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The Effect of Honeybee Propolis on *Escherichia coli* Biofilm Formation *in vitro*

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ABSTRACT

Background: Colibacillosis is an infectious disease that affects livestock such as cattle, pigs, goats, sheep, horses and poultry caused by the bacterium *Escherichia coli*. *E. coli* can form biofilms when environmental conditions are unfavorable. The purpose of this research is to determine the effect of honeybee propolis on the formation of *E. coli* biofilm *in vitro*.

Methods: The research began with the dilution of propolis using the microdilution technique and determination of concentration values with a modified minimum inhibitory concentration (MIC). Biofilm inhibition assays were conducted in microplates with five propolis concentration treatments: 0.39 mg/mL, 0.78 mg/mL, 1.56 mg/mL, 3.125 mg/mL and 6.25 mg/mL, along with negative and positive controls, with eight replications. Subsequently, crystal violet quantification and absorbance value readings were done using an ELISA reader with a wavelength of 595 nm.

Result: The results showed that between concentrations of 3.125 mg/mL, 6.25 mg/mL and the negative control, there was no significant difference with mean values of 0.09963, 0.078857 and 0.079375, respectively. Honeybee propolis extract has the potential to act as an antibiofilm against *E. coli in vitro*, with the minimum concentrations capable of inhibiting biofilm formation being 3.125 mg/mL and 6.25 mg/mL.

Key words: Biofilm, *E. coli*, Honeybee propolis, Infectious disease.

INTRODUCTION

Colibacillosis is an infectious disease that affects livestock such as cattle, pigs, sheep, horses, goats and poultry caused by the bacterium *Escherichia coli* (Singh *et al*., 2023). *E. coli* is a gram-negative bacterium belonging to the order Enterobacterales (Sora *et al*., 2021). *E. coli* can cause infectious diseases in livestock such as cattle, pigs, goats, sheep, horses and poultry which is called colibacillosis. Colibacillosis in pigs causes diarrhea, dehydration, acidosis and even death (Castro *et al*., 2022; Li *et al*., 2021). Colibacillosis infection in chickens and poultry can be divided into two types, localized and systemic infections (Thakre *et al*., 2017). In poultry, the infection is characterized by coliform cellulitis, hemorrhagic septicemia, swollen head syndrome, colisepticemia, omphalitis, peritonitis, salpingitis, coligranuloma, orchitis, osteomyelitis or synovitis, panophthalmitis and enteritis (Kabir, 2010). In horses, *E. coli* is the main cause of urinary tract infections, reproductive organ infections, respiratory diseases and other tissue infections (Nielsen *et al*., 2022).

E. coli is a rod-shaped bacterium with virulence genes fim, krl, csg and agn43, allowing it to form biofilms during its growth phase. Biofilm formation occurs when environmental conditions are unfavorable (Basavaraju and Gunashree, 2021; Zhou *et al*., 2022; Rabins *et al*., 2018). A biofilm is a collection of bacterial cells that adhere to surfaces and bind with secreted exopolymer matrix (Zhao *et al*., 2023). According to Zhou *et al*. (2022), five steps are typically involved in the creation of *E. coli* biofilms: colony formation, biofilm maturation, dispersion, reversible

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adhesion and irreversible adhesion. Biofilm formation in *E. coli* makes these bacteria more resistant to antibiotics than when they are not in a biofilm state.

As antibiotics lose their ability to effectively stop bacterial development, microorganisms develop the ability to withstand the effects of antimicrobial drugs (Reygaert, 2018; Chakraborty *et al*., 2020). According to Wang *et al*. (2023), *E. coli* has been reported to exhibit resistance to 17 types of antibiotics. Antimicrobial agents diffusing through a limited biofilm matrix, communication between the biofilm matrix (polymers and cells) and the antimicrobial agents, enzyme-mediated resistance, levels of metabolic activity within the biofilm, genetic adaptation, efflux pumps and outer membrane structure are the causes of antibiotic resistance in *E. coli* (Singh *et al*., 2017).

The use of antibiotics for the treatment of colibacillosis has been widely practiced. Indonesia is one of the countries with the highest antibiotic usage, with a percentage of 202% (Dewi *et al*., 2023). Due to indiscriminate antibiotic use, various antibiotic resistance issues arise, rendering the treatment of colibacillosis ineffective. This issue needs to be addressed through research on how to inhibit biofilm formation and development with the emergence of antibiofilm activity. However, research on testing honeybee propolis extract as an antibiofilm against *E. coli* has not been conducted. Honeybee propolis is a safe natural and readily available substance. Honeybee propolis extract is known to inhibit biofilm formation in Staphylococcus bacteria. This occurs because honeybee propolis contains flavonoid, saponin, tannin, alkaloid, terpenoid, phenol and glycoside that can act as antibiofilm agents (Almuhayawi, 2020; Aziz *et al*., 2021).

