Jurnal Pijar MIPA
https://jurnalfkip.unram.ac.id/index.php/JPM

ISSN 1907-1744 (Print)
ISSN 2460-1500 (Online)

Total Flavonoid Content and In Vitro Anti-Inflammatory Potentials of Kombucha with Enrichment of Butterfly Pea (*Clitoria ternatea*) Flower Extract

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Received: December 15, 2023. Accepted: January 5, 2024. Published: March 25, 2024

Abstract: Kombucha is a fermented beverage from tea (*Camellia sinensis*) that is claimed to be beneficial for several medical ailments. Several studies have conducted nutritional enrichment of kombucha to optimize its nutritional benefits. One of the native Indonesian plants that can be utilized as nutritional enrichment material is the butterfly pea (*Clitoria ternatea*) flower. Its nutrition is expected to increase the health benefits of kombucha *Camellia sinensis*. Kombucha has beneficial compounds such as flavonoids that have potential applications in medical chemistry due to its anti-inflammatory properties. This study aims to enrich the valuable nutrition and test the in vitro anti-inflammatory potentials of kombucha, conducted by formulating kombucha*Camellia sinensis* with *Clitoria ternatea* flower extract addition (0; 0.5; 1; 1.5; 2)% (m/v), the chemical characterization including pH value test using digital pH meter and total flavonoid content (TFC) using colorimetric ($AlCl_3$) assay) with quercetin as its standard, and in vitro anti-inflammatory activity test using the red blood cell (RBC) membrane stabilization method. The result showed that the pH value of kombucha had met the consumption quality standard (3-4), starting from 3,30 $\pm$ 0,00 (0%) to 3,66 $\pm$ 0,01 (2%). The flavonoid content increased from 38,89 $\pm$ 0,05 mgQE/g (0%) to 126,73 $\pm$ 0,13 mgQE/g (2%). Kombucha samples showed anti-inflammatory activity potentials with the best IC50 value of 140,22 ppm (2% addition). The data from this study were analyzed using a one-way ANOVA statistical test. It shows there is an influence from the addition of *Clitoria ternatea* to the TFC result ($p<0,05$), and it continued to Duncan's *Multiple Range* test (DMRT) that showed sample F0 to F4 were significantly different from each other ($p<0,05$). In conclusion, the enrichment of kombucha (*Camellia sinensis*) using *Clitoria ternatea* flower extract has been proven to increase the health-beneficial compound (flavonoids) and have potential anti-inflammatory activity.

Keywords: Anti-Inflammatory; Butterfly Pea Flower; Kombucha; Flavonoid; HRBC Stabilization

Introduction

Kombucha is a fermented beverage from tea that developed through a consortium of bacteria and yeast, which acts as a starter or initial culture that helps the tea fermentation process[1]. Kombucha traditionally uses the tea (Camellia sinensis) solution and sugar as its base material[2]. The method of making kombucha is by fermenting sweetened tea using a mixture of yeast and bacteria (generally *Acetobacter xylinum*, *Gluconobacter*, and *Saccharomyces cerevisiae*) that will form cellulose plates called SCOBY (Symbiotic Culture of Bacteria and Yeast) or tea fungus that placed on the surface area of the tea during fermentation process[3].

Currently, kombucha is claimed to be beneficial for several medical ailments. The chemical content of kombucha is essential in supporting its benefits, such as its flavonoid contents. Kombucha reported to have flavonoids total around 83,8 to 146,8 mg/L depending on its materials (*Camellia sinensis various*) and their fermentation time (7 to 14 days)[4]. Flavonoids are phytochemical compounds commonly present in plants with potential applications in medical chemistry[5]. Further studies show that flavonoids are anti-inflammatory [5]. Several mechanisms of action explain flavonoids as anti-inflammatory properties such as their anti-oxidative and radical scavenging activities, modulation of the production of other pro-inflammatory molecules, modulation of pro-inflammatory gene expression, regulation of cellular activities of inflammation[6].

Several studies have conducted nutritional enrichment of kombucha to optimize its nutritional benefits. Kombucha with a combination of rosella flower[7], robusta coffee leaves[8], and *Muntingia calabura* leaves [9] stated to have a potential as probiotic-antioxidant drinks. One of the native Indonesian plants that can be utilized as a nutritional enrichment material is the butterfly pea (*Clitoria ternatea*) flower, whose nutrition is expected to increase the benefits of kombucha. *Clitoria ternatea* also has pharmacological activity, such as anti-inflammatory activity[1], related to its flavonoid content. Some studies reported that the flavonoid content of *Clitoria ternatea* is about 63,09 to 187,05 mgQE/g[10], [11]. Therefore, *Clitoria ternatea* has the potential to be a nutritional enrichment ingredient in kombucha.

