Effect of Fermentation to Total Titrable Acids, Flavonoid and Antioxidant Activity of Butterfly Pea Kombucha

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Effect of Fermentation to Total Titrable Acids, Flavonoid and Antioxidant Activity of Butterfly Pea Kombucha

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Abstract : *Butterfly Pea (*Clitoria ternatea L.*) has been utilized as traditional medicine to cure many kind of diseases. Previous study indicated that Butterfly Pea contains bioactive compounds that can be used as antioxidant, such as flavonoid. Butterfly Pea can be used as alternative substance for kombucha. This research aims to study about the effect of fermentation length towards total titrable acids content, total flavonoid content and antioxidant activity of butterfly pea kombucha. This research was conducted from March until May 2018 at the Laboratory of Biology and Pharmacy Department, Sanata Dharma University. This research used Completely Randomized Design (CRD) with the treatment of six different fermentation length, which was 0 day, 4 days, 8 days, 12 days, 16 days and 20 days. Each data obtained from this research were analyzed using correlation and regression statistic test. The results of the test showed that the treatment of fermentation length gave significant and positive correlation towards total titrable acids content and total flavonoid content. It means that the longer of fermentation length leads to higher of total titrable acids content and total flavonoid content. Whereas, the statistic test of antioxidant activity data showed that there was no significant correlation between the treatment of fermentation length to the levels of antioxidant activity of Butterfly Pea kombucha.*

1. Introduction

Human activities sometimes can be harmful for health. Along with the advancement in science and technology, the use of motor vehicle and industrial growth cannot be avoided which then following by the increase of air pollutions. This is very dangerous because air pollution is one of the source of free radicals. UV radiation, consumption of unhealthy diets, high fat foods and artificial food colorings are the other factor that can increase the production of free radicals. Currently there are still many people who are not aware of the influence of free radicals on health, especially the role of free radicals to the emergence of various kind of chronic diseases such as cardiovascular disease, cancer, diabetes mellitus and many others. The activity of free radicals can be minimized or even be prevented by the presence of antioxidants, which can be obtained by consuming various kind of vegetables and fruits that contain antioxidant compounds.

Clitoria ternatea L. which also known as Butterfly Pea is a tropical plant from Asia which is currently popular. This plant is a perennial climber which belongs to the family of Fabaceae. This plant has been used as traditional medicine to cure various kind of disease for over centuries. Almost every part of this plant has been reported to have number of pharmacological activities. Roots, seeds and leaves of *C. ternatea* were used as one of the ingredients in the traditional Ayurwedic medicine system. The roots, stems and flowers also been used for the treatment of snake bites and scorpion string. It also has been reported that this plant contains many kind of bioactive compounds that have a role as antioxidant, such as flavonoids, tannins, saponins and phenols [8]. According to the previous study on *C. ternatea* showed that the flower of this plant also contain flavonoids, which is one of the strongest antioxidants [6]. The flower of *C. ternatea* currently being consumed by people in form of infusion. This plant seems to be more attractive because of its blue color as the result of the presence of antochyanins, which is also has a role as antioxidant. The infusion of *C. ternatea* flower can be modified into various kind of beverages in order to increase its nutritional values.

Kombucha beverage has been known for centuries as one of the traditional beverages, especially by people who lived around China. The name kombucha was formed from the word "Kombu" which was the name of a traditional doctor from Korea and the word "cha" which means "tea" in Chinese language [5]. Kombucha beverage is made by sugared tea fermented by microbial association of bacteria and yeasts which is also known as SCOBY (Symbiotic Culture of Bacteria and Yeast). As a result of these microorganisms activity, some kind of organic acids, vitamins and floating layer of cellulose on the surface of the tea broth are produced during the fermentation process. Kombucha fermentation occur in two stages: alcoholic fermentation by yeasts and acetic acid fermentation by acetic acid bacteria. The fermentation begins when sucrose was hydrolyzed into glucose and fructose using invertase which then used to produce ethanol by yeasts through glycolysis pathway. The ethanol then used by acetic acid bacteria to produce some kind of organic acids such as acetic acids and gluconic acids, so that it tastes slightly acidic [3].

