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(RESEARCH ARTICLE)

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Ethanolic extract of propolis from Indonesian stingless bee (*Tetragonula biroi*) is ineffective against ESBL-producing *Klebsiella pneumoniae*: An in vitro study and a brief review of propolis antibacterial and anti-inflammatory activity.

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Abstract

The number of antimicrobial resistance which is increasing every year is a big problem that attracts attention. The prevalence of infections due to bacterial resistance is quite high, especially from nosocomial infections. This encourages the search for new materials that have the potential to fight resistant bacteria. Propolis is a product of bees that is not only used for structural support for their hives but also as an antiseptic to protect their hives from microorganisms. This study aims to determine whether the ethanolic extract of propolis (EEP) from the stingless bee *Tetragonula biroi* has an antibacterial effect against extended spectrum beta-lactamase (ESBL) producing *Klebsiella pneumoniae*. This study used the broth dilution test and agar-well diffusion test to test for antibacterial activity. EEP is generally inactive against ESBL-producing *K. pneumoniae*. It could not produce an inhibition zone in the agar-well diffusion test. The results of the dilution test for the minimum inhibitory concentration (MIC) are difficult to determine. Phytochemical qualitative test found EEP of *T. biroi* contains alkaloids, flavonoids, and phenols.

Keywords: Propolis; Stingless bees; *Tetragonula sp*; ESBL; Resistant bacteria; *Klebsiella pneumoniae*

1. Introduction

Antimicrobial resistance (AMR) is a global problem which are increasingly worrying. AMR is a group of pathogenic bacteria, viruses and fungi that have acquired resistance to antibiotics, antivirals and antifungals. This is caused by the emergence of multidrug-resistant (MDR) bacteria [1]. Inappropriate use of antibiotics is one of the many causes that leads to bacterial adaptation and mutation that gives them the ability to survive against antibiotics and becoming resistant. One of the most commonly found resistant bacteria is *Klebsiella pneumoniae*. It is often found in nosocomial infection, especially hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP) [2]. Nosocomial infections refer to infections that happen when one is receiving health care in hospital or primary care that appears 48 hours or more after being admitted into hospital or within 30 days after receiving health care [3]. In the United States and Europe, nosocomial infections have a prevalence of 3.2% and 4.4% respectively [4], while worldwide prevalence seems to be much higher, contributed by the developing countries and low-middle income countries (LMIC) [5]. In time, the number of resistant bacteria will only increase, therefore, it is necessary to look for and develop components that have antibacterial properties, which has the potential as therapeutic agents.

Propolis is a glue-like, sticky and resinous bee product. It has been found to be able to not only act as antibacterial but also antioxidant, anti-inflammatory, anti-tumoral, and even plays a role as an immunomodulator [6–9]. There has been less interest on studies regarding stingless bees. Studies on honey bees far exceed the amout of studies on stingless bees

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[10]. Even though there are only small number of studies done on stingless bees, some produce promising results. This study aims to see the antibacterial effect of ethanolic extract propolis (EEP) from the stingless bee (*Tetragonula biroi*) towards clinical strain of ESBL-producing *Klebsiella pneumoniae* with the agar-well diffusion method and broth dilution method.

2. Materials and methods

2.1. Ethical clearance

This study obtained the permission from the Health Research Committee of Soetomo General Hospital, Surabaya, East Java, Indonesia with the appointment number: 1096/LOE/301.4.2/X/2022.

2.2. EEP preparation

Propolis samples were obtained from farm raised *T. biroi* in Gowa, South Sulawesi, Indonesia. It is plucked of any dust and debris and cut into smaller pieces. It is then weighed (230 g), transferred into a bigger container, and was added 2.5 liters of 96% ethanol for the maceration and left as it is for 7 days. During that time, it is regularly stirred. After a week of maceration, the material is then filtered and evaporated using rotary evaporator. The crude extract is then oven-dried and the final extract yielded 71 g of EEP.