In vitro, the anti-inflammatory assay is commonly used in the human red blood cell membrane stabilization method. The red blood cells are analogous to the lysosomal membrane that contains inflammatory mediators[12]. Red blood cells are known to be susceptible to free radicals, so the antioxidant activity of the sample is expected to support the anti-inflammatory activity in protecting cells from hemolysis. In addition, flavonoids (antioxidant properties) can prevent oxidative stress and affect red blood cell stability [13].

How to Cite:
Wahyanto, K. N., & Agustini, R. (2024). Total Flavonoid Content and In Vitro Anti-Inflammatory Potentials of Kombucha with Enrichment of Butterfly Pea (Clitoria ternatea) Flower Extract. *Jurnal Pijar Mipa*, *19*(2), 254–259. https://doi.org/10.29303/jpm.v19i2.6320

Based on this description, this study aims to increase the beneficial nutrition and test kombucha's in vitro anti-inflammatory activity so that its potential as a natural anti-inflammatory drug can be utilized. This study was conducted by making black tea (*Camellia sinensis*) kombucha added with *Clitoria ternatea* simplisia (powder) using variations in mass concentrations and chemical characterization including pH value, total flavonoid content (TFC) using colorimetric ($AlCl_3$) assay, and followed by the potential anti-inflammatory activity (in vitro) with human red blood cell (HRBC) membrane stabilization method. The TFC and anti-inflammatory activity test were analyzed using a spectrophotometer UV-visible.

Research Methods

This research uses the true experimental method. Some of the tools used in this study are stove (Maspion), glass bottle or jar, spatula, thermometer, test tube (Pyrex), analytical balance (Denver Instrument), pH meter (Mediatech), volumetric flask (Pyrex), beaker glass (Pyrex), micropipette (Eppendorf), autoclave (Hirayama), incubator (Memmert), vortex (Labnet), centrifuge (Eppendorf), waterbath (Labtech) Spectrophotometer UV-Vis (Shimadzu UV-1800).

The materials used in this study were water (Cleo), sugar (Gulaku), black tea bag (Tongtji), *Clitoria ternatea* powder (Ryouindonesia), SCOBY and kombucha starter (Rosellaku), ethanol p.a, quercetin (Sigma), potassium acetate (CH_3COOK) 1M, $AlCl_3$ 10%, sodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$), sodium diclofenac, 10% red blood cells (HRBC) suspension, distilled water.

This research was conducted in steps. The first one is the preparation of samples and pH value test. Next, the total flavonoid contents (TFC) are analyzed; the last is an in vitro anti-inflammatory test.

Samples were prepared by mixing tea solution with sugar 15% (m/v), starter SCOBY 10% (m/v), and fermented for five days. After that, *Clitoria ternatea* powder was added with various masses (0; 0.5; 1; 1.5; 2)% per 200 mL first fermented tea solution (F0 – F4). The fermentation process continued for two days. pH values were checked to ensure that kombucha samples qualified for consumption. Samples were extracted using a rotary evaporator (45°C).

The total flavonoid content was measured by colorimetric ($AlCl_3$) assay with quercetin as the standard for the calibration curve [9]. 0,5 mL of extract (1000 ppm) was added to the test tube. Then, 1,5 mL ethanol p.a, 0,1 mL $AlCl_3$ 10%, 0,1 mL CH_3COOK 1M, and 2,8 mL distilled water were added and mixed thoroughly. The absorbance was determined at 440 nm using a spectrophotometer UV-Vis. The total flavonoid content of the extract was expressed as mg quercetin equivalents per gram of sample (mgQE/g).

In vitro, the anti-inflammatory potential was determined using an HRBC membrane stabilization assay with sodium diclofenac as its control positive[14], [15]. Samples were made with various concentrations (ppm). 0,5 mL HRBC suspension, 1 mL sample, 1 mL PBS (pH 7,4), and 2 mL hyposaline were mixed thoroughly and incubated at 37°C using a water bath for 30 minutes. The mixture was centrifuged at 3000 rpm for 5 minutes. The absorbance was determined at 560 nm using a spectrophotometer UV-Vis. The anti-inflammatory activity of the extract was expressed as IC50 (Inhibitory Concentration) value compared to its control positive, sodium diclofenac.

