

Antimicrobial Effects of Ethanolic Extract Propolis of *Geniotrigona thoracica* against *Escherichia coli* Producing Extended-Spectrum Beta-Lactamase (ESBL)

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Abstract

The increasing incidence of antimicrobial drug resistance is a major problem in the health sector, and this has encouraged the discovery of novel antibacterial agents present in natural products that can overcome this problem. Since ancient times, propolis has been widely used as a traditional medicine for various diseases, particularly those caused by bacteria. Propolis is a bee product composed of a mixture of resin derived from plant exudates and enzymes derived from bee saliva. Stingless bees produce more propolis than honey and are widely distributed in tropical areas like Indonesia. The aim of this study was to evaluate the antibacterial activity of ethanolic extract propolis (EEP) produced by *Geniotrigona thoracica* (*G. thoracica*) from West Sumatera against extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* (*E. coli*). In vitro antimicrobial assays were performed by well diffusion and serial dilution methods on six EEP concentrations: 100%, 50%, 25%, 12.5%, 6.25%, and 3.125%. No inhibitory zones were observed in the agar diffusion for any of the propolis concentrations tested. Similarly, neither the minimum inhibitory concentration (MIC) nor the bactericidal concentration (MBC) for each propolis concentration could be determined. These findings indicate that *G. thoracica* EEP has no antibacterial activity against ESBL-producing *E. coli*. On the other hand, phytochemical analyses revealed that the total phenolic, flavonoid, and alkaloid contents of *G. thoracica* EEP were 0.008 mg/mL, 0.015 g/g, and 0.084 g/g, respectively. These results suggest that *G. thoracica* propolis contains active compounds, which are thought to be components that contribute to its pharmacological activity.

Keywords: antimicrobial drug resistance, ESBL, *Escherichia coli*, propolis, stingless bee, *Geniotrigona thoracica*

1. Introduction

Antibiotic resistance has shown an increasing trend in recent years, from 2003 to 2018 (Bezabih *et al.*, 2021). The most critical problem regarding bacterial resistance is the ability of Gram-negative bacteria to produce extended-spectrum beta-lactamase (ESBL) (Naelasari *et al.*, 2018). *Escherichia coli* (*E. coli*) was shown to be the most common cause of ESBL-producing bacterial infections at the Dr. Soetomo General Hospital in Surabaya, accounting for 80% of other ESBL-producing bacterial infections (Naelasari *et al.*, 2018). As a tropical country with diverse flora and fauna, Indonesia has great potential to develop antibiotics from natural sources.

Propolis is a substance collected by bees from plants, which is a resin that is sticky and resembles wax mixed with enzymes from bee saliva (Damodaran, 2021; Wagh, 2013). Naturally, propolis is found in

beehives as a substance that plays a role in defense mechanisms against threats of physical damage or microbial infections (Wagh, 2013). Propolis has been known and used in traditional medicine for centuries because of its pharmacological properties against various diseases (Król *et al.*, 2013). The Meliponini, or stingless bee family, is widely distributed in Indonesia as its habitat is in tropical and subtropical areas (Kek *et al.*, 2014). One species of stingless bee that beekeepers widely cultivate is *Geniotrigona thoracica* (*G. thoracica*).

In contrast to honey bees (*Apis spp.*), stingless bees produce more propolis than honey, and it is also believed that the propolis produced by stingless bees is more potent than honey bees (Ibrahim *et al.*, 2016). The antibacterial therapeutic effect of propolis is mainly due to its active compounds and varies based on the plant source used by bees to collect resin, geographical location, and is influenced by seasonal factors (Righi, Negri and Salatino, 2013). Therefore, differences in chemical composition affect the variability of the effects produced, which in this study aims to test the antimicrobial effect of ethanolic extract propolis (EEP) of stingless bee species, *G. thoracica* against ESBL-producing *E. coli* by well diffusion and serial dilution methods.