The most popular kinds of tea used as material to make kombucha are black tea, green tea and oolong tea. Although the preparation of kombucha was originally using tea, but it also possible to use various kind of materials such as mint, lemon balm, jasmine [7]. In this study we used Butterfly Pea infusions as the base substance to made kombucha. The effect of kombucha fermentation under the treatment of different time of fermentation length towards quantitative amounts of titrable acids, flavonoid and antioxidant activity of Butterfly Pea in form of kombucha were measured in this study.

2. Materials and Methods

2.1. Collection of Plant Materials

Flowers of Butterfly Pea were collected from surrounding area of Kalasan, Yogyakarta on March 2018. The flowers were dried for 2 days under the shade condition so they are not exposed to direct sunlight in order to avoid the color damage.

2.2. Preparation of Butterfly Pea Kombucha

The 24 grams of dried Butterfly Pea flowers were brewed in a hot sugared water solution while stirring it and then cooled to temperature 20 – 30°C. Then, 24 pieces of transparent glass jars were labelled with the treatment and the solution which has been cooled were transferred into the transparent glass jars which was 200 ml for each jars. After that, 10% of SCOBY (b/v) and liquid starter (v/v) were added into each jars and then covered with a cloth tied by a rubber band. The kombucha were kept in a dust free room during fermentation process until several days following the treatment used in this study, which was 0 day (control), 4 days, 8 days, 12 days, 16 days and 20 days. After reaching each day limits of the fermentation length, the total titrable acids, total flavonoid, and antioxidant activity of Butterfly Pea kombucha were measured.

2.3. Determination of Total Titrable Acids

The content of titrable acids was estimated using acid-base titration method. The total titrable acids was expressed in the percentage (%) of acetic acid, since acetic acid is the final product in kombucha fermentation. Sodium hydroxide (NaOH) used as standard solution was standardized using 0,1 M acetic acid solution to estimate the normality of NaOH before used as titrant to determine the concentration of acetic acid. The indicator used for titration was 1 % phenolphthalein. Then, 10 ml of each sample of Butterfly Pea were diluted into 100 ml with distilled water. After that, 25 ml of diluted solution of kombucha samples was added into Erlenmeyer flask for titration. The concentration of acetic acid was calculated by using the following equation [2] :

$$TTA = \frac{Vt \times N \times BE \times P}{V_s \times 1000} \times 100\ \%$$

TTA = the percentage of titrable acids
Vt = volume of titrant used in titration
N = the normality of the standardized NaOH
BE = equivalent weight of NaOH
Vs = volume of sample

2.4. Determination of Flavonoid

Aluminum chloride colorimetric method was used to determine the flavonoid content. Quercetin solutions of various concentrations were used to make the standard calibration curve. The 25 grams of quercetin was dissolved with methanol in a 25 ml volumetric flask and then diluted to 0,02 ; 0,03 ; 0,04 ; 0,05 ; 0,06 mg/ml using methanol. The calibration curve was made by measuring the absorbance of each dilutions using Shimadzu UV-1240 Spectrophotometer at 415 nm. The test solutions was prepared by added 0,5 ml of each kombucha sample solutions, 1,5 ml of methanol, 0,1 ml of 10 % aluminum chloride, 0,1 ml of 1 M potassium acetate and 2,8 ml of distilled water into the test tubes and mixed well using vortex, then incubated at 25°C for 30 minutes. The sample blank was prepared in the similar way by replacing the aluminum chloride with distilled water. The absorbance of all the prepared test solutions were measured using Shimadzu UV-1240 Spectrophotometer at 415 nm against the sample blank solutions. The calculation of total flavonoid content was expressed in the percentage (%) of mg equivalent quercetin / gram of dried flowers, which was determine with the following equation :

$$TFC = \frac{R \times 10^{-3}}{W} \times 100\ \%$$

TFC = total flavonoid content
R = the result obtained from standard curve (mg/ml)
W = the weight of dried plant on each sample (g)