MATERIALS AND METHODS

Study area and sample collection

The research was conducted over a period of July 2023 to November 2023 at the Bacteriology and Mycology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga. The reading of OD value was conducted in the Molecular Biology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga. The materials used in this research include aluminium foil, the ATCC 25922 *E. coli* culture from the Bacteriology and Mycology Laboratory of the Faculty of Veterinary Medicine of Universitas Airlangga, Noble Broth (NB) MERCK media, MacConkey Agar (MCA) media, honeybee propolis from Melia, Phosphate Buffer Saline Solution (PBS), Paraformaldehyde (PFA), Crystal Violet 0.1% and Acetic Acid.

Preparation of noble broth

First, Noble broth media for the cultivation of *E. coli* bacteria and the dilution of honeybee propolis extract was created. The medium was dissolved in 100 mL of distilled water to make 0.8 grams of Noble broth. Second, the dissolved medium in distilled water was slowly stirred around over a flame on the stove. Third, the medium was then transferred into wide-mouth laboratory bottles type I Borosilicate 100 mL. Forth, the bottles were sealed and covered with aluminum foil. Fifth, the medium was autoclaved for 15 minutes at 121°C and 1-2 atm of pressure to sterlize it.

Preparation and microdilution of honeybee propolis

The honeybee propolis used in this research is a product of Melia Propolis purchased from a pharmacy in Surabaya. The propolis underwent microdilution in 7 microtubes that had been labeled 1 to 7. Microtube 1 was filled with 1000 µl of honeybee propolis, while microtubes 2 to 7 were initially filled with 500 µl of culture. Dilution was performed by transferring 500 µl of honeybee propolis from microtube 1 to microtube 2. This process was sequentially repeated for the solutions in microtubes 2 through 7 to achieve appropriate dilution results.

The concentration value of the result of the dilution was determined through the modification and the application of the Minimal Inhibitory Concentration (MIC), recommended by the NCCLS, while maintaining sterile conditions throughout the experiment (Clinical and Laboratory Standards Institute, 2015). The lowest concentration of an antibacterial drug expressed in mg/L (µg/mL) is known as the MIC. The concentration values of propolis were determined based on the previous research, namely 0.39 mg/mL, 0.78 mg/mL, 1.56 mg/mL, 3.125 mg/mL and 6.25 mg/mL (Kaligis and Mokosuli, 2022).

Preparation of *E. coli* inoculum

Inoculation of *E. coli* bacteria into Noble broth media was performed by retrieving bacteria from the bacterial culture on MacConkey agar using a needle, then transferring it into the Noble Broth media. The mixture was then incubated for one day at a temperature of 37°C. Successful bacterial inoculation was indicated by a change in the media color to cloudy yellow.

Biofilm inhibition assay

This testing included positive and negative control, which were prepared in planktonic form. The testing was conducted in triplicate as described by Kayanadath *et al.* (2019) with modifications, comprising positive control, negative control and treatment groups. The positive control contained 100 µl of NB media and 100 µl of *E. coli* bacteria, the negative control contained 200 µl of NB media and the treatment group contained 100 µl of *E. coli* bacteria and 100 µl of various concentrations of propolis (0.39 mg/mL, 0.78 mg/mL, 1.56 mg/mL, 3.125 mg/mL and 6.25 mg/mL). Wells in the positive control and treatment group were inoculated with *E. coli* bacteria. Then, wells in the treatment group were provided with honey propolis according to the concentration of each treatment. Wells filled with bacteria and honey propolis were subsequently shaken using an orbital shaker. The microtiter plate was then incubated for 3 days at a temperature of 30°C.

