

DEVELOPMENT OF KOMBUCHA AND ITS FUNCTIONAL PROPERTY FROM AGRICULTURAL WASTE (FERMENTED TEA BROTH)

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Abstract:

The aim of this project was to develop a functional product from fermented tea broth which is a by-product of fermented tea (Miang). The broth that was from the 15th day fermented tea was used. The broth was boiled with the additional of 10% sugar for brix adjustment to make original and pineapple kombucha. Original fermented broth kombucha were produced from mixing with water and fermented broth (1:1.5) and add (1:1) pineapple for pineapple favor. After that, they were mixed with scoby and 3% of previous kombucha which produced from fermentation of *Komagataeibacter saccharivorans*, *Zygosaccharomyces bailii*, *Dekkera bruxellensis*. The analysis of antioxidant activity, total phenolic content and flavonoids content were performed at interval of 0, 1, 3, 5, 7, 9, 11 day. The result showed that total phenolic of both original and pineapple kombucha were reduced. Total phenolic contents of original kombucha was in ranged of 32.1 - 42.05 $\mu\text{mol GAE/ 100ml}$. Total phenolic contents of pineapple favour was in ranged of $23.1 \pm 0.67 - 29.57 \pm 1.19 \mu\text{mol GAE/ 100 ml}$. While antioxidant (FRAP & ORAC) slightly increased in both batched. Antioxidant activity on FRAP of original kombucha was $311,527.8 \pm 38,978.9 \mu\text{mol TE /100 ml}$. Antioxidant activity on ORAC of original kombucha and pineapple favor were highest at day 7 ($620,442.1 \pm 79,695.4$ and $506,842 \pm 212,017.1 \mu\text{mol TE/ 100 ml}$ respectively). Total flavonoids were decreased from 0th day fermented tea kombucha as it formed dimer and complex structure ($10.15 - 6.92 \mu\text{mol CE /100 ml}$). Therefore, per serving size the developed kombuchas contained high amount of both phenolics and flavonoids. Fermented broth can be developed into Kombucha that containing functional property compounds.

Keywords: Fermented tea, kombucha, antioxidant, value added

Introduction

Fermented tea (Miang) is an ethnic fermented tea leaf which made from Assam tea or (*Camellia sinensis* var. *assamica*). It is commonly grown in the mountainous areas of Nan, Chiang Rai, Chiang Mai, Phayao and Phrae in northern parts of Thailand. Based on the number of tea plantation areas, Chiang Mai is the largest area for tea plantation; nevertheless, the largest of Miang production is established in Nan. Miang is frequently consumed after meals like a snack. Moreover, Miang is commonly served at ceremonial occasions by the northern Thai people such as wedding ceremony, a celebration party to a funeral (Khanongnuch, C. *et al*, 2017; Somsong, P. *et al*, 2018). Miang is often consumed during working hours as consumers claim that it helps refilling strength required during working activities.

Fermented broth is a liquid by-product from fermented tea (Miang) production and is usually discarded. Estimate of the broth was 2,500 ml per 1 kilogram of fermented tea production that was throw away, leading to environmental pollution. For example, air pollution and water pollution when they were thrown away in river and household area. In addition, leaching for bioactive compounds into fermented broth occur during fermentation process. Total phenolic content in fermented water was 1,450.86 mg/100 ml (Phromrukachat *et al.*, 2010).

Furthermore, local people produce Miang for a living, it can be value added product by using fermented broth. They will increase income, reduce waste and save environment. Therefore, kombucha can be that value-added product. It is fermented beverage because of the health benefits. This product has slightly acidic, carbonated, and sweet taste. Fermentation process is formed the symbiosis of bacteria and yeasts as kombucha culture. The mainly common substrate is black tea or green tea (Sinir *et al.*, 2019). *Komagataeibacter* spp., *Acetobacter* spp., and *Lactobacillus* spp., among bacteria; *Zygosaccharomyces* spp. and *Brettanomyces* spp., are the most abundant yeasts (Marsh *et al.*, 2014; Fessard *et al.*, 2016). During fermentation acetic acid bacteria produced the cellulose pellicle layer and biofilm (Vitas, J. *et al.*, 2018). Therefore, kombucha have been reported as beneficial health-promoting effects, such as prevention of cancer, high blood pressure, and improving digestion function (Tu, C. *et al.*, 2019). Thus, the aim of this study was to develop a healthy product from fermented tea broth and kombucha was chosen because the physical appearance is similar to Miang while containing the health benefit compounds.

