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Effect of Sugar Concentration in Kombucha *Clitoria ternatea* on the Growth of Extended-Spectrum Beta-Lactamase *Escherichia coli*

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Abstract

Background: Kombucha made from butterfly pea flowers (*Clitoria ternatea* L.) is a fermented beverage rich in bioactive compounds, including flavonoids, alkaloids, saponins, tannins, and phenols, which are known to possess antibacterial properties. The increasing number of cases of Extended-Spectrum Beta-Lactamase (ESBL)-producing *Escherichia coli* resistance necessitates the development of alternative antibacterial agents based on natural ingredients. **Objective:** To determine the effect of sugar concentration variation during butterfly pea flower kombucha fermentation on the inhibitory power against ESBL *E. coli*. **Methods:** The study employed a laboratory experimental design with sugar concentrations of 10%, 20%, and 30%. Phytochemical tests were conducted to identify bioactive compounds. Total phenol content was analyzed using the Folin-Ciocalteu method, and antibacterial activity was evaluated by disc diffusion on Mueller-Hinton agar. **Results:** The butterfly pea flower kombucha contained flavonoids, alkaloids, saponins, and tannins. The total phenol content increased with increasing sugar concentration, specifically at 939.43 mg Gallic Acid Equivalent (GAE)/L (10%), 1203.9 (20%), and 1253.87 (30%). Antibacterial tests showed inhibition zone diameters of 18.24 mm, 21.06 mm, and 26.46 mm at concentrations of 10%, 20%, and 30%, respectively ($p < 0.05$). The positive control meropenem yielded an inhibition zone diameter of 18.53 mm. **Conclusion:** Increasing sugar concentration in butterfly pea flower kombucha fermentation affects higher phenol content and its antibacterial activity. Butterfly pea flower kombucha at a 30% sugar concentration was effective treatment for inhibiting the growth of ESBL-producing *E. coli*.

Keywords: Antibacterial agent, Butterfly pea flower, ESBL *Escherichia coli*, Kombucha, Total Phenol

Original Research Article

INTRODUCTION

Diarrhea is one of the most common health problems occurring in both developing and developed countries. According to statistics from the World Health Organization (WHO), diarrhea is the second leading cause of death among children. The number of deaths caused by diarrheal disease in children under five was 370,000 cases in 2019 and increased to approximately 443,832 child deaths each year. Diarrhea is defined as an excessive loss of fluids and electrolytes through stool. The etiology of diarrhea

encompasses infectious and non-infectious causes, including food allergies, malabsorption, inflammatory bowel disease, medications, and endocrinopathies. Infectious etiologies include viruses, bacteria, and parasites, which can cause secretory diarrhea, often associated with the bacterium *Escherichia coli*.

Escherichia coli is part of the normal flora of the human body, but its presence in food or drink is a contaminant that can cause disease or become pathogenic if its number reaches 10^6 cells/mL. Hazardous pathogenic strains include Shiga toxin-producing *E. coli* (STEC), Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteraggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), and Diffusely Adhering *E. coli* (DAEC). Resistance mechanisms include restricting drug absorption, modifying drug targets, inactivating drugs, and active efflux. One type of bacteria that has developed resistance is Extended-Spectrum Beta-Lactamase (ESBL)-producing *E. coli*, which produces an enzyme that hydrolyzes beta-lactam antibiotics, leading to infections that are difficult to treat.

Strategies to overcome antibiotic resistance are necessary, including developing alternative therapies and using natural ingredients (Rauf, 2021). Kombucha is a probiotic fermented beverage with a symbiotic culture of bacteria and yeast (SCOBY). Kombucha produces organic acids, polyphenols, and enzymes derived from microbial activity, as well as low levels of ethanol and CO₂ (Ovita et al., 2022). Its bioactive components include organic acids, enzymes, vitamins, and phenolic compounds (Burhan, 2022). The butterfly pea plant (*Clitoria ternatea* L.) is known to contain flavonoids, saponins, alkaloids, and tannins with antibacterial, antidiabetic, and antioxidant properties (Agustiansyah, 2022). The combination of butterfly pea with fermented products, such as kombucha, enhances the potential to form organic acids and functional metabolites that may act as antibacterial agents.

