



**ANTIBACTERIAL ACTIVITY OF FERMENTED SINGLE BULB GARLIC (*Allium sativum* L.) ETHANOL EXTRACT AGAINST ESBL-PRODUCING *Escherichia coli***

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**Abstract**

*Extended-spectrum beta-lactamase (ESBL)–producing Escherichia coli is a major cause of multidrug-resistant infections, posing significant challenges to antibiotic therapy. The search for natural antibacterial alternatives has led to growing interest in fermented single bulb Allium sativum (FSBAS), a thermally processed form of garlic with enhanced bioactive properties. This study aimed to evaluate the antibacterial activity of ethanol extracts of FSBAS against ESBL-producing E. coli isolates. FSBAS was produced by fermentation at 70°C for 35 days. FSBAS was extracted using maceration with 96% ethanol. Antibacterial activity was assessed using the disc diffusion method. This experiment consisted of four treatment groups i.e. extract concentrations of 25%, 50%, 75%, and 100%; and two control groups, namely the negative control (1% DMSO) and the positive control (amoxicillin–clavulanate, 30 µg/disc). The ethanol extract of FSBAS showed measurable antibacterial activity against ESBL-producing E. coli. The diameter of the inhibition zone increased proportionally with the extract concentration, with the strongest effect observed at 100%. Statistical analysis confirmed treatment groups with concentrations of 50%, 75%, and 100% showing no significant differences with positive control ( $p > 0.05$ ). The FSBAS extract shows promising antibacterial potential against multidrug-resistant E. coli.*

**Keywords:** fermented single bulb Allium sativum, antibacterial activity, ESBL-producing E. coli.

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## INTRODUCTION

*Escherichia coli* is a normal component of the intestinal flora, yet it is also a Gram-negative pathogen in human infections. *E. coli* commonly causes infections in several anatomical sites, including the urinary tract, bloodstream, and respiratory tract (Wang et al., 2023; Endraswari et al., 2022). A global data reported the incidence of *E. coli* infection in high-income countries estimated at 48 per 100,000 person-years with a case-fatality rate (CFR) of 12.4% (Doua et al., 2023). The widespread presence of *E. coli* in the environment, coupled with its ability to adapt rapidly, makes it a significant microorganism of medical and public health importance.

Currently, antimicrobial resistance is a significant global threat to humanity. The rise of multidrug-resistant (MDR) bacteria, including *E. coli*, has been observed in various countries. Extended-spectrum  $\beta$ -lactamases (ESBLs)-producing and carbapenem-resistant (CR) *E. coli* can impact morbidity and mortality due to the limitations of available therapies (Endraswari et al., 2022; Ludden et al., 2021; Wang et al., 2023). Data reported *E. coli* infection in hospital, 46.80% were ESBLs-producing and 20.57% were Carbapenem Resistance *E. coli* (Wu et al., 2021). This phenomenon is an urge to explore alternative antimicrobial strategies derived from natural sources as potential solutions to combat bacterial resistance.

One potential herbal source exhibiting antibacterial activity against various bacterial species is *Allium sativum* L. The compound allicin contained within it contributes to the inhibition of Gram-negative bacterial growth, particularly when the *Allium sativum* L. is subjected to fermentation become black garlic (Mousa et al., 2025). Fermented *Allium sativum* L are known to have more active compounds than non-fermented *Allium sativum* L. (Bhatwalkar et al., 2021; Sudana et al., 2025). Data revealed that fermented *Allium sativum* L exhibited significantly greater inhibition of bacterial growth than non-fermented (Bhatwalkar et al., 2021). Fermented *Allium sativum* L processed from fresh *Allium sativum* L at different temperatures, relative humidity, and different periods that differed in their physical properties and chemical composition. According to data, phenolic acid contents in fermented *Allium sativum* L increased over five-fold compared to non-fermented and are a significant source of phenolic acids (coumaric, ferulic, and caffeic), flavonols (myricetin, resveratrol, morin, and quercetin), and flavanols (epicatechin, catechin, and epicatechin gallate) (Paulauskienė et al., 2024).