Materials and Methods

Fermented broth preparation

Fresh mature tea leaves (leaves 4-6 from top) were randomly handpicked form Assam tea trees in October 2019 at Ban Si Na Pan located in Nan province. Fermented broth was made in the lab by fermenting of tea leave around 15 day by starter culture from the same area. Mature tea leaves were steamed at 100°C for 1 hours and cooled. Then, steam leaves were packed in containers and covered with white clothes under aerobic conditions for 7 days. Next, the steamed tea leaves which was covered with mature fungal growth are washed with water. After that, 1,000 gram of fungal growth tea leaves, 8 gram of salt ,8 ml of starter and 2,500 ml of DI water were fermented under anaerobic condition for 15 days. This broth was used to make kombucha.

Sample preparation

Kombucha scoby and previous kombucha was purchased form kombucha DIY company which produced from fermentation of *Komagataeibacter saccharivorans*, *Zygosaccharomyces bailii*, *Dekkera bruxellensis*.

- 1) **Fermented broth Kombucha** Fermented broth was diluted with water in ratio at 1:1.5 and add 10% w/v of sucrose. Then, they were boiled until sucrose well dissolved and pasteurized at 73°C 15 min in water bath. After cooling down to room temperature, the solution was poured into a glass jar and were inoculated with 2.5% (w/v) or 13 gram/500 ml and 3% of previous kombucha. The fermentation was controlled at 37°C in incubator for 11day or until biofilm was visible.
- 2) **Fermented broth with pineapple kombucha** Fermented broth was similar to original favour with the addition of pineapple at ratio at 1:1.5 and add pineapple juice.

pH and Total soluble (TSS)

Changes in pH of kombucha was measured using a pH meter (F20, Mettler Toledo, Bangkok, Thailand). Total soluble was measured using a hand refractometer (brix N1, Atago, Tokyo, Japan) at 25°C, respectively

Estimation of total phenolic content (TPC)

The total phenolic content of the samples determined that the method base on Somsong, P *et al* (2017). In brief, samples were diluted 10 times with distilled water and a 150 µL volume was further diluted with 2.4 mL distilled water. This was followed by adding 150 µL of Folin-Ciocalteu reagent (2 N) diluted with distilled water (1:10). The solutions were mixed well for 2 min before adding 7.5% (w/v) of 300 µL NaCO₃ to account for an ascorbic acid correction and mixed again. After incubation for 2 h at room temperature in the dark, the absorbance at 765 nm was determined microplate reader. Gallic acid concentration used to establish the standard curve ranged from 0.01–0.1 mg/mL. The results were expressed as mg gallic acid equivalent/ 100 ml (mg GAE/ 100 ml).

Estimation of the Antioxidant activity (AA)

Antioxidant activity was determined using two assays, FRAP and ORAC. The antioxidant activity of fermented broth samples determined using FRAP assay and ORAC assay from Somsong, P *et al* (2017). The working FRAP reagent prepared by mixing 300 mM acetate buffer, pH 3.6, with 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM of hydrochloric acid and 20 mM of FeCl₃·6H₂O at a ratio 10:1:1. The acetate buffer (pH 3.6) prepared using 3.1 g sodium acetate trihydrate and 16 mL acetic acid in 1 L distilled water. After that, Samples absorbance was then read at 593 nm after 30 min. A standard curve used concentrations between 100 – 600 µM L⁻¹ of 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox).

For ORAC assay, the reaction carried out in 75 mM phosphate buffer (pH 7.0), and the final reaction mixture was 1 mL. The samples or standard (20 µL) and fluorescein (160 µL; 120 mM solutions were preincubated for 15 minutes at 37 °C; then 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH) solution (20 µL; 480 mM, final concentration) will be added rapidly. The fluorescence (485) recorded every minute for 80 minutes in fluorescence spectrophotometer. A blank (PBS) will using methanol instead of the antioxidant solution. After that, Samples absorbance was then read at 485 nm at 37 °C and a standard curve used concentrations between 5 – 50 µM Trolox.