Previous research by Rezaldi et al. (2021) using butterfly pea kombucha with a 40% sugar concentration showed antibacterial activity against various pathogenic bacteria. Additionally, fermentation time affects antibacterial activity, as demonstrated by Sintyadewi et al. (2023). Sugar is a crucial factor influencing microbial metabolism during fermentation, and variations in sugar concentration can impact the production of organic acids and phenols in kombucha. The 14-day fermentation is known to produce optimal acid levels that enhance antibacterial activity (Leal et al., 2018).

While Rezaldi et al. (2021) established the feasibility of sugar-assisted extraction at a fixed concentration of 40%, the study did not explore concentration-dependent effects on phenolic yield or evaluate antimicrobial activity against ESBL-producing pathogens. The present study bridges this gap by systematically investigating a lower concentration range (10–30%) to identify the minimal effective sugar concentration for optimal bioactivity, and by extending the analysis to include both phytochemical profiling and ESBL-specific antimicrobial assays. By using lower concentrations (10%, 20%, and 30%), this study can also focus on the growth of acetic acid bacteria rather than yeast in kombucha, with the hope that this would be more effective and optimal as an antibacterial against ESBL-producing *Escherichia coli*.

MATERIALS AND METHODS

This research is an experimental study conducted in a laboratory with post-test-only group design. The population in this study consisted of pathogenic ESBL-producing *E. coli* grown in culture media that were obtained from the Microbiological Laboratory, Universitas Muhammadiyah Semarang. The culture was then tested in butterfly pea flower kombucha at sugar concentrations of 10%, 20%, and 30%, with 4 replicates per treatment group. The independent variable in this study was butterfly pea flower kombucha (*Clitoria ternatea* L.) with sugar concentrations of 10%, 20%, and 30%, and the dependent variable was the antibacterial activity against ESBL-*E. coli* by measuring the diameter of the inhibition zone formed on Mueller-Hinton Agar (MHA) media. The tools used included a digital scale, spatula, stainless steel pot, measuring cup, Erlenmeyer flask, petri dish, test tube, microscope slide, inoculating loop, dropper, caliper, spirit lamp, light microscope, incubator, autoclave, glass jar, and stirrer. The materials used consisted of granulated sugar, kombucha starter culture (SCOBY), dried

butterfly pea flower, media MHA, sterile aquadest, physiological saline, and control antibiotics for sensitivity testing ESBL-*E. coli* (Meropenem).

The preparation of butterfly pea flower kombucha involves making butterfly pea tea with 1 liter of boiling water and 10 grams of dried butterfly pea flowers, steeped for 10–15 minutes. The tea is then strained and cooled. Sugar is then added at concentrations of 10%, 20%, and 30% w/v. The tea and sugar solution is poured into a fermentation container, and 50 grams of SCOBY were added, along with approximately 10% kombucha starter based on the total volume. Fermentation is carried out for 14 days at 24–28°C. Phytochemical tests be conducted based on the high phenol content and inhibitory power. Phytochemical testing is done by performing a phenol test by reacting 1 mL of kombucha with FeCl₃ reagent to observe color change as an indication of the presence of phenols, as well as quantitative phenol testing using the Folin–Ciocalteu method, by measuring absorbance at a wavelength of 756 nm (Thermo Scientific™ GENESYS™ 20 Visible Spectrophotometer), with the results expressed as mg GAE/L (Schuetz et al., 2025). While, for flavonoid with addition of Magnesium (Mg) and concentrated Chloride Acid (HCl) for saponin and alkaloid tests.