2.5. Determination of Antioxidant Activity

In this study the free radical scavenging activity of Butterfly Pea kombucha was determined using DPPH assay. DPPH solution (200 μM) was prepared in methanol. The mixtured test solution containing 1 ml of each kombucha sample and 1 ml of DPPH solution were made in test tubes. The final volume in the test tubes was made to 5 ml using methanol. The test tube was protected from light using aluminum foil, then incubated in the dark for 30 minutes at room temperature. Control sample was prepared containing 1 ml of DPPH and 4 ml methanol, without the addition of kombucha solution. The absorbance of the solutions were read after the incubation using a spectrophotometer at 517 nm. The percentage of the antioxidant activity was calculated using the following equation :

$$\text{\% Antioxidant activity} = \frac{\text{Absorbance of the control} - \text{absorbance of the test sample}}{\text{Absorbance of the control}} \times 100\%$$

2.6. Statistical Analysis

Each data obtained in this research were analyzed using correlation and regression statistic test.

3. Results and Discussion

3.1. Total Titrable Acids Content of Butterfly Pea Kombucha

Kombucha beverage has been known by its distinctive taste which is slightly acidic, because it contains many kind of organic acids. It means that the titrable acids measured in this study also gave an influence to the taste of the kombucha product. Total titrable acids measured in this study was expressed in the percentage of acetic acid because acetic acid is the final product that produce in the fermentation process. The result of the measurement can be seen on the figure 1.

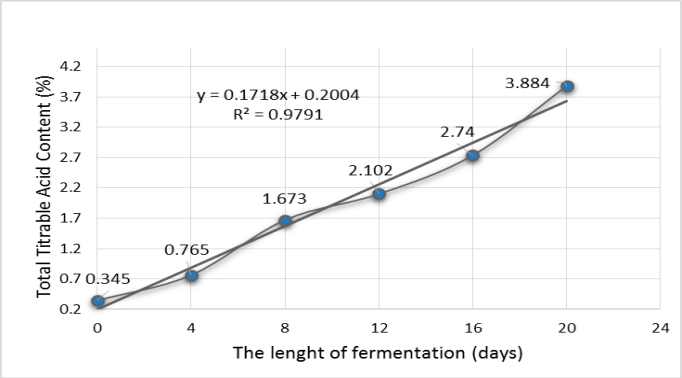

Figure 1. The result of total titrable acid measurements in Butterfly Pea kombucha

Figure 1 shows that the total titrable acids content was increased progressively along with the longer of the fermentation time. The highest concentration of total titrable acid content was found after 20 days of fermentation and the lowest was in the control which was 0 day of fermentation. The change in the concentration of total titrable acid content during fermentation process indicate that there's an increase of microorganisms population during fermentation process, especially the acetic acid bacteria and yeasts which was expected to entering the log phase. The 0 day of fermentation used as the control because at that time the fermentation had not yet begun and the microorganisms were still adapting to the environmental changes, therefore the total titrable acids found in the 0 day of fermentation might come from the organic acids carried by the liquid starter.

During fermentation process the microorganisms contain in SCOBY used the sucrose as the carbon source to produce many kinds of metabolic product such as organic acids. The invertase enzyme excreted by yeasts catalyze the hydrolysis of sucrose into glucose and fructose which was then used to produce ethanol through glycolysis pathway. Meanwhile, the acetic acid bacteria, commonly found as *Acetobacter* and *Gluconobacter*, utilize glucose to produce gluconic acid and ethanol to produce acetic acid [5]. The statistical analysis on the result of total titrable content shows that there's a significant and positive correlation between the fermentation length of kombucha and total titrable acid content (R^2= 0,9791).