These two assays were performed using microplate reader and the results of antioxidant activities were expressed in µM Trolox/ 100 ml.

Estimation of Total flavonoid content (TFC)

The flavonoids content will be determined by aluminium trichloride method which modified from Ahlem Rebaya *et al* (2015). Catechin within the range of 0.01-0.1 mg mL⁻¹ was used as a standard. A volume of 25 µL of standard were added to 15 µL of a 5% NaNO₂ solution. The mixture was allowed to stand for 6 min, then 30 µL of aluminium trichloride (10%) were added and incubated for 5 min, will be followed by the addition of 150 µL of NaOH (1M). After 15 min of incubation the mixture turned to pink and the absorbance were measured at 510 nm using microplate reader. The total flavonoids content was expressed as g E catechin mg/ 100ml.

Statistical analysis

Analysis experiments were done at least in triplicate. Data was processed by a statistical software (IBM SPSS Statistics for Windows Version 18.0, IBM, Armonk, New York, U.S.A.) and presented as mean and standard deviation. Differences between mean values were analyzed by one-way Analysis of Variance and distinguished by Duncan's New Multiple Range Test and the confidence level of 95% ($p \leq 0.05$).

Results and Discussion

Comparison of pH and total soluble (TSS) between fermentation days of fermented broth kombucha (original)

The results of pH and total soluble at interval of 0,1,3,5,7,9,11 day in original kombucha are shown in Fig 1. The changes of pH values and total soluble during fermented broth of Miang fermentation with kombucha was rapidly decreased. The decrease in pH value attributed to the production of acids during fermentation. pH values started at 4.12 ± 0.07 then 4.07 ± 0.05 , 3.87 ± 0.08 , 3.49 ± 0.13 , 3.26 ± 0.10 , 3.12 ± 0.07 and 3.00 ± 0.07 respectively. However, pH values at 0, 3, 5, 7, 9, 11 had a statistically significant decreased ($p \leq 0.05$). There are two stages which are alcoholic and acetic acid fermentation (Li *et al.*, 2015; Li, Li, Li *et al.*, 2015). In alcoholic fermentation stage, yeast hydrolyze sucrose into glucose and fructose, formerly the ethanol is produced that total soluble was decreased. After that stage, it is acetic acid fermentation, the acetic acid bacteria use glucose to produce glucuronic acid and transform ethanol into acetic acid (Ayed, Abid, & Hamdi, 2016). Therefore, TSS are decrease between 10 significant different at day0, 7, 9 and 11.

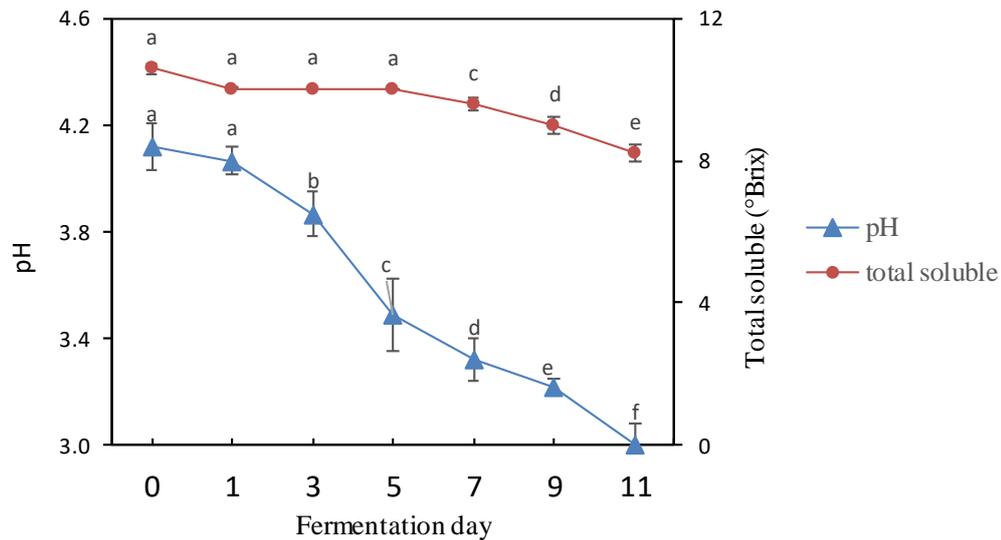


Figure 1: pH and total soluble of different fermentation day in fermented broth kombucha (original) during day 0 to day 11 (▲ = pH, ● = TSS)