Fermented single bulb *Allium sativum* (FSBAS) demonstrated strong quorum quenching activity in *Chromobacterium violaceum*, strong inhibition to *Staphylococcus epidermidis*, Uropathogenic *E. coli* (UPEC), and *Acinetobacter baumannii*, also medium inhibition to *Proteus mirabilis* (Anandhita et al., 2025; Dani et al., 2025; Hutahaeon et al., 2024; Saputra et al., 2023; Sudana et al., 2025).

Given the increasing threat of multidrug-resistant *E. coli*, exploring the antibacterial potential of FSBAS extract represents an important step toward discovering safe, effective, and sustainable therapeutic alternatives. Accordingly, this study aims to assess the inhibitory activity of the ethanol extract of FSBAS against ESBL-producing *E. coli* isolates. To date, no research has specifically evaluated this extract against ESBL-producing *E. coli* derived from clinical samples in Indonesia, highlighting the novelty and significance of the present investigation.

## METHOD

### Research Ethical Approval and Sample Collection

The research was carried out at the Microscopic Laboratory, Faculty of Medicine, Universitas Tanjungpura, from July to December 2024. Ethical approval for this research was granted by the Ethics Committee of the Faculty of Medicine, Universitas Tanjungpura, as stated in the Ethical Clearance Letter No. 11751/UN22.9/PG/2024, dated August 22, 2024., with considerations regarding the use of Stored Biological Materials (BBT) as research subjects. The clinical isolates of *E. coli* used in this study were obtained from the Department of Microbiology, Universitas Tanjungpura.

### **Preparation and Extraction Procedure of FSBAS**

The FSBAS was produced from fresh *Allium sativum* by controlled heating at 70°C with 80–90% humidity for 35 days. The ethanol extract was prepared using a maceration method. Finely crushed FSBAS was soaked in 96% ethanol with a 1:4 ratio (w/v) for three days at room temperature. The filtrate was collected through Whatman No.1 filter paper and concentrated using a rotary evaporator at 40°C. The thick extract was stored in sealed dark glass bottles at 4°C until use (Ahmed & Wang, 2021).

### **Confirmation of Tested Bacterial Isolate**

Bacterial identification was made based on colony morphology on Eosin Methylene Blue (EMB) agar, and the Gram stain procedure. The species confirmation using the standardized biochemical substrate stripe API 20E (bioMérieux). Confirmation of ESBL production was achieved using the combination disk of Amoxicillin-Clavulanic acid in conjunction with cefotaxime and ceftazidime (Mardhia et al., 2025).

### **Preparation of Bacterial Suspension & Antibacterial Activity**

One loop of test bacteria from 24-hour rejuvenation was taken and aseptically suspended in a sterile test tube containing 5 ml of 0.9% NaCl solution. The turbidity was then measured by comparing the tube containing the bacterial suspension to the 0.5 McFarland standard (CLSI, 2025). Antibacterial activity testing was carried out using the disc diffusion method (Kirby-Bauer). The suspension of the ESBLs producing *E. coli* test bacteria was taken by dipping a sterile cotton swab into the test bacterial suspension and inoculating using the swab method on Mueller-Hinton Agar (Merck, USA) media. A sterile disk of paper was soaked in the ethanol extract of FSBAS in every concentration of 25%, 50%, 75%, and 100%, and placed onto the inoculated MHA surface, along with positive controls using an antibiotic disk of amoxicillin-clavulanate (AMC, 30 µg, Oxoid) and negative controls (1% DMSO). Each treatment in the experiment was performed in four replicates. The media inoculated with test bacteria and the test extract discs were incubated at 37°C for 18 hours. Measurements were made on the inhibition zone formed around the discs to determine the antibacterial activity and properties of FSBAS extract using clinical caliper (mm). The level the antibacterial activity of the extract is based on the level of the diameter inhibition zone, categorized into weak (below 5 mm), intermediate (6-10 mm), and strong (over 11 mm) (Yunus et al., 2021).

### **Statistical test**

The statistical test used was Kruskal-Wallis and continued with post-Hoc pairwise comparison to determine significant differences between data from one treatment group of FSBAS extract and other concentrations. The test was performed using SPSS 29.0 version.

