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Dear Authors
Oktira Roka Aji, Nida'a Fauziyyah Putri, Diah Asta Putri

Your manuscript entitled Pancreatic lipase inhibitory activity of butterfly pea flower (Clitoria ternatea) Kombucha, needs to be revised. Kindly follow all reviewer's suggestions. The authors may upload the revision (file docx) through the revision tab until **Thursday, May 18, 2023**

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

ABSTRACT. Preventing the action of pancreatic lipase is believed to be an effective method for treating obesity. Pancreatic lipase inhibitor act by suppressing the activity of pancreatic lipase, leading to a decrease in lipid absorption. Kombucha is a traditional fermented drink believed to have numerous health benefits, including as 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 inhibiting activity of kombucha made from butterfly pea flowers towards 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 % titratable acid. The total phenol and total flavonoid content were also analyzed before and after fermentation. The inhibitory effect on pancreatic lipase of butterfly pea flower kombucha was presented as an IC50 value. 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 found to be greater compared to that of 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 has the ability to inhibit pancreatic lipase in vitro with an IC50 value of 162.83 µg/mL. Compared to butterfly pea flower tea infusion, butterfly pea flower kombucha has been found to be more effective in inhibiting pancreatic lipase. Thus, butterfly pea flower kombucha might be a promising candidate for being developed as an agent for combating obesity.

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

INTRODUCTION

Obesity is a health problem caused by an imbalance between energy intake and energy output. Obesity in terms of health 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 (Riskesdas, 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. Pancreatic lipase plays a major role in fat metabolism, where this enzyme is responsible for 60% of fat absorption (Kim *et al.*, 2016). Currently, commercial anti-obesity drugs that inhibit pancreatic lipase are orlistat. However, there are side effects such as oily stools, stains on underwear, bloating, and inhibition of vitamin K absorption (Liu *et al.*, 2020). In addition, this drug is contraindicated for pregnant women and people with malabsorption syndrome and can only be used for adults and teenagers aged 12-18 and above. Noncompliance with regular medication and fear of serious side effects due to long-term use are factors that hinder the success of this drug (Behl and Misra, 2017). Therefore, the search for complementary and alternative treatment methods such as the use of functional foods for obesity treatment is needed (Asgary *et al.*, 2018; Payab *et al.*, 2020).

An example of a 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 relationship between yeast, fungus, and acetic acid bacteria, commonly referred to as SCOBY (Symbiotic Culture of Bacteria and Yeast), kombucha is widely consumed as a beverage believed to enhance overall health (de Miranda *et al.*, 2022). Research has indicated that kombucha possesses properties such as antibacterial, anticancer, anti-diabetic, antioxidant, can prevent cardiovascular disease, has a hepatoprotective effect, improves digestive function, stimulates the immune system and reduces cholesterol levels (Zubaidah *et al.*, 2019; Watawan *et al.*, 2015). 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 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 unknown. Therefore, the purpose of this research is to determine the lipase inhibitor activity of butterfly pea flower kombucha.

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MATERIALS AND METHODS

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

Kombucha preparation and fermentation process. The production of butterfly pea flower kombucha refers to Ahmed et al., (2020), with modifications. 1 gram of butterfly pea flowers are brewed with 200 mL of boiling water for 15 minutes, 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. 1 gram of butterfly pea flowers are cursed with 200 mL of boiling water for 15 minutes, then poured and filtered into five sterilized glass bottles, each containing 40 mL. 20 grams of sugar (10% w/v) is 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 grams of solid culture (SCOBY) are 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. The growth density of the fermented culture (O.D) was determined by using a spectrophotometer at 600 nm. The dry weight of the kombucha mushroom was determined by following these steps: first, the mushroom was separated from the culture using filter paper, then washed three times with distilled water, and finally dried at 80°C until a constant weight was achieved.