Comparison of total phenolic, antioxidant activity (FRAP, ORAC) and total flavonoid of different fermentation day in fermented broth kombucha (original)

The change in TPC value of original kombucha with fermentation day was oscillate during fermentation day (figure 2a). The value of TPC was decrease significantly at day 3. After fermentation at day 5, the TPC was increased and the highest at day 5. Then, it was slightly decreased at day 7, 9 and slightly increase at day 11. However, they weren't significantly. The oscillation of TPC might be caused by some complex phenolic compounds. They might be degraded under the acidic environment or enzymatic by bacteria or yeast in SCOBY. In particular, *Saccharomyces* sp, *Lactobacillus* sp., and *Acetobacter* sp. which have ability to excreted tannase, an enzyme that could degrade tannins. (Naland, 2004; Dwiputri & Feroniasanti, 2019)

TFC of original kombucha was slightly decreased (Figure 2b). The TPC varied from 10.47 ± 1.18 to 10.47 ± 1.18 mg CE/ 100 ml. TFC value was tuned point at day 5 (8.13 ± 0.68 mg CE/ 100 ml) significantly. Consequently, the first day is the day that was reported to contain highest catechin in fermented tea leaves (Sampanvejsobha, S *et al.*, 2013; Phromrukachat *et al.*, 2010). After that, during fermentation of kombucha, the catechins formed dimeric catechins called theaflavins, larger compounds called proanthocyanidins, and very large oligomers and polymers called thearubigens. The chemical structures of these flavonoid oligomers in tea are very complex and have yet to be entirely characterized. (Dwyer & Peterson, 2013, Subramanian *et al.*, 1999)

However, the antioxidant activity was slightly increase (Figure 2c and 2d). They were a fluctuation of antioxidant activity occur during fermentation day. The content of antioxidant activity was decreased at day 3 ($271,250.00 \pm 46,550.73$ $\mu\text{mol TE} / 100 \text{ ml}$, $563,320.26 \pm 125,456.15$ $\mu\text{mol TE} / 100 \text{ ml}$ on FRAP and ORAC respectively). After day 5 of fermentation the antioxidant activity was slightly increased and the highest content at day 9 ($314,305.56 \pm 15,426.5273$ $\mu\text{mol TE} / 100 \text{ ml}$) significantly on FRAP. For the highest content of ORAC was $620,442.09 \pm 79,695.4173$ $\mu\text{mol TE} / 100 \text{ ml}$ at Day 7 then the fermentation period of ORAC was insignificantly. The fluctuation of antioxidant might be due to the activity of the microorganism during fermentation process and the stability of some antioxidant compounds. Especially, the microorganisms have the competence to degrade polyphenols compound

(Dwiputri & Feroniasanti, 2019). Zhao & Shah (2016) reported that after fermentation, TPC was increase in phenolic acids, derivatives, and flavan-3-ols and decrease in flavonols (Zhao & Shah, 2016). Therefore, in current study, we found that TPC was increase but total flavonoid was deceased.

Comparison of pH and total soluble (TSS) between fermentation days of pineapple favor kombucha

The results of pH and total soluble at interval of 0,1,3,5,7,9,11 day in pineapple favor kombucha are shown in figure 3. The changes of pH values and total soluble solid during fermentation was rapidly decreased. The decreasing in pH value is attributed to the production of acids during fermentation. pH values started at 3.95 ± 0.01 then 3.88 ± 0.00 , 3.75 ± 0.00 , 3.44 ± 0.01 , 3.29 ± 0.01 , 3.23 ± 0.01 and 3.17 ± 0.00 , respectively. However, pH values were changed value significantly at day 5. Therefore, TSS was decreased 16.18 ± 1.67 °brix to 7.56 ± 0.64 °brix and total soluble value was significantly at day5 (12.62 ± 0.60 °brix). Several authors have the same behavior (Chakravorty *et al*, 2016 ; Sun *et al*, 2015).