Results And Discussion

Kombucha with enrichment of *Clitoria ternatea* (m/v) is shown in Figure 1. It shows that the more *Clitoria ternatea* added, the more intense its color formed. The glass with the number 0 is a kombucha without any addition, and glass numbers 1 to 4 are kombucha with various additions of *Clitoria ternatea.* The *Clitoria ternatea* is originally blue due to its anthocyanin content under a pH of 6 to 7 (dissolved in water)[16]. In this study, the color of the kombucha changed to purple because the pH value of the samples is around 3 to 4. The anthocyanin was reported to be purple under pH values around 3 to 5[17].

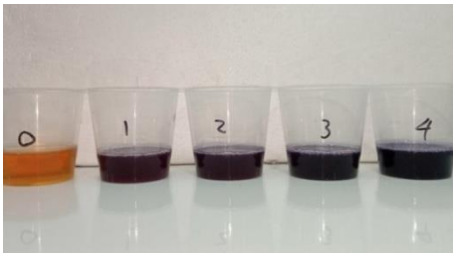

Figure 1. Kombucha: (0) 0%, (1) 0,5%, (2) 1%, (3) 1,5%, (4) 2% extract addition per 200 mL.

The maximum addition of *Clitoria ternatea* extract in this study was 2%. This formulation is based on the pre-laboratory test that showed the sample with 2,5% (m/v) addition of *Clitoria ternatea* has a pH value of over 4, which is not qualified for the consumption standard. Kombucha's pH value that is safe for consumption ranges from 3 to 4 [18]. When the addition exceeds 2% (m/v), the pH value of the sample increases, exceeding the predetermined consumption limit in previous studies. Therefore, the formulation of kombucha in this study is limited to a 2% (m/v) addition of *Clitoria ternatea* flower powder.

Table 1. pH Value

Sample	pH
F0	3.30 ± 0.005^{a}
F1	3.36 ± 0.005^{b}
F2	3.48 ± 0.005^{c}
F3	3.57 ± 0.005^{d}
F4	3.66 ± 0.011^{e}

*Notes: the numbers following with the same letters mean no significantly different ($p<0.05$)

The data in Table 1 showed that the pH value of kombucha increased with the addition of Clitoria ternatea. Compared to other studies, kombucha has various pH values with the same range (3 to 4) and the same time of fermentation (7 to 10 days) [19]. With the addition of fruits

rich in vitamin C or acidic taste, Kombucha is reported to have a lower pH value [20]. In this study, the original pH value of *Clitoria ternatea* is around 6 to 7 when it dissolved in water (Amalia et al., 2020), which can affect the pH value of kombucha when added. So, the more *Clitoria ternatea* powder added, the higher the pH value of the samples could get.

The pH value data were analyzed using the *Kruskal-Wallis* statistical test. It shows an influence of adding Clitoria ternatea to the samples' pH value ($p<0,05$). Next, the data were further analyzed with the *Mann-Whitney* test to see the difference between every sample and another. The statistical test result showed in Table 1 that samples F0 to F4 were significantly different ($p<0,05$), followed by other letters.

TFC Test Result

The total flavonoid content (TFC) of kombucha with enrichment of *Clitoria ternatea* was measured by colorimetric ($AlCl_3$) assay because flavonoids contain conjugated aromatic systems that show strong absorption bands in the UV-visible spectrum. Quercetin was used as a standard for the calibration curve of this test because quercetin is the most significant part of the flavonol group and presents about 60-75% of flavonoids [6]. The basis for determining TFC in the colorimetric assay is forming a complex between $AlCl_3$ with the ketone group on the C-4 atom and hydroxyl group on the C-3 and C-5 atom from the flavone and flavonol group. Figure 2 shows the mechanism of TFC with colorimetric $AlCl_3$ methods.

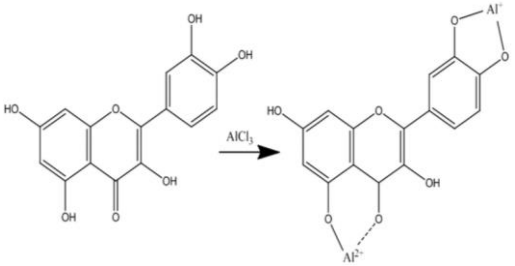

Figure 2. Mechanism of complex quercetin-aluminum chloride formed[6]

The regression equation calculates total flavonoid content (TFC) based on equivalence to quercetin (mgQE/g extract). The calibration curve obtained linear regression equation $y = 0,0057x + 0,0082$ with correlation coefficient $R^2 = 0,9877$. The calibration curve of quercetin is shown in Figure 3, and the result of the TFC test can be seen in Table 2.