Chemical determination. The pH levels were determined by utilizing an electronic pH meter that had been calibrated at pH 4.0 and 7.0. The titratable acidity (TA) is determined by taking a 10 mL sample of the fermentation broth after removing the CO2 by heating it in a water bath at 100 °C for 10 minutes. The sample is then diluted to 250 ml. 25 ml of this solution is added to a 100 ml Erlenmayer flask and 3-5 drops of Phenolptalein (PP) indicator are added. The solution is then titrated with a 0.1 N standard NaOH solution until it turns light pink. The total acidity is expressed as acetic acid (BM = 60). The method of Nelson-Somogyi was used to determine the amount of reducing sugars present in the kombucha sample (Somogyi, 1952). 1 ml of kombucha sample was taken and then diluted to 100 ml. 1 ml of sample was added with 1 ml of Nelson reagent and heated for 20 minutes in a boiling water bath. It was then cooled to 25℃ in running water then 1 mL of Arsenomolibdat reagent was added. The mixture was agitated until the Cuprooksida sediment dissolved then 7 ml of distilled water was added. The absorbance was measured using a spectrophotometer at a wavelength of 540 nm. The amount of reducing sugar in kombucha was determined based on a standard curve of glucose. 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 taken and added to a 100 mL Erlenmeyer flask, and the volume was adjusted to 46 mL with distilled water. Then, 1.0 mL of Folin-Ciocalteu reactive solution was added and the mixture was left to incubate at room temperature for 3 minutes. Next, 3 milliliters of 2% w/v sodium carbonate was mixed in, and the absorbance was measured at 760 nm after 30 minutes. The total phenol content was reported as gallic acid equivalents using a calibration curve. The flavonoid content of the sample was determined using a colorimetric method (19). The process began by adding 100 microliters of the extract to 4 milliliters of distilled water. Afterwards, 0.3 milliliters of 5% sodium nitrite was added

and allowed to sit for 5 minutes. Next, 0.3 milliliters of 10% aluminum chloride was added and left for another 6 minutes. Then, 2 milliliters of 1M sodium hydroxide was added to the mixture, which was immediately diluted by adding 3.3 milliliters of distilled water and mixed thoroughly. The absorbance was then measured at 510 nanometers against a blank. A quercetin standard was used to create a calibration curve. The results were expressed as the amount of milligrams of quercetin equivalents per gram of sample (mg/g).

In vitro assay for lipase inhibitory activity. The in vitro Lipase Inhibitory Activity test was conducted according to the method performed by Aji *et al*., (2020) with modifications. The activity of lipase 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 minutes at 37℃, and then 0.1 mL of 50 mM pNPP was added and incubated for another 30 minutes 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 kombucha of butterfly pea flower was measured. Orlistat was used as a positive control, while DMSO 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 a temperature of 37 C. 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.

Data analysis. All the analyses were conducted in a triplicate. Data were represented as mean ± standard error. The lipase inhibitory activity was presented as the IC50 value, which is the concentration required to reduce enzyme activity by 50%. This value is determined by plotting the results of substrate concentration and percent inhibition on a regression equation.

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RESULTS AND DISCUSSION

Biological activity of Butterfly pea flower Kombucha. In this experiment, Kombucha was produced using butterfly pea flower as the substrate. During the initial 2 or 4 days of fermentation, both Kombucha cultures and mushrooms exhibited exponential growth, as seen in Figure 1. The data demonstrates that as the fermentation time increased, the growth of the Kombucha cultures, as measured by OD600, also increased, reaching a peak after 9 days. During the fermentation process, the O.D600 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 mushroom increasing as the fermentation period progresses is linked to the expansion of a cellulosic structure made up of acetic acid bacteria (Ahmed, 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, 2022). The number of bacteria present in the liquid portion of Kombucha was found to be higher than in the solid portion (SCOBY) (Watawana *et al*., 2016). The partnership between the acetic acid bacteria and yeast in kombucha is effective in preventing the contamination of spoilage and pathogenic microorganisms (Kapp *et al*., 2019).