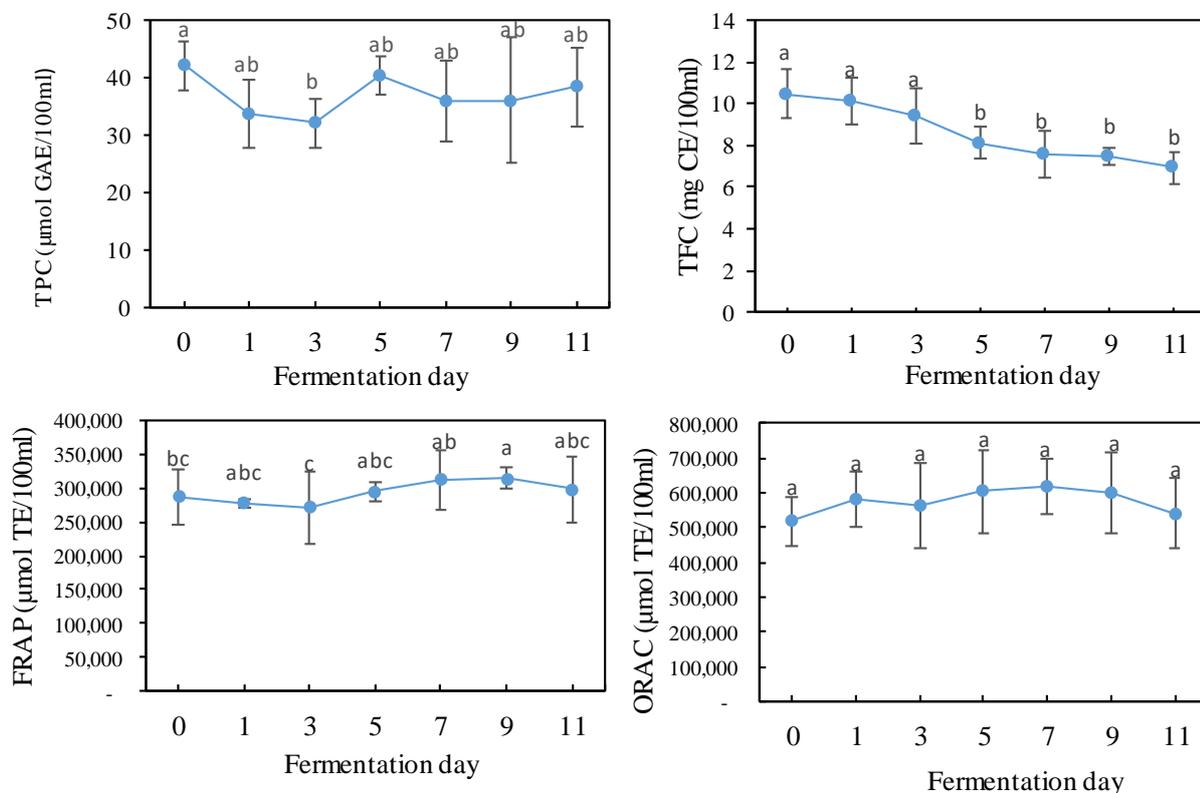


Figure 2: Total phenolic content (a), Total flavonoid (b), FRAP (c), and ORAC values (d) of original kombucha in different fermentation day

