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Isolation of bacteria from *Apis cerana* hive, their antibacterial potency and cytotoxicity

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Abstract. *Yasmin Y, Fitri L, Fauziah, Wasliyah F. 2019. Isolation of bacteria from* Apis cerana *hive, their antibacterial potency and cytotoxicity. Biodiversitas 20: 2733-2738.* This study aimed to identify bacteria from *Apis cerana* hive; to determine their antibacterial activity, and cytotoxic effect of hive extract as well as bacterial extract. Identification of bacteria isolated from the hive was carried out based on morphological and biochemical characters. The antibacterial assay of beehive bacteria isolates was done by disk diffusion method against *Staphylococcus aureus* and *Escherichia coli*. Toxicity test of beehive extract and beehive bacterial extract were carried out using Brine Shrimp Lethality Test (BSLT) with six final concentrations (32 ppm, 64 ppm, 126 ppm, 250 ppm, 500 ppm, and 1000 ppm). The LC_{50} value was determined by probit analysis using SPSS. Bacterial isolation showed there were four bacterial isolates from *A. cerana* hive were the genus *Bacillus* (BSL1, and BSL3), the genus *Micrococcus* (BSL2) and the genus *Neisseria* (BSL4). The antibacterial assay showed that BSL4 isolate has the highest antibacterial activity against *S. aureus* with the diameter of the inhibitory zone was 32.6 mm. Toxicity test showed that *A. cerana* hive extract and bacterial ethanol extract from *A. cerana* hive had LC_{50} value of 67,744 ppm and 86.985 ppm respectively and categorized as toxic.

Keywords: Antibacterial, *Apis cerana*, BSLT, hive bacteria, LC_{50}

INTRODUCTION

Apis cerana hive had been studied for its biological activities as antimicrobial, anticancer, antioxidant, anti-inflammatory, respiratory tract treatment (Banskota et al. 2001). *A. cerana* hive can be found on trees in open areas. According to Koetz (2013), bees (*A.cerana*) could be found nesting in all habitats ranging from primary forests, secondary forests, agricultural areas, residential, and open areas. *A.cerana* tends to nest at relatively low altitudes, with an average height of one to two meters. Hadisoesilo and Otis (1998) stated that *A. cerana* had 1-2 cm body length. *A. cerana* was smaller than *Apis dorsata* and *Apis mellifera* which had a body length twice the size of *A. cerana.* Abdominal lines of *A. cerana* was more prominent than *A. mellifera*, and worker bee of *A. cerana* had four abdominal lines, while the worker bee of *A. mellifera* had three abdominal lines. The tip of *A. cerana* abdomen was rounded and blunt. According to a study conducted by Damus and Otis (1997), the front wing length of *A. cerana* was 7.64 mm, the rear wing length was 2.66 mm, and the rear leg length was 6.57 mm.

Beehive is one of the bee products that is widely used for medical purposes. Beehive had mutualistic associations with various types of microbes, namely bacteria, molds, yeasts, and mushroom. This microbial in hive likely to produce secondary metabolites (Promnuan et al.. 2009). Bioactive compounds produced by microbes are very potential to be developed into drugs. According to Lihan et al. (2014), bacteria, as any other microbes, especially bacteria have the ability to produce biologically active secondary metabolites such as useful antimicrobial.

Many research has been conducted on *A. cerana* products, but the study on their microbiological analysis was still very limited (Piccini et al. 2004). Piccini and Zunino (2001) reported that *Paenibacillus larvae* and *Melissococcus plutonius* was obtained in adult larvae, bee, and honey of *A.cerana*. Several studies related to bacteria in beehive have been done. Snowdon and Cliver (1996) reported that *A. cerana* microbes were commonly found in beehive and adult bee. Another study discovered 32 actinobacteria isolated from the beehives of three species of *Apis* spp. and two stingless bee species in Thailand. These actinobacteria were capable of producing antibacterial compounds against pathogenic bacteria *P. larvae* and *M. plutonius* in *A. cerana* (Promnuan et al. 2009). Therefore, bacteria from beehive had the potential as natural antimicrobial sources which might be able to overcome the problem of resistance from commercial antimicrobials.

Therefore, it is necessary to conduct a study to determine the antimicrobial activity produced by bacteria isolated from *A. cerana* hive. This study aimed to isolate and determine the antibacterial activity and cytotoxicity of bacteria isolated from the beehive.