2. Materials and Methods

2.1. Ethical Clearance

This research has received a certificate of ethical clearance from the Health Research Ethics Committee, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia, with appointment number: No.237/EC/KEPK/FKUA/2022

2.2. EEP Preparation

Raw propolis was obtained from *G. thoracica* beekeepers in Sawahlunto City, West Sumatra province, Indonesia. The extraction process involved macerating 650 g of raw propolis in 96% ethanol for a week. Following this period, the mixture underwent filtration to separate the residue from the filtrate. The filtrate obtained is then concentrated using a rotary evaporator to evaporate the solvent through low-pressure distillation. This process yielded 77 g of EEP, equating to an extraction yield of 11.85%.

2.3. Determination of Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and Alkaloid of Propolis

TPC levels were determined using the UV-Vis spectrophotometric method. Initially, a standard curve was established by employing gallic acid as the standard solution. The absorbance of the gallic acid was then measured at a wavelength of 770 nm. These absorbance measurements were plotted to generate a curve, allowing the derivation of a linear regression equation. This equation serves as the basis for calculating the overall phenolic compound levels in the propolis extract. The measurement of the absorption value of EEP begins by dissolving the propolis extract with distilled water and adding the Folin-Ciocalteu reagent to the solution. After letting it sit for 5 minutes, 10% sodium carbonate solution was added to the mixture. The absorbance of the samples was measured at the maximum absorption wavelength of 770 nm. The TPC is expressed as gallic acid equivalent (GAE).

The determination of TFC followed a spectrophotometric procedure similar to the TPC test. This test begins with sample preparation to obtain the ethyl acetate fraction from the EEP. Quantifying the TFC begins

by mixing the extraction results from the ethyl acetate fraction with a solution of AlCl_3 and glacial-methanol acetic acid. After allowing the sample mixture to settle for 30 minutes, the absorbance was measured at a maximum wavelength of approximately 425 nm.

The quantification of alkaloid compound levels was conducted using the gravimetric method. In this method, EEP is combined with 10% acetic acid in ethanol, and the resulting mixture is allowed to sit in a sealed container for 4 hours. Subsequently, the extract is filtered to collect the filtrate. The filtrate obtained is then evaporated and added to a concentrated ammonia solution until it can settle completely. The resulting precipitate is filtered and rinsed with a diluted ammonia solution to obtain the alkaloid compound. The alkaloid content was calculated after the sediment was dried and weighed.

2.4. Confirmation of Bacterial Resistance

The ESBL-producing *E. coli* bacterial isolate was acquired from the Microbiology Laboratory Department of Medical Microbiology, Faculty of Medicine, Universitas Airlangga. A double-disc synergy test (DDST) was conducted using the disc diffusion method on Mueller-Hinton (MH) agar to confirm the resistance of bacteria-producing beta-lactamase enzymes. Initially, a sterile cotton swab was immersed in a suspension of ESBL-producing *E. coli* with the turbidity adjusted visually to the 0.5 McFarland standard and then inoculated on the agar surface. An amoxicillin disc (30 μg) was placed in the center of the agar, surrounded by an aztreonam (ATM) disc, as well as three 3rd generation cephalosporin discs: cefotaxime (CTX), ceftazidime (CAZ), and ceftriaxone (CRO) placed around the amoxicillin disc. Following this, the plate was incubated at 37°C for 24 hours.

2.5. Antibacterial Activity

The antibacterial activity test was carried out using serial dilution and well diffusion. The groups tested consisted of 6 variations of EEP concentration, 100%, 50%, 25%, 12.5%, 6.25%, and 3.125% (w/v) obtained by dissolving EEP (100%) with 10% ethyl acetate.