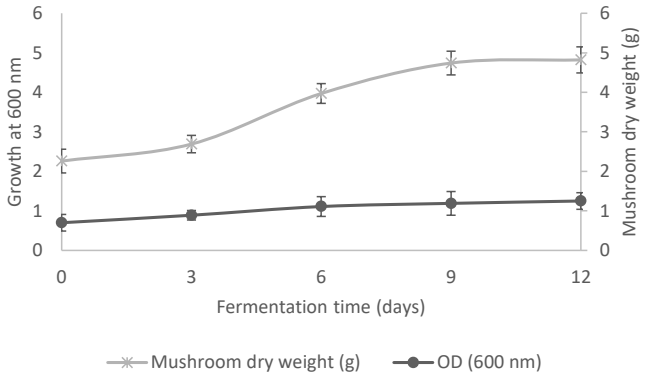

Fig. 1. Optical density (OD600) and mushrooms dry weight patterns during Butterfly pea flower Kombucha fermentation.

Chemical composition of Butterfly pea flower Kombucha. Data shown in Fig. 2 indicated that the pH values decreased as the fermentation period prolonged and reached its lowest point at 2.04 on the 12th day. In contrast, the total acidity, represented as acetic acid concentration, increased throughout the fermentation process and reached 0.34% on the 12th day. These results align with Ahmed (2020), who observed that Kombucha's pH steadily declined as the fermentation progressed. This decline is due to the presence of sucrose, which is metabolized into organic acids 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, resulting in a reduction in pH (Amarasinghe *et al.*, 2018).

The data on reducing sugar concentration, as presented in Fig. 2, demonstrates that there was a decrease in the concentration at the start of the fermentation process after two days, and the levels remained relatively stable after six days. 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 is a result of the sugar being 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).

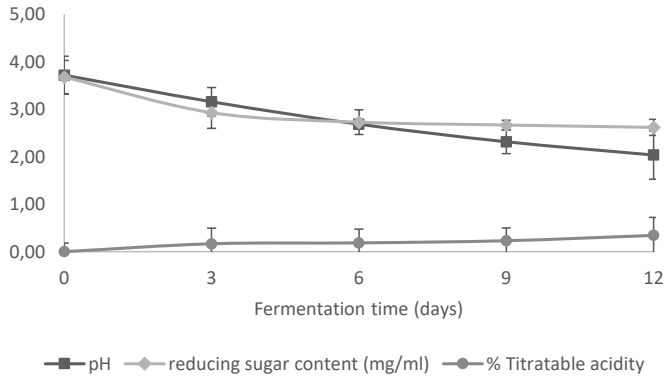

Fig. 2. pH, reducing sugar content, and % titratable acidity patterns during Butterfly pea flower Kombucha fermentation.

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 Figure 3, compared to the content prior to fermentation. The findings of this study are consistent with prior research that has found kombucha fermented drinks to contain a higher amount of total phenolics compared to the initial fermentation process (Vitas *et al*., 2018). Another investigation demonstrated a rise in total phenolic content as the duration of fermentation progressed (Ayed and Hamdi, 2015). The enhancement of phenolic compounds in Kombucha was observed after three days as a result of an increase in the kinetics growth of microorganisms (Antolak *et al*., 2021). The variety of tea utilized in the production of kombucha affects the amount of polyphenols present (Bishop, 2022).

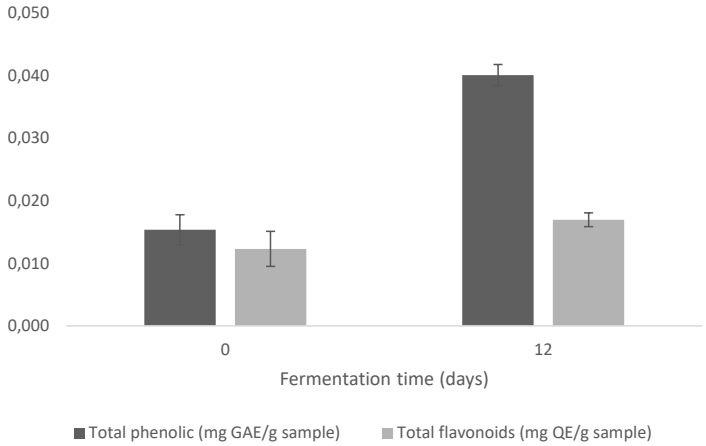

Fig. 3. Total phenolic and total flavonoid compound of Butterfly pea flower Kombucha before and after fermentation (day 12)

The total flavonoid content also shows a similar trend, as seen in Figure 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 present in the mixture. These microorganisms produce various bioactive compounds, including phenols and flavonoids, which contribute to the characteristic flavor and health-promoting properties of the kombucha. 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 owing to the presence of 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 Figure 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 IC50 of 239.39.9 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 sweetleaf) using kombucha culture as a functional beverage. The research found that kombucha made from these

substrates can 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).