2.3. EEP phytochemical analysis

EEP is tested qualitatively for the following phytochemicals: alkaloids, flavonoids, phenols, saponin, and triterpenoids. Alkaloid test was done by adding 10 ml of chloroform ammonia to EEP and it was filtered. The filtrate is then added 2M of H_2SO_4, and then thoroughly mixed until 2 layers are formed. The upper acidic layer is taken and was added Dragendorff's reagent and Mayer's reagent respectively. A positive test is indicated by white or yellow color in Mayer's reagent and a reddish orange color in Dragendorff's reagent. Phenol is tested by adding several drops of 1 % $FeCl_3$. A positive test is indicated by dark violet color. Flavonoid test is done by adding 100 ml of boiled water to EEP and then filtered. The filtrate is added magnesium powder and 1 ml of HCl and then thoroughly mixed. A yellow to orange color indicates Flavonoid. Saponin test is done by further extracting the EEP with diethyl eter for 3 times. There will be fraction that is soluble and not soluble in diethyl eter. The insoluble fraction is added 5 ml of water and mixed. A positive test is indicated by foam of at least 3 cm in height and lasts for at least 15 minutes. Triterpenoid test uses the fraction that is soluble and was added glacial CH_3COOH and H_2SO_4.

2.4. Antibacterial activity of EEP to ESBL *K. pneumoniae*

Clinical isolates of *K. pneumoniae* were collected from various samples from patients in Soetomo General Hospital, Surabaya. It is then transferred into the Medical Microbiology laboratory in Faculty of Medicine, Airlangga University. ESBL-producing strains are tested prior, phenotypically, using double disc synergy test (DDST) using the following disc: Amoxicillin-clavulanate (30µg), Ceftriaxone (30µg), Ceftazidime (30µg), Cefuroxime (30µg), and aztreonam (30µg). The antibacterial activity is tested using agar-well diffusion and broth dilution method. Each method is replicated 6 times.

The EEP is scooped using metal spatula and taken into small sterile bottles and weighed. EEP is then made into 6 concentrations; 100%, 50%, 25%, 12.5%, 6.25%, and 3.125% (w/v). The solvent used is 5% ethyl acetate.

The bacterial concentration is adjusted to the 0.5 McFarland turbidity standard and are inoculated into mueller-hinton agar (MHA) (Oxoid) plates. 7 wells are then made (respective to the EEP concentration and negative control) using sterile borer of 6 mm width, along the circumference of the agar. EEP extract is then added to their respective wells and concentrations. The negative control used is the solvent, 5% ethyl acetate. The positive control, a 10 µg meropenem disc (Oxoid) is placed in the middle of the agar. It is then incubated at 37°C (Binder, Germany) for 1x24 h.

The broth dilution method used in this study is a macrodilution of twofold serial dilution. The bacterial cell concentration is adjusted into 0.5 McFarland turbidity standard. 0.5 ml of Mueller-hinton (MH) broth is added into sterile tubes (6 to their respective EEP concentration) and 2 tubes as broth control and growth control. Serial dilution is then performed. Broth control contains MH broth and EEP, while growth control contains the bacteria. The tubes are then incubated for 24h at 37°C, and is looked for turbidity. Afterwards, each tube is then streaked onto nutrient agar plates and further incubated to confirm bacteria presence.

3. Results

3.1. Qualitative phytochemical result of EEP

Alkaloids, phenols, and flavonoids are found in EEP. Triterpenoids and saponins are not present in EEP.