Extended-Spectrum Beta-Lactamase *E. coli* sensitivity testing was performed to confirm resistance to beta-lactam antibiotics. Bacterial suspensions were adjusted to a 0.5 McFarland standard, inoculated onto MHA medium, and antibiotic discs were placed with a minimum distance of 24 mm. Incubation was carried out at 35–37°C for 16–18 hours. Inhibition zones were measured and compared with CLSI 2024 interpretation standards (Clinical and Laboratory Standards Institute, 2018). Extended-Spectrum Beta-Lactamase confirmation was performed using ceftazidime/cefotaxime discs with and without clavulanic acid, where a difference in inhibition zone of more than 5 mm was considered ESBL positive (Kothari, 2020). The antibacterial test of butterfly pea flower kombucha was conducted using the disc diffusion method. An extended-spectrum beta-lactamase *E. coli* suspension was prepared according to the McFarland 0.5 standard, evenly inoculated onto the MHA, and discs containing 100 µL of kombucha (10%, 20%, 30%) were placed on the medium surface. Meropenem was used as the positive control, which is often referred to as a third-line antibiotic or a last-resort antibiotic for hard-to-treat Gram-negative bacterial infections. Meropenem belongs to the carbapenem class, which is not destroyed by ESBL enzymes, so it almost always remains sensitive. Distilled water served as the negative control. Incubation was carried out at 35–37°C for 16–18 hours, then the diameter of the inhibition zone was measured using a caliper. The strength of inhibition was assessed using inhibition zone categories: ≤ 5 mm (weak), 6–10 mm (moderate), 11–20 mm (strong), and ≥21 mm (very strong) (Aslamiyah et al., 2023; Izzulhaq et al., 2023).

Data processing was carried out using SPSS, including the Shapiro–Wilk normality test, Levene's homogeneity test, one-way ANOVA, and Tukey HSD post hoc test, with p-values < 0.05 considered significant.

RESULTS

Phytochemical Analysis

Analysis of kombucha components was conducted through phytochemical tests and total phenol measurements to identify the bioactive compounds produced during butterfly pea flower kombucha fermentation. Phytochemical tests revealed the presence of flavonoids, alkaloids, saponins, and tannins, which contribute to kombucha's biological activity. (Table 1).

Table 1. Phytochemical analysis result of butterfly pea flower kombucha

Phytochemical Analysis	Reagent	Result	Result description
Flavonoids	Mg + concentrated HCl	+	Color red
Alkaloids	HCl 1%+ Dragendorff	+	Red precipitate
Saponins	HCl 1 N	+	Stable Foam

Phytochemical Analysis	Reagent	Result	Result description
Flavonoids	Mg + concentrated HCl	+	Color red
Tannins	FeCl ₃ 10%	+	Blackish green

Based on Table 1, the flavonoid test showed a red color change in the butterfly pea flower kombucha extract after the addition of Magnesium (Mg) and concentrated Chloride Acid (HCl), due to the formation of flavylum salt from the reduction reaction of the flavonoid carbonyl ring by Mg in an acidic environment (Astuti et al., 2025). Red precipitate in the alkaloid test appears because basic alkaloids react with Dragendorff's reagent, which contains potassium bismuth iodide (Ahlan et al., 2020). Saponin tests yielded positive results, indicating the formation of a stable foam. Saponins have both polar and nonpolar components that form micelles upon shaking. The foam's stability after the addition of HCl confirms the presence of saponins (Burhan et al., 2022). The greenish-black color in the tannin test with FeCl₃ occurs because Fe³⁺ ions form a colored complex with the phenol groups in tannin, thus producing a characteristic color as a positive indicator. The presence of these bioactive compounds confirms the potential of butterfly pea flower kombucha as a functional beverage with biological activities, including antibacterial activity (Ahmad, 2015; Alimuddin, 2023).