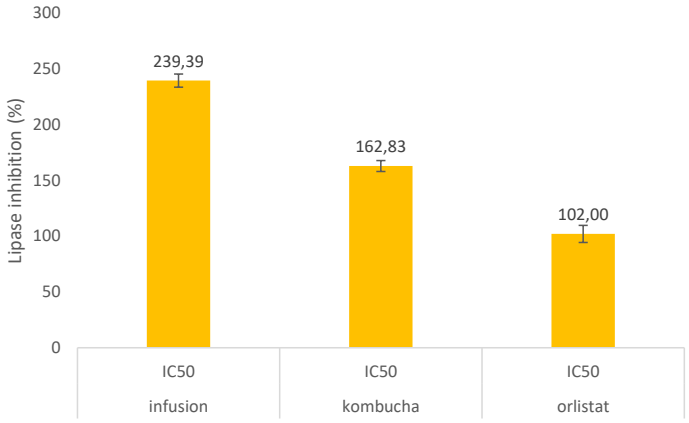

Fig. 4. Total phenolic and total flavonoid compound of Butterfly pea flower Kombucha before and after fermentation (day 12)

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. A number of studies have shown that subclasses of polyphenols, including flavonoids, and phenolic acids, can effectively inhibit pancreatic lipase (Buchholz & Melzig, 2015). Kombucha contains phenol compounds, which are the main bioactive compound group in kombucha and responsible for the health benefits of the drink (Cardoso *et al*., 2020). These compounds play an important role in inhibiting the activity of pancreatic lipase (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*, 2022). 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 (Velicanski *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 (Cetojevic-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 % 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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[bio] Editor Decision

1 message

Devi Armita <journal@uin-alauddin.ac.id> Sat, May 20, 2023 at 8:15 AM
To: Oktira Roka Aji <oktira.roka.aji@gmail.com>, Nida'a Fauziyyah Putri <nidafauziahputri@gmail.com>, Diah Asta Putri <diah.putri@bio.uad.ac.id>

LETTER OF ACCEPTANCE

Dear Authors,
Oktira Roka Aji, Nida'a Fauziyyah Putri, Diah Asta Putri

On behalf of Biogenesis: Jurnal Ilmiah Biologi editorial team, we are pleased to inform you that your manuscript with ID number 35724 entitled "Pancreatic lipase inhibitory activity of butterfly pea flower (Clitoria ternatea) Kombucha" has been accepted and will be published it on next issue.

The authors will receive a payment invoice through email as soon as possible.

Thank you for submitting your work to Biogenesis: Jurnal Ilmiah Biologi. We hope to receive it in the future too.

Editor in Chief

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Dear Authors,
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We are contacting you regarding the latest invoice for your manuscript entitled Pancreatic Lipase Inhibitory Activity of Butterfly Pea Flower (Clitoria ternatea) Kombucha, which will be published in Biogenesis: Jurnal Ilmiah Biologi Vol 11 No 2, 2023 SINTA 2 (http://journal.uin-alauddin.ac.id/index.php/biogenesis/) as declared in the publication charge tab. You can make your payment before ***Monday, October 23, 2023***. You can find detailed information in the invoice attached.

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Kepada Yth. Editor Biogenesis: Jurnal Ilmiah Biologi

Berikut kami kirimkan bukti pembayaran.
Atas perhatian dan bantuannya, kami ucapkan terima kasih.
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On behalf of Biogenesis: Jurnal Ilmiah Biologi, we would like to thank you all for considering and trusting our journal as the platform for publishing your valuable work. We hope to receive it in the future too.

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Isna Rasdianah Aziz

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