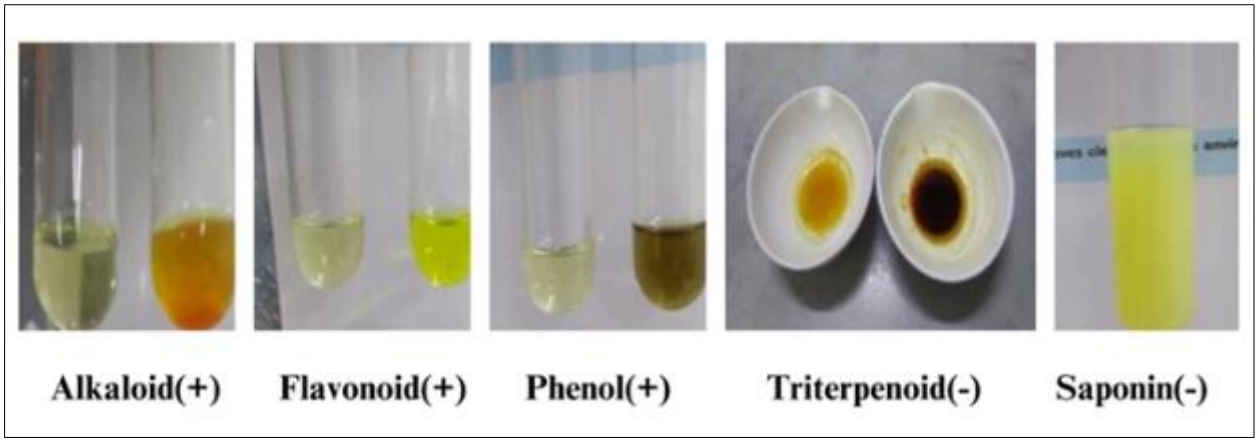

Figure 1 The qualitative phytochemical test result of EEP found a positive alkaloid, phenol, and flavonoid. Triterpenoid and saponin were negative.

3.2. Antibacterial activity of EEP against ESBL *K. pneumoniae*

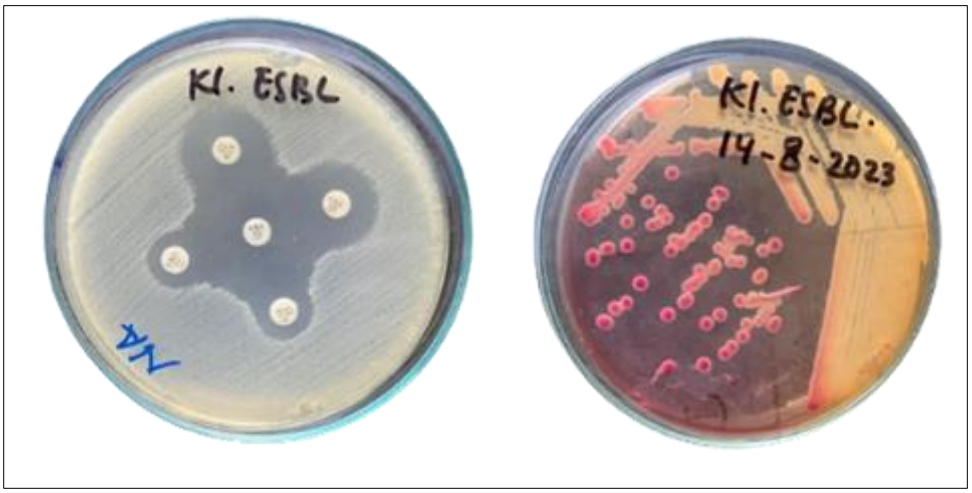

Figure 2 The DDST result confirms ESBL presence phenotypically. It is shown that the bacteria are resistant towards third generation cephalosporins such as Ceftriaxone, Ceftazidime, and Cefuroxime and also towards Monobactams (Aztreonam). The inhibition zone is only made towards the middle where a *B*-lactamase inhibitor (Clavulanate) is present.

On dilution test, after 24h incubation at 37°C, it was hard to determine the MIC as the color of EEP prevents visual confirmation of turbidity.

Figure 3 The broth dilution test after 24h incubation at 37°C. From left to right: 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, negative control, and positive control. The color of EEP hinders the turbidity visual confirmation.

The tubes were then streaked onto nutrient agar plates and after another 24h of incubation, it was found that the tube containing 100% EEP had no bacterial growth on the agar. The concentration below 100% all had bacterial growth except the broth control (negative control).