Analysis of Total Phenol Content

The total phenol content was analyzed to determine the number of phenolic compounds produced during butterfly pea kombucha fermentation at various sugar concentrations. Measurements were performed using the spectrophotometric method with Folin–Ciocalteu reagent, based on a phenol standard curve, and the results are expressed in milligrams of gallic acid equivalent per liter (mg GAE/L). The Folin–Ciocalteu method involves reacting phenolic compounds with reagents in an alkaline medium, yielding a blue complex that is measured by spectrophotometry. The higher the phenol content, the stronger the color formed and the greater the absorbance value (Lapailaka, 2023). The phenol standard curve yielded a linear regression equation of $Y = 0.1094X - 0.0796$ with an R^2 value of 0.9987, indicating a positive linear relationship between phenol standard concentration and absorbance. The R^2 value, which is very close to 1, indicates an excellent model fit, meaning that almost all variation in absorbance can be explained by variation in phenol concentration.

Table 2. Total phenol content of butterfly pea flower kombucha with 10–30% sugar concentration variation during fermentation process

Sugar Concentration (% w/v)	Y (Absorbance) Mean ± Std. Dev	Dilution	X (Phenol Concentration) Mean ± Std. Dev
30	0.263±0.033	400	1253.87±121.72
20	0.250±0.041	400	1203.90±150.94
10	0.177±0.023	400	939.43±84.52

The analysis of total phenol content in butterfly pea flower kombucha shows that the 30% sugar concentration has the highest average phenol content, at 1253.87 mg GAE/L (Table 2). These findings indicate that total phenol content tends to increase with increasing sugar concentration during fermentation.

Results of Antibacterial Activity Test

Antibacterial activity tests showed that butterfly pea kombucha at 10%, 20%, and 30% w/v sugar inhibited the growth of ESBL *E. coli*. The 30% sugar concentration in butterfly pea flower kombucha produced the largest inhibition zone, 26.46 ± 0.80 mm (very strong) (Table 3). The technical control and negative control showed no inhibition zone, indicating that the antibacterial activity originated from the butterfly pea kombucha. These results indicate that the inhibition zone diameter increases with increasing sugar concentration. The positive control produced an inhibition zone of 18.53 ± 0.57

mm (strong category), which was still lower than those observed at 20% and 30% concentrations. This finding demonstrates that variations in sugar concentration affect the antibacterial activity of butterfly pea kombucha against ESBL-*Escherichia coli*.

Table 3. Zone inhibition categorization of butterfly pea flower kombucha against extended-spectrum beta-lactamase *Escherichia coli*

Treatments	N	Mean±Standard Deviation	Inhibition Categorization
Kombucha 10% Sugar	4	18.24±0.55	Strong
Kombucha 20% Sugar	4	21.06± 0.14	Very Strong
Kombucha 30% Sugar	4	26.46±0.80	Very Strong
Meropenem (10 µg)	4	18.53±0.57	Strong

The normality test was performed using the Shapiro–Wilk method, as the sample size was less than 50. A significance level of $p < 0.05$ was used to determine the statistical significance of the data distribution. The Shapiro–Wilk normality test results show that all treatment groups have significance values ($p > 0.05$), namely kombucha 10% sugar ($p = 0.454$), kombucha 20% sugar ($p = 0.964$), kombucha 30% sugar ($p = 0.502$), and the positive control, meropenem ($p = 0.625$). Thus, the data in each group are normally distributed and meet the assumptions for proceeding to the parametric ANOVA test.

The homogeneity test was performed using the F-test for homogeneity of variance in the one-way ANOVA analysis. Data is declared homogeneous if its significance value is greater than 0.05. The homogeneity test results using Levene's Test show a significance value of 0.366 ($p > 0.05$). This means the data have homogeneous variance between treatment groups. Thus, the assumption of homogeneity is met, so the analysis can proceed using the one-way ANOVA test.

The results of the One-Way ANOVA test show a significant F-value of 179.398, indicating a significant ratio of variations, with a significance level of $p < 0.000$ ($p < 0.05$). This indicates a substantial difference in the average diameter of the inhibition zone between the treatment groups (Table 4).