3.2. Total Flavonoid Content of Butterfly Pea Kombucha

Flavonoid is secondary metabolite compound widely found in plants. This compound can be found in almost every parts of plants such as roots, seeds, flowers and leaves. Flavonoid belongs to the phenolic compounds which has a big role as an antioxidant. The protective effects of flavonoid in the biological system comes from its ability to act as an electron donor to inhibit the oxidation of free radicals and active oxygen species [1].

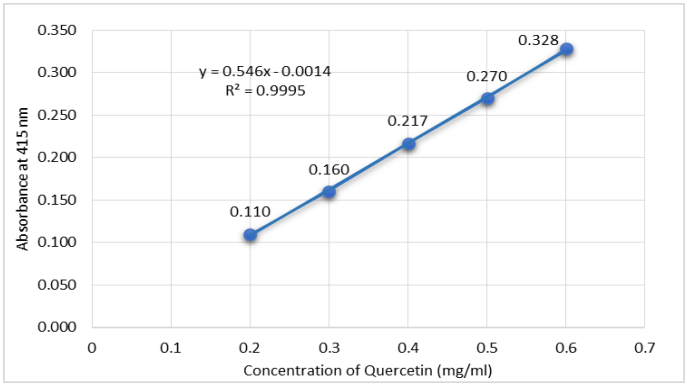

Figure 2. Standard curve of Quercetin

Figure 2 shows the standard calibration curve for the determination of flavonoid in Butterfly Pea kombucha. From the equation found in standard curve (y = 0.546x – 0.0014), concentration values of the total flavonoid during kombucha fermentation were measured. Total flavonoid content is expressed in the percentage (%) of mg quercetin equivalent / gram of dried Butterfly Pea flowers that contained in each samples. The results of the measurement of total flavonoid content during kombucha fermentation can be seen in figure 3.

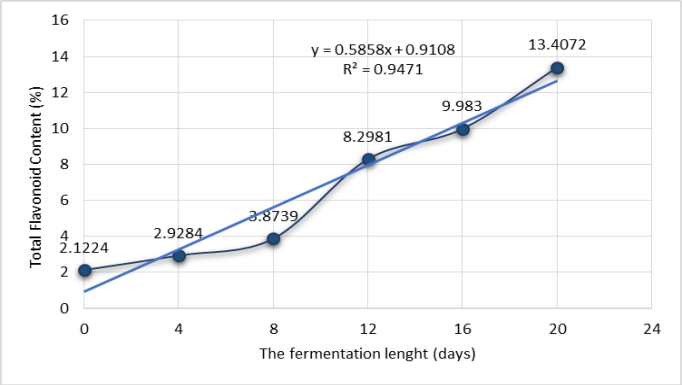

Figure 3. The result of total flavonoid content measurements in Butterfly Pea kombucha

Interestingly, as can be seen from the figure 2, we found that the flavonoid content in Butterfly Pea kombucha increase as the fermentation progressed. The highest concentration of total flavonoid found at 20 days of fermentation, and the lowest was found in the 0 day of fermentation. The increase of the total flavonoid content might be due to the activity of some microorganisms contained in SCOBY which have the ability to degrade the polyphenols compound in the Butterfly Pea flower, since flavonoid may be produced from the degradation of other polyphenol compounds [4]. Some species of lactic acid bacteria has been known to have the ability to degrade polyphenols such as *Lactobacillus plantarum* and *L. acidophilus* which commonly found in kefir [13] and *L. hilgardii* which commonly found in wine [10][4].

Some microorganisms other than acetic acid bacteria and yeasts has been reported can be found in kombucha, including some species of lactic acid bacteria [5]. Unfortunately, in this research we did not identified the culture of the microorganisms contains in the SCOBY, but according to the result that we have found indicated that there must be some of the microorganisms which can excreted some kind of enzymes to degrade polyphenols might contained in the SCOBY. However, this result indicating that there is a possibility of the secondary metabolite conversion stimulated by fermentation. The statistical analysis on the result of total flavonoid content showed that there's a significant and positive correlation between the fermentation length of kombucha and total flavonoid content (R^2= 0,9471).