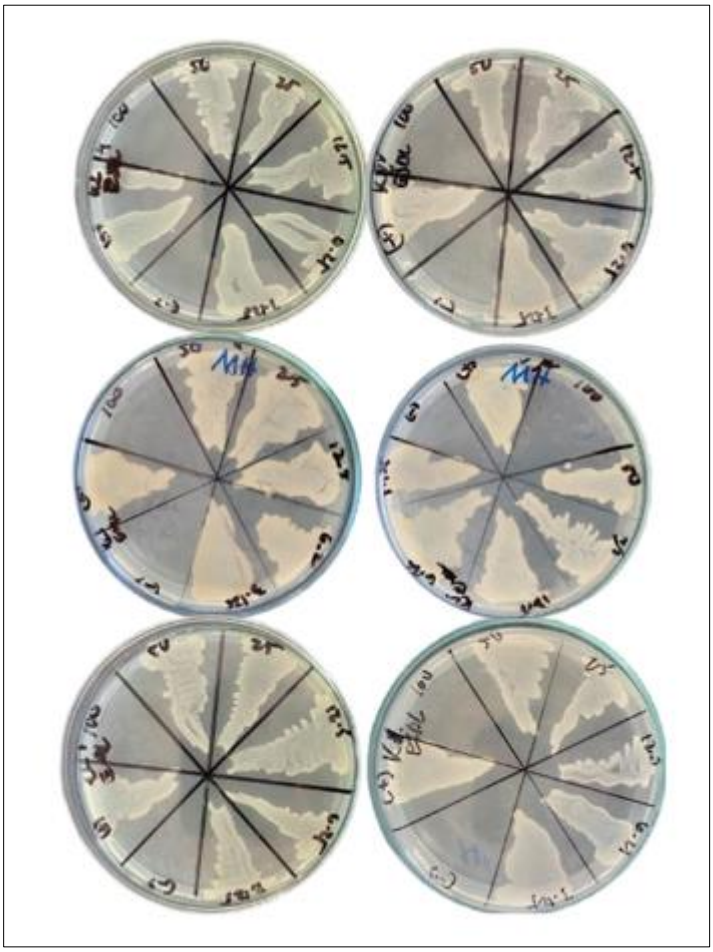

Figure 4 The result of the subcultured dilutions onto nutrient agar.

Agar-well diffusion test had the same result. EEP fails to inhibit the growth of ESBL-producing *K. pneumoniae.* The meropenem disc showed strong inhibition zones across all replicates (>20 mm). The average inhibition zone produced is 25.1 mm.

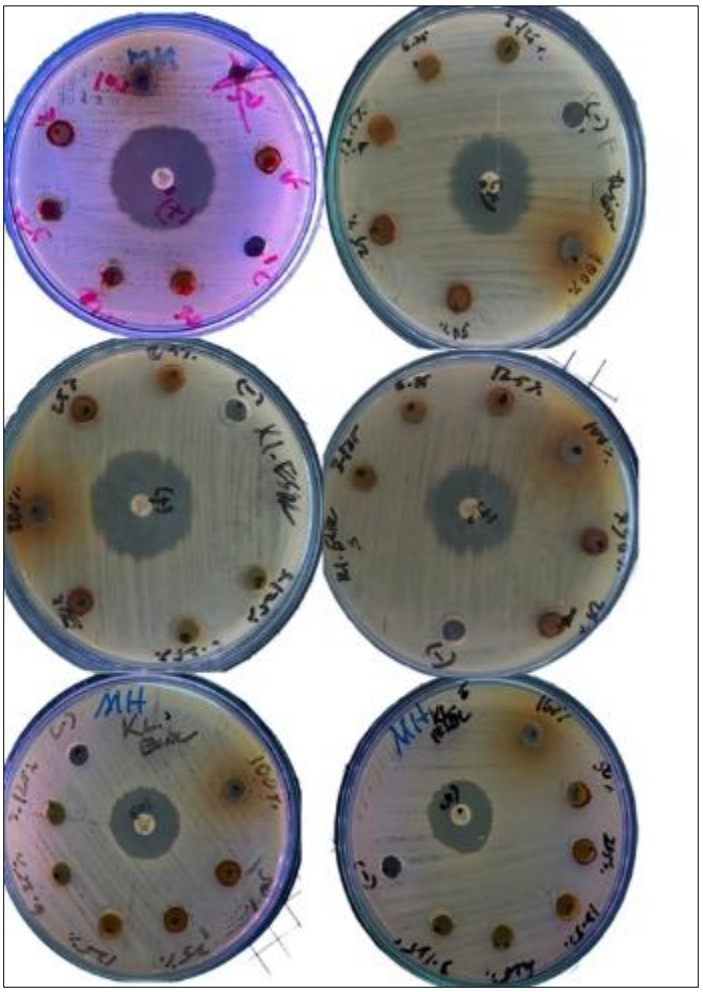

Figure 5 The result of agar-well diffusion test.