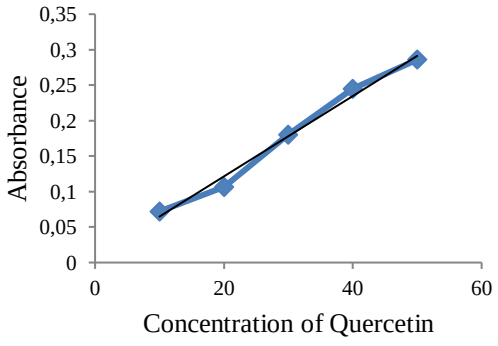

Figure 3. Calibration curve of quercetin

Table 2. TFC Result

Sample	Absorbance	TFC (mgQE/g)
F0	0.1210	38.89 ± 0.05^a
F1	0.1341	87.13 ± 0.06^b
F2	0.1559	101.81 ± 0.06^c
F3	0.1769	117.14 ± 0.06^d
F4	0.1935	126.73 ± 0.13^e

*Notes: the numbers following with the same letters mean no significantly different ($p>0.05$)

The addition of *Clitoria ternatea* extract increases the total flavonoid content of kombucha. The F0 kombucha without any addition was stated to have a TFC of 38,89 ± 0,05 mgQE/g and raised along with the addition of *Clitoria ternatea* powder. Compared to another study, the TFC value is higher. Kombucha made from white tea has a TFC value of 83,8 mgQE/g [4], and other studies with the addition of *Muntingia calabura* leaves have a TFC value of 44,026 mgQE/g [9]. The highest TFC value reached 126,73 ± 0,13 mgQE/g on sample F4. In conclusion, adding Clitoria ternatea has proven to enrich the beneficial compound (flavonoids) from the original kombucha (*Camellia sinensis*) and some previous studies.

Flavonoids can be found in foods or beverages made from plants, fruits, flowers, etc., like kombucha originally made from *Camelia sinensis* or other substances such as *Clitoria ternatea*. Flavonoid is a phenolic compound reported to have antioxidant and anti-inflammatory properties [9], [21]. There have been several studies explaining the anti-inflammatory activity of flavonoids. Flavonoids possess anti-oxidative and radical scavenging activities that could regulate the cellular activities of the inflammation-related cells. Flavonoids can modulate the enzyme that metabolizes arachidonic acid (AA), such as phospholipase A_2 (PLA_2), cyclooxygenase (COX), lipoxygenase (LOX), and the enzyme that produces nitric oxide (NO), nitric oxide synthase (NOS). This ability of flavonoids can reduce the production of those crucial mediators of inflammation [22].

The data were analyzed using a one-way ANOVA statistical test, which shows an influence from the addition of *Clitoria ternatea* to the TFC result ($p<0,05$) of the samples. Next, the data were further analyzed with *Duncan's Multiple Range* test to see the difference between every sample and another. The statistical test result showed in Table 2 that samples F0 to F4 were significantly different ($p<0,05$), followed by other letters.

In Vitro Anti-Inflammatory Activity Test Result

The anti-inflammatory activity potentials of kombucha with enrichment of *Clitoria ternatea* extract were analyzed using an in vitro assay and the red blood cell (RBC) membrane stabilization method. This method is commonly used to determine the anti-inflammatory activity since the membrane of RBC is similar to the lysosome membrane, which means stabilization of the lysosomal membrane is essential in the limitation of the inflammatory response by preventing the release of enzyme in the

lysosome from the activation of neutrophils such as protease enzymes during the inflammatory process in extracellular tissue and fluid [23]. Samples exhibited membrane stabilization effect by inhibiting hypotonicity-induced lysis of RBC membrane.

The anti-inflammatory activity of kombucha *Camellia sinensis* (F0), kombucha with enrichment of *Clitoria ternatea* flower extract (F1-F4), and sodium diclofenac expressed in %inhibitory and IC50 value showed in Table 3.