3.3. Antioxidant Activity of Butterfly Pea Kombucha

Antioxidants are compounds that can reduce or even preventing the negative effects of oxidants present in the body by donating electrons to inhibit the free radicals and oxidant activity. The antioxidant activity of kombucha was estimated using DPPH assay. The principle is based on the capability of each kombucha solutions tested in this study against DPPH (*α-diphenyl- β-picrylhydrazyl*) radicals. The result of the measurement of antioxidant activity of kombucha can be seen in figure 4.

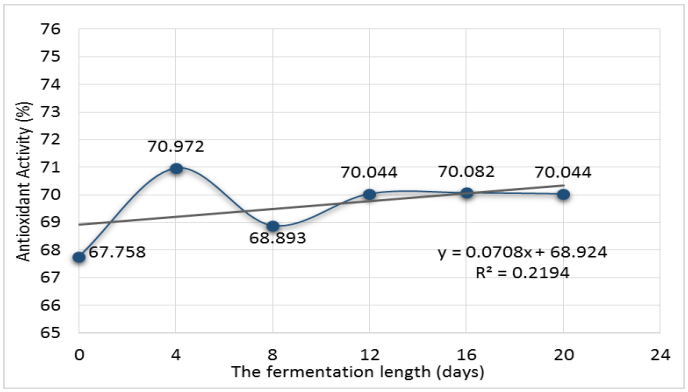

Figure 4. The result of antioxidant activity measurements in butterfly pea kombucha

From the figure 4 shows that there was a fluctuation of antioxidant activity occurs during fermentation process. The percentage of antioxidant activity was increased dramatically at 4 days of fermentation and then continue to dropped on the next 4 days. After 8 days of fermentation the percentage of antioxidant activity rise gradually, then remain to almost stable until the end of the fermentation. The fluctuation of antioxidant activity found in this study might be due to the presence of some antioxidant compounds contained in Butterfly Pea flowers as well as the activity of the microorganism during fermentation process, especially the microorganisms that have the capability to degrade polyphenols compound. The antioxidant compounds that might be responsible in the change of antioxidant activity of kombucha during fermentation are flavonoids, tannins and phenols, as it was reported in the previous study that they can be found in Butterfly Pea flower [8].

In addition, some of the complex phenolic compounds contained in Butterfly Pea flower might be degraded under the acidic environment or by the enzymes excreted by bacteria and yeast in SCOBY. Some bacteria and yeasts such as *Acetobacter* sp., *Lactobacillus* sp., and *Saccharomyces* sp. which was known to have the ability to excreted tannase, an enzyme that could degrade tannins [9] possibly become one of the factor that can cause the fluctuation of antioxidant activity of Butterfly Pea kombucha. Although in this study we found that the flavonoid content (as one of the antioxidant compounds) was increased progressively during fermentation, there is a possibility that the other antioxidants compound that contained in Butterfly Pea flowers may have the higher levels of content compared to flavonoid, so it was more affected to the levels of antioxidant activity if it was degraded.

However, as it was seen in the figure 4, the antioxidant activity measured after the fermentation process was found higher compared to the result of antioxidant activity found in control (0 day). This may be due to the accumulation of other antioxidants that also can be produced during kombucha fermentation such as organic acids, especially ascorbic acid which produced by *Acetobacter xylinum* and DSL (d-saccharic acid 1,4 lactone) which can be produced under the symbiosis between lactic acid bacteria and *Gluconacetobacter* sp. [5][11]. The result of antioxidant activity found in Butterfly Pea kombucha which was between 60 % - 70 % were categorized to the high level of antioxidant activity [12]. The result of the statistical analysis of antioxidant activity of Butterfly Pea kombucha showed that there was no significant correlation between the treatment of fermentation length to the levels of antioxidant activity of Butterfly Pea kombucha ($R^2 = 2194$).