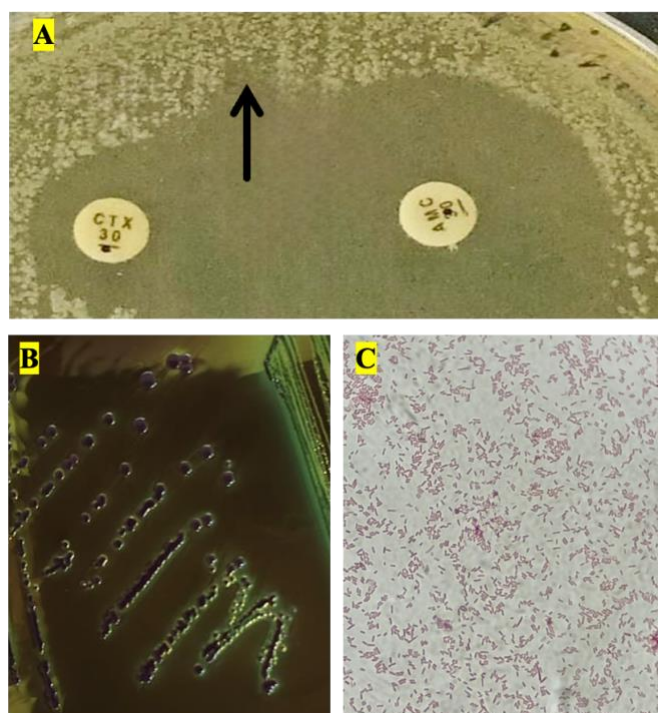
## **RESULT AND DISCUSSION**

### **Preparation and Extraction of FSBAS**

The FSBAS preparation was conducted in the Non-Microscopic Laboratory, Faculty of Medicine, Tanjungpura University. A total of 4000 g of single bulb garlic obtained from Fatuneno Village, East Nusa Tenggara, was processed through thermal fermentation in an oven at 70°C for 35 days. This process produces FSBAS with a distinctive dark color, dense texture, and a distinctive sweet aroma. FSBAS extraction was carried out using a maceration method with 96% ethanol solvent. After being crushed, cleaned, and ground, a homogenized FSBAS paste of 1735 g was obtained. The maceration process was carried out for three days, followed by concentration of the filtrate, resulting in 202 g of thick ethanol extract of FSBAS.

### Pathogenic Bacteria Confirmation

The bacterial isolate used in this study was a clinical isolate of ESBLs-producing *E. coli* obtained from the Microbiology Laboratory, Faculty of Medicine, Tanjungpura University. The ESBLs confirmation through double disc synergy test that showed the increasing of inhibition zone of cefotaxime that combined with Amoxicillin-Clavulanic acid (Figure 1A). The isolate was reconfirmed macroscopically and microscopically before use. On eosin methylene blue (EMB) agar, the bacterial colonies exhibited a metallic green sheen with a round morphology and smooth colony edges (Figure 1B), characteristic of *E. coli*. Microscopic observation using Gram staining revealed rod-shaped, red-stained cells (Figure 1C), indicating that the isolate was Gram-negative. This finding verified that the tested bacteria were indeed *E. coli*, ensuring the validity of subsequent antibacterial tests.