Table 4. One-Way ANOVA test for inhibition zone diameter analysis of various treatments against extended-spectrum beta-lactamase *E. coli*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	174.096	3	58.032	179.398	0.000
Within Groups	3.882	12	0.323		
Total	177.978	15			

After the ANOVA test revealed a significant difference, the analysis continued with the Post Hoc Tukey HSD test to determine which treatment groups differed significantly in the diameter of the inhibition zone against ESBL-*E. coli*. Overall, the higher sugar concentration, the greater antibacterial inhibitory power of butterfly pea kombucha, with 30% w/v sugar concentration being the most effective treatment against ESBL *E. coli*. The control antibiotic meropenem had the same inhibitory power as 10% sugar (w/v) butterfly pea flower kombucha but was weaker than 20% and 30% sugar butterfly pea flower kombucha (Table 5).

Table 5. Tukey HSD post-hoc test for inhibition zone diameter analysis of various treatments against extended-spectrum beta-lactamase *E. coli*

Treatment Groups		Sig.	Note
Kombucha 10%	Kombucha 20%	0.000	Significant
	Kombucha 30%	0.000	Significant
Kombucha 20%	Kombucha 30%	0.000	Significant
Kombucha 10%	Meropenem	0.894	No Significant
Kombucha 20%	Meropenem	0.000	Significant
Kombucha 30%	Meropenem	0.000	Significant

DISCUSSION

The research results indicate that butterfly pea flower kombucha contains various bioactive compounds, including flavonoids, alkaloids, saponins, and tannins, each of which is known to have distinct yet complementary antibacterial mechanisms. The presence of these bioactive compounds explains the ability of butterfly pea flower kombucha to inhibit the growth of ESBL-*E. coli*. Flavonoids, for example, can damage bacterial cell membranes and form complexes with extracellular proteins, leading to the release of intracellular compounds and disruption of membrane stability. Alkaloids work by inhibiting peptidoglycan synthesis in bacterial cell walls and blocking topoisomerase enzymes, which are essential for bacterial DNA replication, thereby causing the bacteria to lose their ability to survive. Saponin has surface-active properties that decrease the surface tension of bacterial cell walls, leading to membrane leakage and ultimately, cell death (Muliasari, 2020). Meanwhile, tannins bind to proteins, forming stable complexes that can damage cell wall structures and inhibit essential bacterial enzymes. Thus, the combination of these bioactive compounds yields a synergistic effect, resulting in strong antibacterial activity in butterfly pea kombucha.

This research is consistent with the study by Rezaldi et al. (2021), which showed that butterfly pea kombucha has antimicrobial activity against various pathogens. An increase in sugar concentration during fermentation has been shown to enhance the antibacterial activity of kombucha, as demonstrated in this study, in which kombucha with a 30% w/v sugar concentration exhibited a significantly larger inhibition zone than kombucha at lower sugar concentrations. This can be explained by the increased metabolism of symbiotic microorganisms in SCOBY when sugar availability is higher. Microorganisms, such as yeast and acetic acid bacteria, utilize sugar as the primary substrate to produce various secondary metabolites, including organic acids, phenols, converted flavonoids, and short-chain alcohols (Villarreal-Soto et al., 2018). These compounds directly contribute to the formation of the antibacterial inhibition zone.

Additionally, the increase in total phenol content at higher sugar concentrations aligns with the findings of Agustiansyah et al. (2022), who reported that increasing the concentration of fermentation substrates, such as honey, can increase kombucha's phenol content. In this study, a 30% w/v sugar concentration yielded the highest phenol content of 1253.87 ± 121.72 mg GAE/L. Phenols play a crucial role in antibacterial mechanisms by denaturing membrane proteins, disrupting permeability, and damaging bacterial cytoplasmic structures (Gyawali & Ibrahim, 2014). This indicates that the antibacterial activity of butterfly pea kombucha is not solely determined by the presence of flavonoids and alkaloids but is also influenced by the phenol content, which increases during fermentation.