4. Discussion

4.1. Propolis and bees

Propolis is made from plant sap which is then processed by bees with their salivary enzymes. In nature, they are hard and rough in consistency. When it is heated up, it becomes soft and very sticky. Propolis also varies by their color, ranging from yellow, brown, to black color, based on the resin the bees used and the propolis age [8]. The rough composition of propolis consists of mostly resin and wax with essential oils, pollen and various organic components [8]. The chemical composition of propolis is roughly 50% plant resin, 30% waxes, 10% essential oils with small number of pollens and other organic substances [11]. Propolis itself is used by bees to protect their nest, as cement for the nest structures. It is also used as antiseptic material for the bees when they go back inside their nest from the outside world. This hygiene process is important for them to protect the larvae who are prone to infections and also to protect their product, mainly honey from going bad due to microbials. Due to these antimicrobial effects, the bees also put propolis on places where their nest makes contact with an object [12]. It is also important for them to prevent an infection amongst one of them, as they live in one colony and a disease can be spread quickly [6,12].

The present taxonomic classification delineates bees into two primary categories: honey bees (e.g., *Apis mellifera*) and stingless bees (e.g., *Trigona* and *Meliponia*). Honey bees such are found to be more widely distributed in the world compared to stingless bees which are more commonly found in tropical and sub-tropical regions. Some stingless bees produce less honey compared to honey bees and combined with the fact that it is less distributed, it is more common for honey bees to be farmed and studied. The number of studies done for honey bee product far exceed the number of studies on stingless bee product [10], even though several studies report that stingless bee product potentially has better nutritional value and greater number of active compounds [10,13]. It is thought that it is attributed to the distribution of the stingless bees that live in tropical areas which have a rich vegetation [10]. The content of the bee

product also depends on the season it was harvested; on what kind of vegetation grows during said season. The propolis used in this study comes from farm raised *T. biroi*, located in Gowa, South Sulawesi. The farm itself is laid on forest and woods, therefore the bees take their materials from multifloral vegetation.

4.2. Propolis antibacterial mechanism

Propolis contains polyphenols which are secondary metabolites from plants that are produced when plants are exposed to stressors such as pathogens or extreme temperatures. These polyphenols are further divided into flavonoids and non-flavonoids based on their molecular structure [9]. However, the content of these polyphenols, especially flavonoids, depends on the ecosystem and types of plants in the bee's environment. Therefore, the properties and content of propolis are closely related to the local vegetation in the bee habitat [6]. It is known to have antimicrobial effects such as antibacterial, antifungal, antiviral and antiprotozoal due to its phytochemical components [6,12]. Flavonoids can act directly on bacteria through cell walls and membranes [6]. Some type of flavonoids in propolis are also known to increase bacterial membrane permeability and inhibit nucleic acid synthesis by inhibiting enzymes such as DNA gyrase and topoisomerase, or even inhibit protein synthesis [6,7]. The alkaloids found in propolis can also inhibit cell division and nucleic acid synthesis by inhibiting topoisomerase and dihydrofolate reductase [14]. Phenols such as p-coumaric and gallic are also known to damage bacterial membranes by inducing the entry of protons and the release of important ions from bacterial cells [10]. Terpenoids and saponins also damage bacterial cell walls and membranes [15,16]. Propolis is known to have a synergistic effect on with several drugs against multi-drug resistant bacteria [9]. Flavonoids such as quercetin when co-administered with amoxicillin against MRSA can reduce MRSA resistance [6].

