcUA). The conjugation of phenols with GlcUA leads to greater transportability and bioavailability of the substances.

Numerous studies have demonstrated that the kombucha fermentation process can enhance the health benefits of herbal infusions (de Miranda *et al.*, 2022). The antioxidant and antimicrobial properties of herbal infusions are improved after being fermented with kombucha culture. The antioxidant activity of lemon balm (*Melissa officinalis* L.) kombucha is higher compared to its herbal infusion (Velićanski *et al.*, 2014). Similarly, in the case of winter savory (*Satureja montana* L.), the antioxidant and antimicrobial effects against both gram-positive and gram-negative bacteria were found to be stronger after fermentation compared to its herbal infusion (Četojević-Simin *et al.*, 2012).

Furthermore, this study demonstrates that, in contrast to butterfly pea flower infusion, kombucha fermentation results in an increase in lipase inhibitor activity. Therefore, butterfly pea flower kombucha has the potential to be developed as an anti-obesity agent that may also offer many health benefits, such as antioxidants and antimicrobials.

CONCLUSION

During the fermentation process of butterfly pea flower kombucha, it was observed that the pH and reducing sugar levels showed a decrease, while there was a noticeable increase in cell density (OD600), dry weight of SCOBY, and percentage of titratable acid. The findings from this study indicate that butterfly pea flower kombucha contains various bioactive compounds such as polyphenols and flavonoids, and can inhibit pancreatic lipase *in vitro*. It is believed that these active compounds play a role in the lipase inhibitory activity of kombucha, thereby presenting potential as an anti-obesity agent. However, it is necessary to obtain further evidence to identify bioactive compounds, performance of in-vivo studies, and comprehend the underlying mechanisms of the results obtained.

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