The broth macro-dilution technique was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). EEP was distributed through two-fold serial dilution into six test tubes using 10% ethyl acetate as a diluent. The final concentration of propolis ranged from 100% to 3.125%. Each propolis sample tube received an inoculum suspension of ESBL-producing *E. coli* calibrated to the 0.5 McFarland standard (1.5×10^8 CFU/mL). Two additional test tubes containing 10% ethyl acetate served as controls: one with bacterial inoculum suspension as the positive control and the other with only liquid medium or broth as the negative control. All tubes were incubated at 37°C for 24 hours and then observed for visible turbidity. The lowest concentration that appears clear is interpreted as the MIC. MBC was determined by inoculating a test tube containing propolis concentrations equal to or higher than the MIC onto agar media. MBC is defined as the lowest concentration of EEP without the growth of bacterial colonies on agar media.

The well diffusion method was performed on Mueller-Hinton agar, which had been inoculated with a suspension of ESBL-producing *E. coli* equivalent to the 0.5 McFarland standard. Seven 6 mm wells were made using a sterile cork borer. A total of six wells were filled with 100 μL of EEP at varying concentrations (ranging from 100% to 3.125%). The remaining well was filled with 100 μL of 10% ethyl acetate as a negative control. A 10 μg meropenem disc was placed in the center of the agar as a positive control. The test

plate was then incubated at 37°C for 24 hours to observe the formation of the inhibition zone. The inhibition zone appears as a clear area surrounding the well after incubation, indicating the absence of bacterial growth. The diameter of the inhibitory zone was measured in millimeters using a caliper.

According to Federer's formula, each treatment group should ideally undergo a minimum of four repetitions. Nevertheless, in this study, the total replication for every dilution and diffusion method is six.

3. Results

3.1. Total Phenolic, Flavonoid, and Alkaloid Content of Propolis

Table 1. TPC, TFC, and alkaloid content of ethanol extract of *Geniotrigona thoracica* propolis

Compound	Concentration	Yield % (w/w) (mean $\pm$ RPD)
Total phenolic content (TPC)	0.008 mg/mL propolis	0.08 $\pm$ 0.02
Total flavonoid content (TFC)	0.0015 g/g propolis	1.52 $\pm$ 0.15
Alkaloid	0.084 g/g propolis	8.43 $\pm$ 1.5

RPD = Relative Percent Difference

3.2. Antibacterial Activity

The DDST test results confirmed the bacteria phenotypically produced ESBL. This was observed through a visible expansion of the inhibition zone on the side exposed to amoxicillin, originating from the four surrounding antibiotic discs, which could be interpreted as ESBL-producing bacteria.

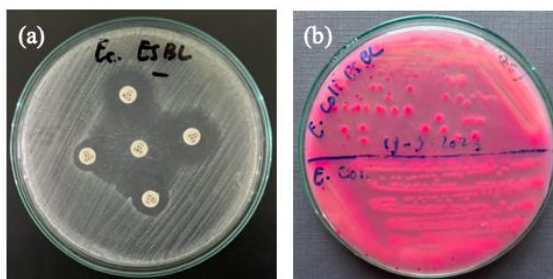


Fig. 1. (a) DDST test results of ESBL-producing *E. coli* bacterial isolates to confirm their resistance; (b) ESBL-producing *E. coli* isolates

In the macro-dilution test method, after the test tube is incubated, the turbidity of the tube contents is observed visually to determine the EEP concentration as the MIC value. All tubes appeared cloudy except for the negative control tube. Consequently, based on visual observation alone, the MIC value cannot be determined for any EEP concentration. The streak plate method on nutrient agar (NA) is subsequently employed to confirm the findings and to determine the MBC value.

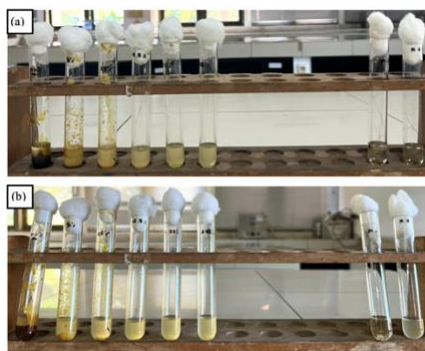


Fig. 2. (a) Macro-dilution test tubes before incubation; (b) Macro-dilution test tubes after incubation