Table 3. Anti-inflammatory Activity

Sample	Concentration (ppm)	% inhibitory (%)	Reg. linear equation	IC50 (ppm)
F0	12.5	5.360 ± 0.11	y = 0.2204x + 3.4872 R^2 = 0.9852	242.68
	25	10.155 ± 0.05		
	50	13.840 ± 0.66		
	75	20.952 ± 0.08		
	100	24.971 ± 0.04		
F1	12.5	7.676 ± 0.023	y = 0.2872x + 6.1393 R^2 = 0.9778	195.47
	5	15.279 ± 0.940		
	50	21.499 ± 0.044		
	75	26.977 ± 0.050		
	100	34.647 ± 0.198		
F2	12.5	8.051 ± 0.120	y = 0.3326x + 5.8158 R^2 = 0.9866	167.82
	25	15.670 ± 0.524		
	50	23.576 ± 0.079		
	75	30.611 ± 0.050		
	100	38.480 ± 0.080		
F3	12.5	8.490 ± 1.070	y = 0.3873x + 7.0089 R^2 = 0.968	147.20
	25	18.736 ± 0.161		
	50	28.868 ± 0.160		
	75	36.69 ± 0.097		
	100	43.924 ± 0.102		
F4	12.5	7.119 ± 0.081	y = 0.3917x + 4.925 R^2 = 0.9808	140.22
	25	17.200 ± 0.094		
	50	25.265 ± 0.398		
	75	34.809 ± 1.331		
	100	43.059 ± 0.116		
Sodium diclofenac	10	7.131 ± 0.299	y = 1.1955x – 4.491 R^2 = 0.993	45.58
	20	21.292 ± 0.524		
	30	30.059 ± 0.082		
	40	41.677 ± 0.227		
	50	56.714 ± 0.418		

% inhibitory value indicates the ability of a sample to stabilize the red blood cell membrane from hemolysis [24]. A previous study stated that when a sample's %inhibitory is greater than 20%, it is considered an anti-inflammatory activity property and can be used for anti-inflammatory drug development[25]. The data in Table 3 showed that kombucha without any addition (F0) has %inhibitory over 20% at a concentration of 75 ppm, and kombucha with various additions of *Clitoria ternatea* (F1-F4) starts at 50 ppm. Meanwhile, sodium diclofenac (anti-inflammatory drug) has a % inhibitory over 20% at a concentration of 20 ppm. This %inhibitory in various concentration of the samples used to determine the regression linear equation by comparing the concentration of sample with its %inhibitory. From that equation, IC50 value can be measured. The IC50 value referred to the effectivity of the sample in inhibiting 50% hemolysis of RBC membrane.

The data in Table 3 revealed that the more *Clitoria ternatea* extract added, the greater the IC50 value of samples could get. F0, kombucha without any addition of extract has an IC50 value of 242,68 ppm, and it decreased to 140,22 ppm in 2% addition of *Clitoria ternatea*. Clitoria ternatea extract, also proven in this study, can increase kombucha's total flavonoid contents. *Clitoria ternatea* flower extract were analyzed in the previous research and identified using HPLC-MS to have quercetin, its glycosides, and ternatin anthocyanin [21]. Both compounds are stated to have antioxidant and anti-inflammatory activity[5], [21]. Figure 4 below presents a correlation between TFC's test result and the IC50 value from the samples. It showed that the higher the TFC's result, the greater the IC50 value from the sample.

In addition, flavonoids, especially in this study, were represented as quercetin, essential to the sample's anti-inflammatory activity. This compound protected the RBC membrane (lysosome membrane analog) significantly in preventing the release of pro-inflammatory constituents. Thus, samples with higher TFC results have stronger anti-inflammatory activity potentials.

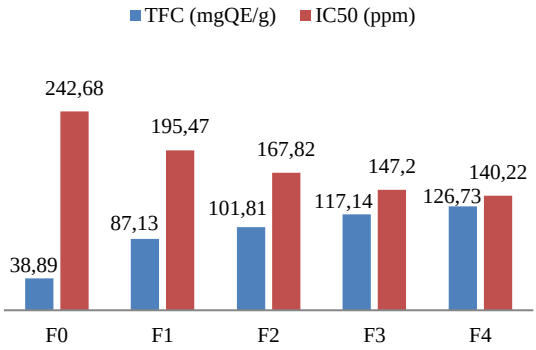

Figure 4. TFC and IC50 Result

Conclusion

Based on this study's results, kombucha with enrichment of *Clitoria ternatea* extracts was proven to increase the total flavonoid content of kombucha *Camellia sinensis*. Kombucha samples showed in vitro anti-inflammatory activity potentials with the best IC50 value on sample F4, kombucha with 2% addition. In conclusion, the enrichment of kombucha (*Camellia sinensis*) using *Clitoria ternatea* flower extract has been proven to increase the health-beneficial compound (flavonoids) and have potential anti-inflammatory activity. Thus, kombucha can be utilized as a natural anti-inflammatory drug.

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