Biofilm quatification of crystal violet assay

After the incubation period, planktonic cells were removed by carefully aspirating the supernatant. Biofilm fixation was done at room temperature with 150 µl per well of 4% paraformaldehyde (PFA) diluted in phosphate buffer saline (PBS) for 30 minutes. Subsequently, the PFA was removed and the wells were rinsed twice with 200 µl per well of PBS. Evaluation of biofilm formation required staining with 175 µl per well of 0.1% crystal violet pipetted into the wells and incubated for 10 minutes (O'Toole, 2011). Unbound staining was removed and the wells were washed with 200 µl per well of PBS. The microtiter plate was air-dried at room temperature for approximately 3 hours. Remaining color residues in the wells were dissolved by adding 200 µl per well of 30% acetic acid, then 100 µl of the solution was transferred to a new microtiter plate for analysis.

Reading of optical density values using an ELISA reader

The microtiter plate was subjected to optical density (OD) readings at a wavelength of 595 nm using a microplate spectrophotometer or ELISA Reader (Wahyudi and Wael, 2021).

Data analysis

To identify significant differences among mean pair, the collected data were subjected to analysis using the Post Hoc Dunn Test.

RESULTS AND DISCUSSION

This research employs honeybee propolis extract as an antibiofilm agent against *E. coli in vitro*. The decrease in absorbance at 595 nm in the presence of the inhibitor confirms the inhibitory effect of honeybee propolis. It has been proven that biofilm formation by some bacteria is a crucial defense strategy against xenobiotics. There are three antibiotic resistance mechanisms associated with biofilm-forming bacterial strains: the biofilm matrix reduces antibiotic penetration, the chemical composition of the biofilm protects bacterial cells and biofilm-forming bacteria can grow and form spores (Zhao *et al*., 2023). The bacterium *E. coli* was treated with concentrations of honeybee propolis extract and incubated for 3 days. Then, the wells were washed to remove planktonic cells and residue. Propolis is a compound known to exhibit antibiofilm activity against Gram-negative bacteria. This is because propolis contains high levels of flavonoids. This study focuses on *E. coli*, which is a pathogen capable of expressing various virulence factors. Honeybee propolis extract was able to prevent biofilm formation, as indicated by the low optical density values compared to the control group.

The average OD values, which vary across each research group, are presented in the following Table 1 and Fig 1. The results presented in Table 1 indicate that the OD values and the average OD values for each treatment and control group. The treatments with concentrations of 0.39 mg/mL, 0.78 mg/mL, 1.56 mg/mL, 3.125 mg/mL and 6.25 mg/mL yielded average values of 0.1715, 0.142375, 0.115,

Table 1: The mean of optical density values

Group	Mean (OD±SD)
Negative control	0.07938±0.012939[a]
Positive control	0.20513±0.038391[e]
0.39 mg/mL	0.17150±0.009243[d]
0.78 mg/mL	0.14238±0.009054[c]
1.56 mg/mL	0.11500±0.011314[bc]
3.125 mg/mL	0.09963±0.047901[ab]
6.25 mg/mL	
0.078857±0.036608[a]	

Note: Different notations in the same column indicate significant differences ($p<0.05$)

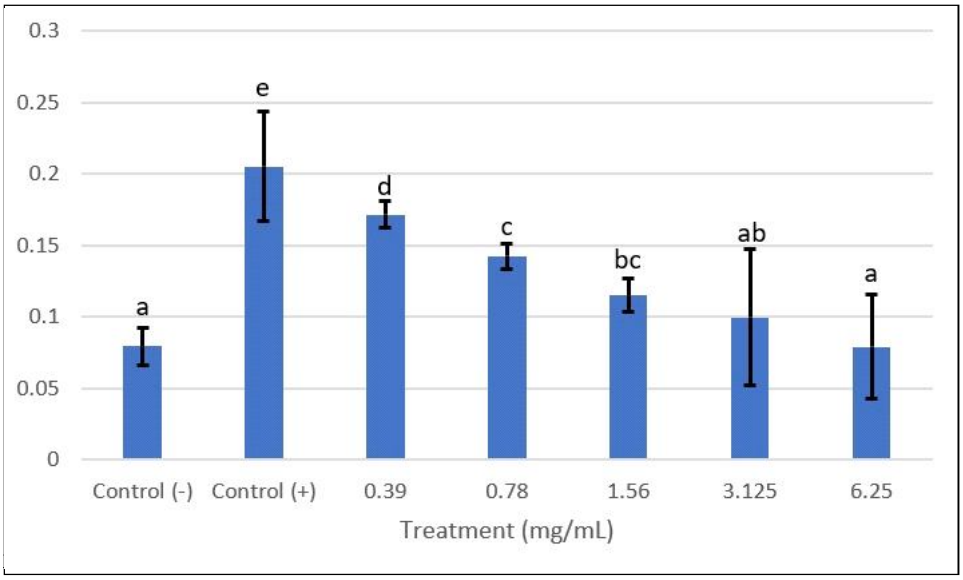


Fig 1: Bar graph of OD mean values.