The results of subcultured dilution on agar media showed bacterial growth at all EEP concentrations. Thus, no MBC value was obtained

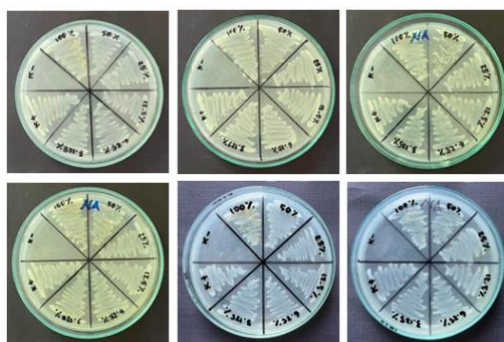


Fig. 3. The results of subculture dilution to ensure the growth of bacteria on agar media

The same results were obtained in the well diffusion test. After the test plate was incubated, the results from 6x replicates showed that none of the wells containing 100% - 3.125% EEP concentrations and 10% ethyl acetate as negative control showed any inhibition zones. The zone of inhibition was only present in 10 µg meropenem disks as a positive control, with a mean diameter of 28.12 mm from 6 replicates.

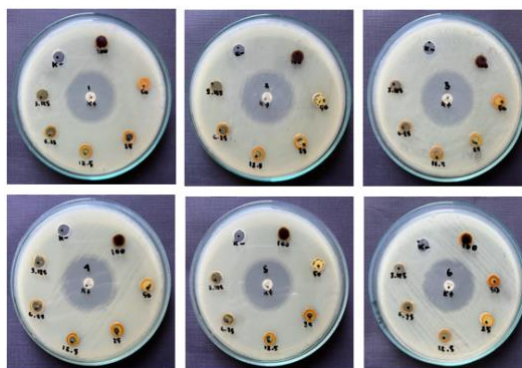


Fig. 4. The results the well diffusion test after incubation

4. Discussion

4.1. Total Phenolic, Flavonoid, and Alkaloid Content of Propolis

The physical characteristics of *G. thoracica* EEP show a thick, dark brown appearance and lack of solubility in water due to its hydrophobic nature, primarily attributed to its high-fat content. In Abdullah *et al.*'s study (2020), the fat content of *G. thoracica* propolis was found to be 47.86%, notably higher (3-5 times greater) than the fat content in propolis produced by *Apis mellifera* or honey bees. Increased fat content enhances hydrophobic or non-polar characteristics. Hence, solvents other than distilled water, a non-polar solvent, are necessary to achieve lower EEP concentrations (50%, 25%, 12.5%, 6.25%, and 3.125%). In this research, 10% ethyl acetate was selected as the solvent because of its absence of antibacterial activity against ESBL-producing *E. coli*, ensuring that the antibacterial test outcomes accurately represent EEP.

Propolis is a bee product that contains complex chemical compounds. The compound content of propolis varies depending on many factors. In Brazil, the quality standard for commercialized propolis extract is based on the content of the main class of phenols (Contieri *et al.*, 2022). Phenolic compounds play an important role in antioxidant activity (Abdullah *et al.*, 2020), as do flavonoids (Awang *et al.*, 2018). These two compounds are also related to the antimicrobial activity of propolis (Abdullah *et al.*, 2020). Phenols and flavonoids act as antibacterial agents by causing structural damage to bacterial cell walls and membranes. This damage leads to the leakage of cellular contents and eventual cell death, a process influenced by the lipophilic nature of these compounds (Echeverría *et al.*, 2017). In this study, the TPC and TFC values tended to be lower than those obtained in other studies. As presented in Awang *et al.*'s study (2018), the differences observed in propolis flavonoids primarily stem from the varied plant preferences of stingless bees in different regions. On the other hand, the total phenol content is affected by both the extraction technique and the type of propolis. However, Escriche and Juan-Borrás (2018) highlight that the propolis type plays a more significant role in determining the phenol content. The diminished TPC and TFC might result from the prevalence of hydrophobic compounds, primarily beeswax and resin within propolis (Przybyłek and Karpiński, 2019). This explanation relates to the use of 96% ethanol (absolute) as an extraction solvent. Ethanol, as a polar solvent, contrasts with propolis's non-polar nature. Consequently, using ethanol is deemed unsuitable for dissolving propolis since it doesn't adequately interact with the propolis compounds, most exhibiting non-polar characteristics. This is explained by the solubility rule "like dissolves like," a theory regarding the polarity of two liquids (Zhuang *et al.*, 2021). Cunha *et al.* (2004) research also highlighted that the yield of bioactive compounds in propolis extract increased proportionally with higher ethanol concentrations, preferably around 70% or above, but this does not mean using absolute ethanol. The low concentration of TPC and TFC within propolis extract could be one of the factors contributing to the absence of antibacterial activity against *E. coli*-producing ESBL.