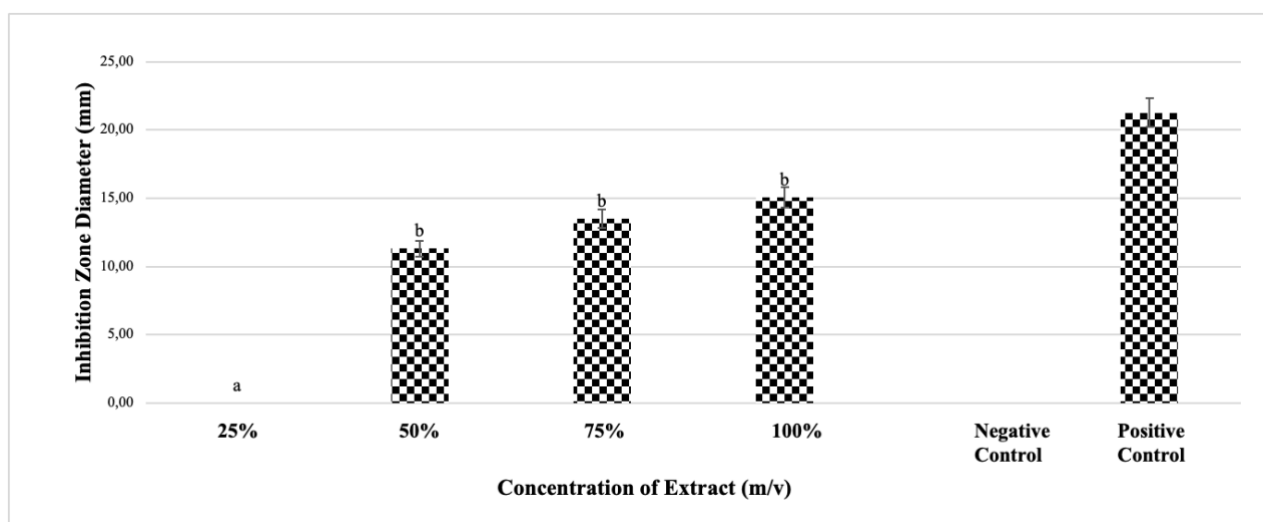


**Figure 1.** A. an increasing of inhibition zone diameter between Amoxicillin-Clavulanic acid (AMC) and cefotaxime (CTX) B. *E. coli* bacterial colony on EMB media showing a metallic green sheen with a round morphology and smooth colony edges. C. Gram staining of *E. coli* bacteria is classified as a Gram-negative bacterium with rod-shaped cells and examined at 1000x magnification under an oil immersion microscope.

### Antibacterial Activity of FSBAS

The antibacterial activity of the FSBAS ethanol extract against ESBL-producing *E. coli* was evaluated using the disc diffusion method. The diameter of the inhibition zone around the paper disc indicated the growth inhibition capacity of the test bacteria. These results provide initial evidence that the FSBAS extract possesses antibacterial properties capable of inhibiting the growth of *E. coli*. These results provide initial evidence that the FSBAS extract possesses antibacterial properties capable of inhibiting the growth of *E. coli*. The average measurement results of the inhibition zones of ESBL-producing *E. coli* against FSBAS at concentrations of 25%, 50%, 75%, 100%, the negative control (1% DMSO), and the positive control (amoxicillin-clavulanate) were 0 mm, 11.74 mm  $\pm$  SD 0,11, 14.67 mm  $\pm$  SD 0,21, 15.4 mm  $\pm$  SD 0,33, 0 mm, and 21.84 mm  $\pm$  SD 0,45, respectively. The Shapiro-Wilk normality test indicated that the data were not normally distributed ( $p < 0.05$ ), and the Levene's test for homogeneity of variance also indicated non-homogeneity ( $p < 0.05$ ). Therefore, parametric

testing was not met. Transformations using the square root and logarithmic methods were applied; however, the data remained non-normally distributed ( $p < 0.05$ ). Based on these results, the non-parametric Kruskal–Wallis test was applied. The analysis revealed a significant difference between the treatment groups ( $p = 0.001$ ), indicating that the antibacterial effects of the tested concentrations differed significantly. Post-hoc analysis confirmed which specific treatment groups exhibited significant differences in inhibitory activity. Overall, the findings indicate that the FSBAS ethanol extract exerts a measurable antibacterial effect against clinical isolates of ESBLs-producing *E. coli*. The findings demonstrated that the inhibitory effect increased proportionally with the concentration of the extract, with the highest activity observed at 100%. Moreover, the study revealed that the inhibitory capacities of the 50%, 75%, and 100% extract concentrations were not significantly different with positive control (Figure 2).



**Figure 2.** Bar chart of the antibacterial activity of FSBAS against ESBL-producing *E. coli*, where (a) showed a significant difference compared with the positive control, and (b) showed no significant difference compared with the positive control.