4. Conclusions

The study demonstrated that as the fermentation getting longer, the number of titrable acids content and total flavonoids content increased progressively, while the antioxidant remain to leveled off after reach a peak on day 4. It also can be said that through kombucha fermentation, the nutrition values of the Butterfly Pea infusion modified as kombucha could be increased. It was proved by result of the measurements which showed that the level of total titrable acids content, total flavonoid content and antioxidant activity was increased compared to the result found on control (0 day of fermentation).

5. References

[1.] Alzand K I and Mohamed M A 2012 Flavonoids : Chemistry, Biochemistry, and Antioxidant Activity *Journal of Pharmacy Research* **5(8)** pp 4013 – 20
[2.] Gandjar I G and Rohman A 2007 *Kimia Farmasi Analisis* (Yogyakarta: Pustaka Pelajar)
[3.] Hasruddin and Pratiwi N 2015 *Mikrobiologi Industri* (Bandung: Alfabeta)
[4.] Hunaefi D, Divine N A, Riedel H and Smemstanska I 2012 The Effect of *Lactobacillus plantarum* ATCC and *Lactobacillus acidophilus* NCFM Fermentation on Antioxidant Properties of Selected In Vitro Sprout Culture of Orthosiphon aristatus (Java tea) as Model Study *Antioxidants* **1** pp 4 – 32
[5.] Jayabalan R, Radomir V M, Eva S L, Jasmina S V, and Muthuswamy S 2014 A Review on Kombucha Tea – Microbiology, Composition, Fermentation, Beneficial Effects, Toxicity, and Tea Fungus *Comperhensive Reviews in Food Science and Food Safety* **13** pp 538 – 550
[6.] Lakshmi C H N D, Raju D B P, Madhavi T and Sushma N J 2014 Identification Of Bioactive Compounds By FTIR Analysis And In Vitro Antioxidant Activity Of *Clitoria ternatea* Leaf And Flower Extract *Indo American Journal Of Pharmaceutical Research* **4(09)** pp 3894 – 3903
[7.] Leal J M, Lucia V S, Jayabalan R, Oros J H and Ascalante-Aburto A 2018 A Review on Health Benefits of Kombucha Nutritional Compounds and Metabolites *Journal Of Food* **16(1)** pp 390 – 9
[8.] Manjula P, Mohan C H, Sreekanth D, Keerthi B and Prathibhadevi B 2013 Phytochemical Analysis Of *Clitoria ternatea* Linn. A Valuable Medicinal Plant *J. Indian Bot Soc* **92** pp 173 – 8
[9.] Naland H 2004 *Kombucha Teh Ajaib Pencegah dan Penyembuh Aneka Penyakit* (Jakarta: PT Agro Media Pustaka)
[10.] Rodriguez H, Curiel J A, Landete J M, Rivas B D L, Felipe F L D, Cordoves C G, Mancheno J M, Munoz R 2009 Food Phenolics and Lactic Acid Bacteria *International Journal of Food Microbiology* **132** pp 79 – 90
[11.] Yang Z, Zhou F, Ji B, Li B, Luo Y, Yang L and Li T 2008 Symbiosis Between Microorganisms from Kombucha and Kefir: Potential Signifincance to the Enhancement of Kombucha Function *Appl. Biochem. Biotecnol* **160(2)** pp 446 – 55
[12.] Wulansari D and Chairul 2011 Penapisan Aktivitas Antioksidan dan Beberapa Tumbuhan Obat Indonesia Menggunakan Radikal 2,2-Diphenyl-Picrylhydrazyl (DPPH) *Majalah Obat Tradisional* **16(1)** pp 22 – 25
[13.] Zheng Y, Lu Y, Wang J, Yang L, Pan C and Huang Y 2013 Probiotic Properties of *Lactobacillus* Strains Isolated From Tibetan Kefir Grains *PLoS ONE* **8(7)** e69868