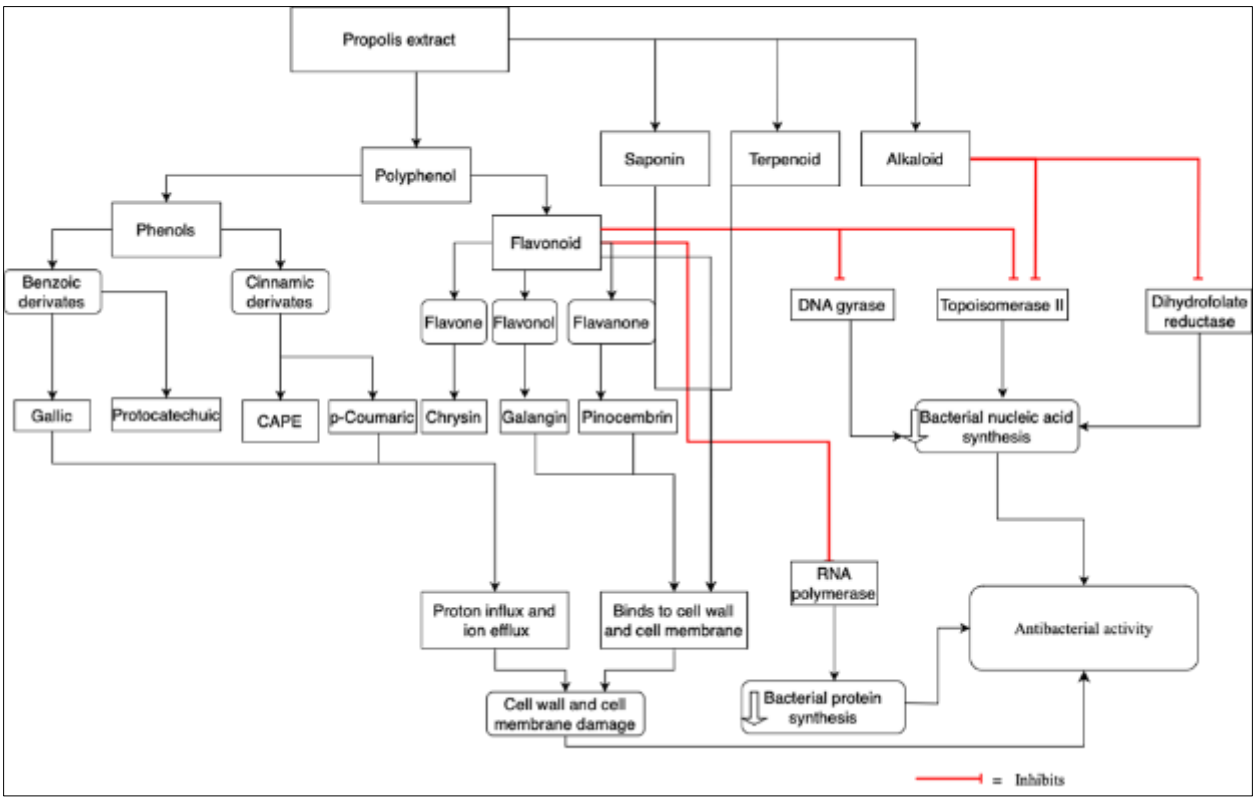

Figure 6 The phytochemical component and their antibacterial mechanism that has been reported to be found in propolis extracts [6,7,9,10,14–16].

4.3. Propolis anti-inflammatory mechanism

Inflammation is the body's response towards a stress be it endogenous or exogenous, caused by physical, chemical, or biological factors, that causes cellular damage. During the acute phase of inflammation, immune cells migrate towards the location of damage. It is then activated and releases reactive oxygen species (ROS), cytokine, and growth factors. Several other inflammatory mediators might also be produced from the cell membrane by the enzyme cyclooxygenase (COX) and lipoxygenase (LOX) [17,18].