The ethanol extract of FSBAS demonstrated antibacterial activity against ESBLs producing *E. coli*, a clinical isolate with known multidrug resistance. The inhibition zones observed confirm that the extract contains bioactive compounds capable of suppressing resistant bacterial growth, consistent with previous findings on FSBAS antimicrobial potential. The process of fermentation *Allium sativum* L. become FSBAS through heating at 70°C for 35 days initiates the Maillard reaction, producing Amadori compounds, aldehydes, hydroxymethylfurfural (HMF), and melanoidins. These compounds contribute to the dark coloration, mild aroma, and enhanced biological activity (Liu et al., 2022; Tamanna & Mahmood, 2015). The process reduces volatile allicin but increases the stability and concentration of secondary metabolites such as phenolics, flavonoids, saponins, alkaloids, terpenoids, tannins, and organosulfur compounds (Agustina et al., 2020; Azizah et al., 2020; Choi et al., 2014; Fadhilah Azhar et al., n.d.; Zhang et al., 2016).

The antibacterial effect observed likely results from the synergistic interaction of these metabolites (Sabila et al., 2019). Phenolic compounds disrupt bacterial membranes, disrupt nucleic acid synthesis, inhibit virulence factors such as enzymes and toxins, and inhibit bacterial biofilm formation (Miklasińska-Majdanik et al., 2018; Takó et al., 2020). Flavonoids inhibit bacterial growth by inhibiting nucleic acid synthesis, cell membrane function, biofilm formation, and energy metabolism (Shamsudin et al., 2022; Yuan et al., 2021). Saponin compounds can damage cell membranes, inhibit protein synthesis, and disrupt bacterial cell metabolism (Alina et al., 2023; M. I. Khan et al., 2018). Alkaloids inhibit bacterial growth by inhibiting nucleic acid synthesis, respiration, bacterial enzyme activity, cell membrane function, and bacterial virulence genes (Yan et al., 2021).

The antibacterial effect of tannins is due to their ability to pass through the bacterial cell wall to the internal membrane, disrupting cell metabolism, and consequently destroying bacterial cells (Kaczmarek, 2020; Mickymaray, 2019). Terpenoids damage porins in the outer membrane of the bacterial cell wall, thereby reducing the permeability of the bacterial cell wall and reducing nutrient absorption and growth (Guimarães et al., 2019; Masyita et al., 2022). In addition, the elevated level of S-allyl cysteine (SAC) that reported to be 4–8 times higher than in fresh garlic (Kimura et al., 2017), enhances the activity of other phytochemicals by improving cell penetration and oxidative stability. Organosulfur compounds such as SAC and diallyl disulfide (DADS) also play key roles by interfering with bacterial enzyme systems and compromising cell wall integrity. Previous studies by Harun et al. reported similar inhibitory effects of black garlic extracts against *Escherichia coli* (Harun et al., 2021). The present results support these findings, showing that inhibition increased proportionally with extract concentration (Khan et al., 2019), with the strongest activity observed at 100%. Furthermore, this study also showed that the ability of the 50%, 75%, and 100% extracts did not differ significantly after statistical analysis, indicating that the 50% concentration already had strong activity that was not significantly different from the 75% and 100% concentrations, also positive control i.e Amoxicillin-Clavulanic acid in inhibiting ESBLs-producing *E. coli*.

The ability of FSBAS extracts to inhibit ESBL-producing *E. coli* suggests potential as an alternative or complementary antibacterial agent, particularly against multidrug-resistant pathogens. The extract's mechanism is likely multifactorial, involving disruption of membrane integrity, inhibition of nucleic acid and cell wall synthesis, and interference with bacterial metabolism (Uzun et al., 2019). Additionally, the main compound in FSBAS, organosulfur compounds (SACs), can help modulate the entry of other compounds and enhance their mechanism of action (Magryś et al., 2021). In summary, the enhanced antibacterial activity of FSBAS extract is attributed to the elevated and synergistic action of its secondary metabolites and organosulfur compounds formed during thermal processing. These findings reinforce the potential of FSBAS as a natural source of antibacterial agents capable of combating resistant clinical isolates.

## CONCLUSION

The ethanol extract of FSBAS showed potent antibacterial effects against ESBL-producing *E. coli*, with inhibition increasing with increasing concentration. Antibacterial activity at concentrations of 50%, 75%, and 100%, as well as the positive control, was not statistically significantly different. Overall, the FSBAS extract shows strong potential as a natural option to help combat multidrug-resistant *E. coli*. Further studies involving purification of active compounds and in vivo antibacterial testing are recommended to validate the clinical applicability of FSBAS.

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