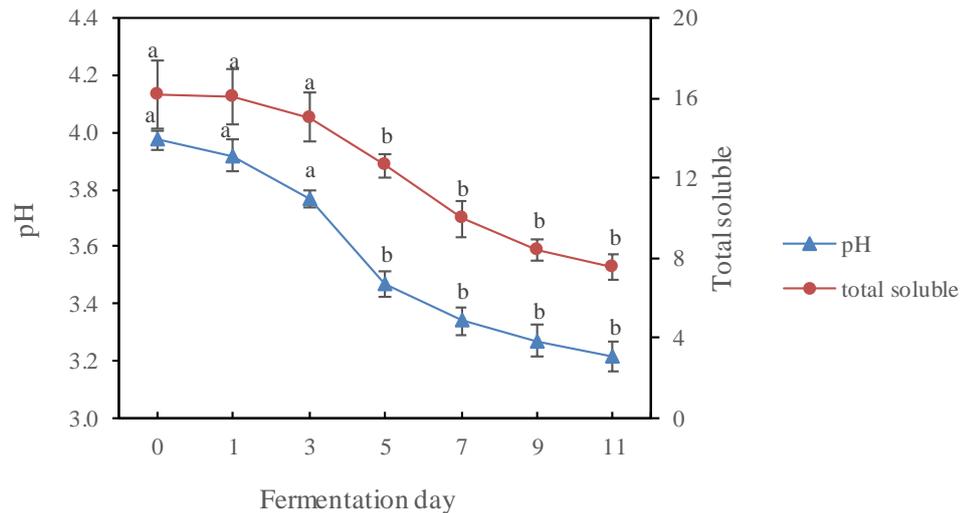


Figure 3: Comparison of pH and total soluble solid of different fermentation day in pineapple favor kombucha (\blacktriangle = pH, \bullet = TSS)

Comparison of total phenolic, antioxidant activity (FRAP, ORAC) and total flavonoid of different fermentation day in pineapple favor kombucha

The change in TPC value of pineapple favor kombucha with fermentation day was slightly increased during fermentation day (Figure 4a). The value of TPC was increased significantly ($29.57 \pm 1.20 \mu\text{mol GAE}/100 \text{ ml}$) at day 11. Likewise, other researchers found rather same results. After fermentation, TPC value was increased (Chakravorty *et al*, 2016, Jayabalan, 2014). Phenolic compounds were increased such as gallic acid, m-coumaric acid and Resveratrol that they were produced from microbiological (Amorim *et al*, 2018).

TFC of original kombucha was slightly decreased (Figure 4b). The TFC varied from 4.83 ± 0.34 to $2.12 \pm 0.31 \text{ mg CE}/100 \text{ ml}$. TFC value was turned point at day 5 ($3.27 \pm 0.18 \text{ mg CE}/100 \text{ ml}$) significantly and other turning point was significantly at day 7 ($2.66 \pm 0.81 \text{ mg CE}/100 \text{ ml}$). Then, values were stable. In the same way, Gaggia *et al* had total flavonoid content decrease that found catechins were decreased (Gaggia *et al*, 2019).

However, the antioxidant activity of FRAP was slightly increased from the Figure 4c. The highest content of FRAP was $139,814.81 \pm 12,633.80 \mu\text{mol TE}/100 \text{ ml}$ at Day 7 then the fermentation period of FRAP was insignificantly. The researcher had report antioxidant activity of FRAP in pineapple was no significant change (Fessard *et al*, 2016). For the antioxidant activity of ORAC during fermentation day was oscillate that shown the Figure 4d. The content of antioxidant activity was decreased significantly at day 1 ($83,390.72b \pm 39,232.59 \mu\text{mol TE}/100 \text{ ml}$ on ORAC, respectively). In current study, day 3 of fermentation the antioxidant activity was increased and the highest content ($594,714.96 \pm 116,029.61 \mu\text{mol TE}/100 \text{ ml}$) significantly. After that, the antioxidant activity content of ORAC was slightly decreased and significantly at day 9 to be stable. The antioxidant activity of ORAC was slightly increased from the Figure 4c. The fluctuation of antioxidant might be due to the activity of the microorganism during fermentation process and the constancy of some antioxidant compounds. Especially, the microorganisms may be used to perform mild or strong, slow or rapid food fermentation that could be ability to modulate antioxidant capacity and enzyme activities. Antioxidant activity of ORAC in pineapple juice was occur that fermented juice is lower than initial stage (Fessard *et al*, 2016, Septembre-Malaterre *et al*, 2018).

Finally, original favor fermented broth kombucha had higher content of total phenolic, antioxidant activity (FRAP, ORAC) and total flavonoid than pineapple favor fermented broth kombucha. Similarly, total soluble of original favor fermented broth kombucha had lower than pineapple favor fermented broth kombucha that is good for health. Moreover, original favor fermented broth kombucha used by-product to add value. On the other hand, pineapple favor fermented broth kombucha used pineapple juice that is normal product.