Propolis exerts anti-inflammatory effects in several ways such as: 1) inhibiting COX; 2) counteract free radicals; 3) inhibit the synthesis of nitric oxide (NO); 4) reduces pro-inflammatory cytokines; and 5) through immunosuppressive activity [7]. Green propolis from Brazil is known to modulate the immune system through regulation of pro-inflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) [19] and flavonoids such as pinocembrin can reduce pro-inflammatory cytokines and chemokines [7]. Flavonoids are also strong antioxidants to ward off free radicals. Apart from that, flavonoid can also regulate the production of leukotrienes and prostaglandins [12] by inhibiting COX. Galangin and Caffeic acid phenethyl ester (CAPE), one of the most frequently researched phenolic acids, is known to inhibit the COX enzyme needed to form prostaglandins, prostacyclin, and thromboxane [7,9,17,20]. CAPE is also known to have many molecular functions through various complex mechanisms such as inhibition of nuclear factor-kappa B (NF-κB), which has the potential to provide healing, antitumor and anti-inflammatory effects [9]. The NF-κB pathway activates and induces several genes for apoptosis and pro-inflammatory cytokines, including TNF-α, IL-1, IL-2, IL-6 and IL-8 [17]. CAPE also exerts its effect on the arachidonic acid pathway by inhibiting the gene expression COX and LOX directly and increase the synthesis of anti-inflammatory cytokines such as IL-10 and IL-14 [17]. Other bioactive compounds such as pinocembrin, chrysin, and artepilin C also has anti-inflammatory activity [18]. An in vivo study using transgenic mice of Alzheimer's model has shown that pinocembrin administration reduces the level of TNF-α, IL-1, and IL-6 [7]. Artepilin C present in south and south-east Brazilian propolis is shown to have strong antioxidant activity and is able to reduce the levels of prostaglandins especially PGE_2 [20].

4.4. Antibacterial activity of EEP towards ESBL-producing *K. pneumoniae*

From the result of the broth dilution test and agar-well diffusion test, it is found that EEP of *T. biroi* is not effective as antibacteria towards ESBL-producing *K. pneumoniae*. Both tests show similar results but the dilution test shows no bacterial growth in the 100% concentration. This might be due to the direct contact with the bacteria as it is more soluble on the broth dilution test. On the other hand, the agar-well diffusion test needs the material to be thoroughly soluble and diffused into the agar to actually make contact and act towards the bacteria within the agar. Furthermore, precipitate of those non-soluble materials can interfere with the diffusion on the agar. This test is not suitable for less polar, water insoluble materials [21–23], while the main component of propolis, resin and beeswax are hydrophobic [11].

Propolis is more effective in dealing with gram-positive bacteria compared to gram-negative bacteria [11,24], or it might even be completely ineffective [25–27]. This is due to the difference in the morphological structure of the bacteria [6,28]. Gram positive bacteria lack the outer membrane. This layer of membrane that is only present in gram negative bacteria can confer resistance by changing the hydrophobic properties and mutations in the porin [29]. In addition, gram-negative bacteria produce hydrolytic enzyme that can hydrolyse the active ingredient of propolis, thereby rendering it ineffective to act [6,11]. This is the reason why propolis is more used for wound care management on the skin and skin disinfection, even dating back to the Middle Ages [6]. The normal flora of the skin comprises mostly of gram-positive bacteria which are more sensitive to propolis. Propolis is also non-toxic, hypoallergenic, and it induces skin cell proliferation to accommodate for wound healing [24]. It has been shown that CAPE also promotes wound healing by accelerating wound healing phase to maturation phase [9]. Propolis demonstrates promising potential as a therapeutic agent for wound healing in the context of burns [24] and eczema [30].

5. Conclusion

EEP of *T. biroi* is ineffective towards ESBL-producing *K. pneumoniae.* This is in alignment with the consensus that propolis is less effective towards gram-negative bacteria. The active ingredient of propolis is largely dependent upon the local vegetation of the bee's habitat. EEP of *T. biroi* is found to contain alkaloid, flavonoid, and phenol. While the phytochemicals are present, the concentration might not be high enough to act as antibacteria. It is also worth noting that gram-negative bacteria are more resistant to propolis by nature and the strain used in this study are clinical strain, ESBL-producers.

Compliance with ethical standards

Disclosure of Conflict of interest

The authors declare that they have no conflicts of interest concerning this article.

Statement of ethical approval

This study obtained the permission from the Health Research Committee of Soetomo General Hospital, Surabaya, East Java, Indonesia with the appointment number: 1096/LOE/301.4.2/X/2022.

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