MATERIALS AND METHODS

Isolation of beehive bacteria

One gram of hive was soaked with sterile distilled water for one minute, then the surface was soaked with 70% alcohol for three minutes and soaked again with sterile distilled water for one minute. Furthermore, the beehive was cut using a sterile knife and mashed using mortar and pestle, then added with 5 mL of sterile distilled water and homogenized using vortex for three minutes. A total of 100 µL of the suspension was taken out using a micropipette and spread onto nutrient agar (NA) medium. The sample was incubated at 37ºC for 48 hours (Promnuan et al. 2009).

Bacteria identification

Bacterial identification was conducted by selecting several bacteria colonies that showed differences in agar medium. Identification was conducted by referring to Bergey's Manual of Determinative Bacteria (Holt et al. 1994) and the Manual for the Identification of Medical Bacteria (Cowan and Steels 1993).

Antibacterial activity test

Staphylococcus aureus and *Escherichia coli* were used for the antibacterial assay. *S. aureus* and *E. coli* were incubated for 24 hours at 37ºC in slanted NA medium. After incubation complete, *S. aureus* and *E. coli* were picked using an inoculating loop and suspended into 0.9% NaCl solution. Furthermore, 1 mL of bacterial suspension was taken out and spread on the surface of sterilized MHA medium and left for 5 minutes.

Beehive bacteria isolates were taken using cork borer and transferred onto Mueller Hinton Agar (MHA) medium; the isolates were put upside down facing the media that has been inoculated with tested bacteria. Each isolate was tested with three replication (triplicate). The agar plates were incubated at room temperature for 24 hours (Pratiwi 2008). Antibacterial activity was carried out in triplicate and determined by measuring the diameter of the clear zone or inhibitory zone formed using a caliper.

Toxicity test

Preparation of beehive ethanol extract

A total of 500 beehives was cut into small pieces and dried using an oven at 50°C. The dried sample was crushed with blender, then macerated with 70% ethanol for 3 x 24 hours. The ethanol extract was dried and concentrated with a rotary evaporator at 50°C. A stock solution of beehive ethanol extract was prepared at a concentration of 2000 ppm. Seawater was added into 20 mg beehive ethanol extract until the volume reached 10 mL to make the solution at a concentration of 2000 ppm.

Preparation of bacteria ethanol extract

Bacteria with the highest antibacterial activity (BSL4) was inoculated into 50 mL of Nutrient Broth medium in Erlenmeyer and incubated in a shaker incubator at 150 rpm for two days at room temperature. Cells were separated by centrifugation at 6000 rpm for 30 minutes; then the supernatant was collected and added with ethanol (1: 1 v/v) and homogenized until two layers were formed. The solvent fraction was concentrated using rotary evaporator at 50°C.

Brine Shrimp Lethality Test (BSLT)

Six concentrations of extract with the concentrations of 1000 ppm, 500 ppm, 250 ppm, 126 ppm, 64 ppm and 32 ppm were prepared by pipetting respectively 2500 µL, 1250 µL, 625 µL, 315 µL, 160 µL, and 80 µL of stock solution into test tubes and added with 2 mL seawater, 10 larvae of brine shrimp and seawater was added again until the volume of each tube reaching 5 mL. The number of dead larvae was counted after 24 hours. Total mortality was obtained by summing the dead larvae in each concentration. Each sample was tested with three replications (triplicate). The control was conducted with the same method as the sample without adding crude extract. Negative control was contained only larvae of brine shrimp and seawater (Kumala and sapitri et al. 2011).

Data analysis

Data from the results were analyzed descriptively presented in the form of tables and images, and the LC50 value was analyzed by probit analysis using SPSS Statistics 23.0.

RESULTS AND DISCUSSION

Isolation of bacteria from the beehive

By culturing *A. cerana* hive on NA medium, it was found a total of 4 bacteria isolates from *A. cerana* hive. The results of previous studies showed a different number of bacteria isolates. Promnuan et al. (2009) reported one isolate from *A. florea* hive, two isolates from *A. cerana* hive, eight isolates from *A. mellifera* hive, eight isolates from stingless bee *Trigona laeviceps* hive, and four isolates from stingless bee *Trigona fuscobalteata* hive. Gilliam et al. (1990) reported three bacteria isolates from stingless bee *Melipona fasciata* hive, while Piccini et al. (2004) reported 16 bacteria isolates from 12 *Apis* sp. hives.

Figure 1. *Apis cerana*

The number of bacteria obtained from beehive was influenced by several factors. According to Promnuan et al. (2009), the factor that influences the number of bacteria to isolate was the geographical or environmental location of the beehive. Bacteria enter the hive through worker bees that collect food or water from the environment outside the hive. Plants that were used as food sources and raw materials for making a hive for each bee was different depending on the environment around the hive. Therefore, the number of bacteria species in each beehive was varied.