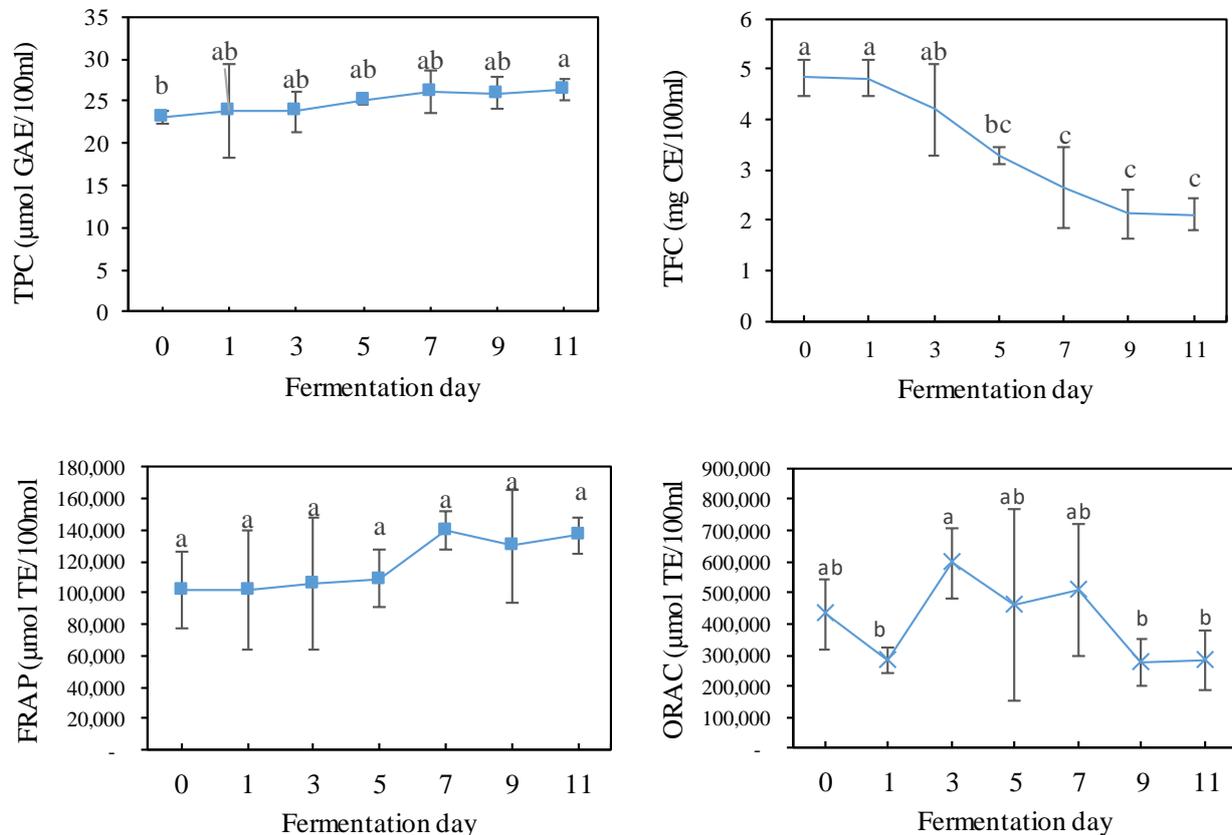


Figure 4: Total phenolic content (a), Total flavonoid (b), FRAP (c), and ORAC values (d) of pineapple favor kombucha in different fermentation day

Conclusion

Kombucha beverages were successfully produced by using fermented tea broth (Miang) that is waste of Miang fermentation. They are suitable for health that have higher significantly total phenolic and antioxidant than day 0. The most suitable period fermentation of original fermented tea kombucha is day 5 because total phenolic, antioxidant activities are highest than other days. Likewise, pH at day 5 is suitable for people as it is either too bitter or sour. For pineapple favor fermented broth kombucha is day 3 since antioxidant activity on ORAC is higher than other days. Nevertheless, fermented broth kombucha should be evaluated for sensory evaluation to confirm characteristic that is suitable for customers. Moreover, various reasons may contribute to the change of these results such as climate, season, and species. In terms of selecting high antioxidant tea or pineapple, attention needs to be paid to plantation, season, and species.

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