0.099625 and 0.078857, respectively. The positive control and negative control exhibited average values of 0.205125 and 0.079375, respectively.

Based on the data in Table 1, it is evident that the growth media utilized in this study lacks antibiofilm activity. This is indicated by the absence of a decrease in optical density values. While, in the group treated with honeybee propolis, antibiofilm activity is observed as demonstrated by the decreasing optical density values compared to the control group.The data were then analyzed using post hoc dunn test. The results of the post hoc dunn test in Table 1 indicate that there were no significant differences between the negative control, the concentration of 3.125 mg/mL and the concentration of 6.25 mg/mL ($p>0.05$). The concentrations of 1.56 mg/mL and 3.125 mg/mL in the treatment group did not change significantly ($p>0.05$). There were no discernible variations ($p>0.05$) between the concentrations of 1.56 mg/mL and 0.78 mg/mL. The concentration of 0.39 mg/mL and the positive control showed significant differences compared to all treatments ($p<0.05$).

Honey propolis at concentrations of 3.125 and 6.25 mg/mL which represents the minimum concentrations in the group is capable of inhibiting *E. coli* biofilm formation *in vitro*. This is indicated by OD values that do not significantly differ when compared to the negative control. Based on Fig 1, it can be concluded that the higher the concentration of extract added to the medium, the greater the ability of the antibiofilm from the extract. This aligns with the findings of De Marco *et al*. (2017), which suggest that the higher the concentration of propolis used, the higher the antibiofilm activity. At a concentration of 100 µg/mL, the biofilm mass was lower than at a concentration of 50 µg/mL. Meanwhile, at a concentration of 50 µg/mL, the biofilm mass was lower than at a concentration of 10 µg/mL.

The antibiofilm activity of honeybee propolis extract is caused by its high flavonoid content. Flavonoids are active ingredients with antibiofilm ability. The presence of flavonoid compounds in honeybee propolis extract disrupts the adhesion and polymerization processes of biofilm-associated proteins (ALaerjani *et al*., 2022). Flavonoids also inhibit the icaA and icaD genes responsible for EPS formation. According to Roy *et al*. (2018), the antibiofilm mechanism involves disrupting bacterial adhesion to surfaces, inhibiting quorum sensing and inhibiting EPS growth. The flavonoids contained in honeybee propolis inhibit the formation of DnaK. DnaK is a protein crucial for biofilm formation, as it enhances the expression of CsgA and CsgB. CsgA and CsgB function to aggregate curli fibers, which is one of extracellular matrix components in biofilm formation (Tursi and Tükel, 2018). According to Buchmann *et al*. (2023), By stopping the production of amyloid structures CsgA, CsgB and CsgD, flavonoids can stop *E. coli* and other Gram-negative bacteria from forming biofilms. CsgD is a gene that functions as a connector for adhesion factor production, transportation, metabolism and gene regulation in biofilm formation (Gualdi *et al*., 2007). CsgA and CsgB are genes involved in forming curli fimbriae. Curli fibers play a critical role in the initial stages of biofilm formation, particularly in the attachment phase. Disruption of curli fiber formation hampers bacterial attachment to surfaces. If the formation of CsgA and CsgB is inhibited, biofilm formation in *E. coli* cannot occur.

According to Bhargava *et al*. (2021), honeybee propolis extract is capable of inhibiting the motility of Gram-negative bacteria. This occurs due to the reciprocal relationship between curli and flagella that mutually inhibit each other. When cells transition to a non-motile state to form biofilms, the intracellular c-GMP levels increase and activate CsgD, which functions to activate the expression of curli genes. CsgD suppresses flagellar gene operons, thereby reducing flagella synthesis. Decreased bacterial motility can increase the force of repulsion to the surface, making adhesion unstable. To achieve stable attachment, bacteria must suppress or decrease their motility after contact with the surface.

CONCLUSION

Based on the conducted research, it can be concluded that honeybee propolis extract holds potential as an antibiofilm agent against *E. coli in vitro*. The concentrations of 3.125 mg/mL and 6.25 mg/mL are the minimum concentrations of honeybee propolis extract capable of inhibiting *E. coli* biofilm formation in vitro.

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Conflict of interest

The authors declare that there is no conflict of interest.

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