The increase in the diameter of the inhibition zone at 20% and 30% w/v sugar concentrations, which exceeded that of the meropenem positive control, is an important finding in this study. Meropenem is a carbapenem antibiotic that is effective against various Gram-negative bacteria. However, in ESBL-producing bacteria such as ESBL-producing *E. coli*, the beta-lactamase enzyme can hydrolyze the beta-lactam ring of antibiotics, thereby reducing their effectiveness (Abdelbary et al., 2026; Benjamin et al., 2011). This condition resulted in the meropenem inhibition zone measuring only 18.53 mm, which was still below the antibacterial activity observed in butterfly pea flower kombucha at 20% and 30% w/v sugar concentrations.

The inhibition zone of meropenem against ESBL-producing *E. coli* only reached 18.53 mm, which based on the Clinical and Laboratory Standards Institute (CLSI) breakpoint is classified as resistant ($R \leq 19$ mm). Interestingly, butterfly pea kombucha extract at sugar concentrations of 20% and 30% w/v produced inhibition zones larger than meropenem, suggesting that bioactive compounds in kombucha have a different mechanism of action and these findings indicate the potential of kombucha as a source of natural antibacterial agents effective against clinical isolates resistant to last-line antibiotics.

Another study by Pertiwi et al. (2022) also supports this study's findings, particularly regarding the effect of sugar concentration on antibacterial activity. They found that fermenting butterfly pea flower kombucha at high sugar concentrations (40%) can produce a larger inhibition zone against several Gram-positive and Gram-negative bacteria. The differences in inhibition zone results between

this study and the previous study are most likely due to variations in the test bacteria, fermentation conditions, and sugar concentrations used. However, overall, the same biological trend is observed: increased sugar levels lead to more optimal fermentation activity and higher production of antibacterial metabolites.

In addition, research by Sintyadewi et al. (2023) on torch ginger flower kombucha showed that fermentation time significantly increases the production of bioactive compounds and antibacterial activity. Torch ginger flower kombucha fermented for 9 days produced a larger inhibition zone than kombucha fermented for shorter periods. This finding aligns with the research that fermentation parameters, such as fermentation time and sugar concentration, play a crucial role in determining the antibacterial effectiveness of kombucha (Sintyadewi et al., 2023).

Research highlighting the antioxidant activity of butterfly pea flowers, as reported by Astuti et al. (2025), who found an IC_{50} of 27.80 ppm, further supports the potential of butterfly pea kombucha as an antibacterial agent. Antioxidant compounds, such as flavonoids and anthocyanins, not only neutralize free radicals but also disrupt bacterial membrane structures and inhibit metabolic enzymes. Thus, butterfly pea kombucha offers two biological benefits simultaneously: antibacterial activity and powerful antioxidant activity.

Overall, this study's results suggest that increasing sugar concentration enhances the antibacterial activity of butterfly pea kombucha against ESBL *E. coli*. Butterfly pea flower kombucha with a 30% w/v sugar concentration proved to be the most effective treatment, with a very strong inhibition zone and the highest total phenol content. This indicates that the production of secondary metabolites during fermentation is significantly influenced by the availability of sugar as an energy source. The combination of bioactive compounds, including flavonoids, alkaloids, saponins, tannins, phenols, and organic acids, exerts a synergistic effect, making butterfly pea kombucha a potential natural antibacterial agent for combating resistant bacteria, such as ESBL-producing *E. coli*.

CONCLUSION

In conclusion, this study provides foundational evidence that sugar concentration significantly modulates the phytochemical profile and anti-ESBL activity of butterfly pea flower kombucha extracts. While the 30% sugar extract shows promise as a natural antibacterial agent, further investigation through *in vivo* models, active compound identification, and formulation studies is essential to translate these findings into practical therapeutic applications.

CONFLICT OF INTEREST

There is no conflict of interest in this research.

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