Apart from polyphenols and flavonoids, alkaloid compounds were also found in the EEP of *G. thoracica*. However, as of now, no prior research has specifically investigated the concentration levels of alkaloids in *G. thoracica* propolis extract. Like polyphenols and flavonoids, alkaloids could indicate potential antioxidant activity (Mulyati *et al.*, 2020). Additionally, these compounds have a wide range of pharmacological activities encompassing antimalarial, antiasthmatic, anticancer, analgesic, and antibacterial properties (Hidayat *et al.*, 2022). Alkaloids are the most abundant phytochemical compounds and contribute significantly to the biological activity of propolis (Kegode *et al.*, 2022). This statement aligns with the phytochemical test results of this study, revealing that alkaloid levels were more significant compared to polyphenols and flavonoids. Nevertheless, the comprehensive quantification of the total alkaloid content in propolis remains relatively underreported.

4.2. Antibacterial Activity

Several research studies, including Ibrahim *et al.* (2016), Abdullah *et al.* (2020), and Przybyłek and Karpiński (2019) have highlighted that propolis extracts exhibit better antibacterial activity against Gram-positive bacteria compared to Gram-negative bacteria. Typically, the antibacterial potency of stingless bee propolis extract against resistant bacteria tends to be lower than that against antibiotic-sensitive bacteria (Okńczyc *et al.*, 2020). The reason behind the reduced activity of propolis extract against Gram-negative bacteria lies in the distinctive structure unique to these bacteria—the presence of an outer lipid membrane, posing greater difficulty for various molecules to penetrate this barrier (Blair *et al.*, 2014). Apart from morphological reasons, Gram-negative bacteria also have the ability to produce hydrolytic enzymes, which cause damage to the active ingredients of propolis (Przybyłek and Karpiński, 2019). This phenomenon elucidates why Gram-negative bacteria often necessitate higher propolis concentrations to inhibit their growth or kill (Xavier *et al.*, 2023) or have no activity against Gram-negative bacteria.

Regarding agar diffusion, the polarity of active compounds within the test material extract significantly influences the test outcomes. Since agar constitutes an aqueous preparation, polar compounds do not diffuse as effectively as non-polar compounds. Interestingly, numerous plant species contain antimicrobial compounds that are relatively non-polar (Eloff, 2019). In the case of propolis, its composition includes plant exudates alongside other elements like bee enzymes, pollen, and wax (Sforzin, 2016).

5. Conclusion

Based on the results of the *in vitro* antibacterial test using the diffusion and dilution test method, it can be concluded that the ethanolic extract propolis of *Geniotrigona thoracica* does not exhibit antibacterial activity against ESBL-producing *E. coli*. Therefore, using propolis against Gram-negative bacteria is not recommended as the sole antibacterial agent; it should be combined with potent antibiotics against Gram-negative bacteria. The results of phytochemical tests revealed that EEP of *G. thoracica* contained low concentrations of active compounds like polyphenols, flavonoids, and alkaloids, which might explain the absence of antibacterial activity.

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