Morphological and physiological characteristics of *A. cerana* Beehive bacteria

Characterization of bacterial isolates was conducted macroscopically and microscopically. Macroscopic characterization was carried out by observing morphological characters of bacteria colonies, i.e., colony form, margin, color, and elevation (Figure 2). The morphology of bacterial isolates presented in Table 1.

Based on the observations, bacteria isolates had different colony forms, margins, colors, and elevations. Morphological characterization of bacteria was carried out based on Prescott's Microbiology (2002). According to Prescott et al. (2002), bacteria that cultured on agar medium would form a colony with different patterns. This pattern varied due to the ability of bacteria to absorb nutrients from the agar medium.

Microscopic characterization was carried out by Gram staining, to observe bacteria cell form and to differentiate bacteria into Gram-positive and Gram-negative bacteria. The results of Gram staining presented in Table 2.

Based on Gram staining (Figure 3), BSL1, BSL2, and BSL3 isolates belonged to Gram-positive bacteria, while BSL4 belonged to Gram-negative bacteria. Based on the observations, it was shown that BSL1 Gram-positive coccobacilli form, BSL2 was Gram-positive coccus form, BSL3 was Gram-positive rod/bacilli, and BSL4 was Gram-negative coccus form.

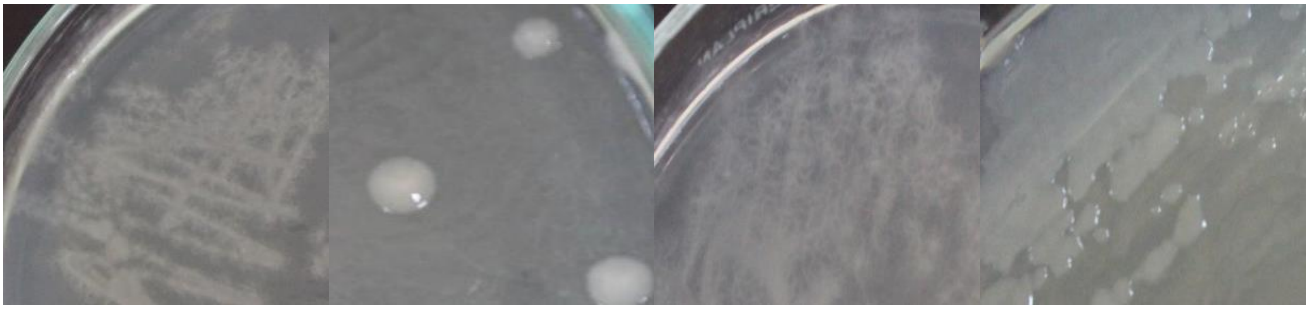
Table 1. Morphology of bacteria isolated from *Apis cerana* beehive

Isolate	Morphology			
	Colony form	Margin	Color	Elevation
BSL1	Filamentous	Thread-like	White	Flat
BSL2	Circular	Smooth	Milky white	Convex
BSL3	Filamentous	Thread-like	White	Flat
BSL4	Circular	Smooth	White	Raised

Note: BSL: bacteria isolated from the beehive

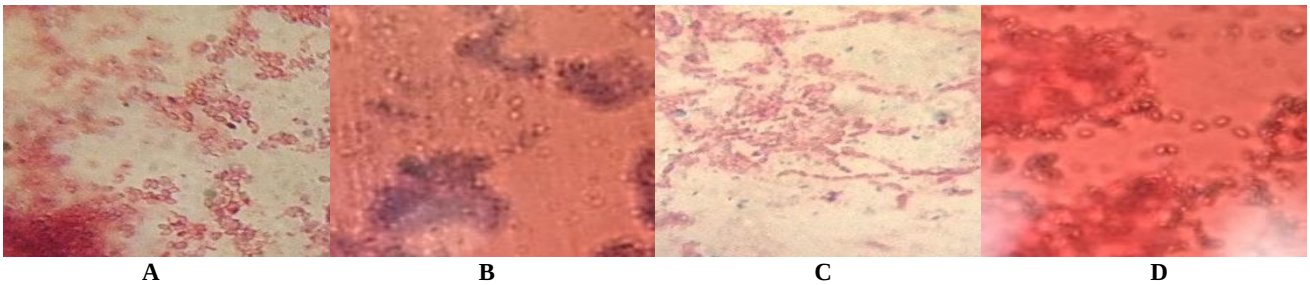
Table 2. Gram staining of *Apis cerana* hive bacteria

Isolate	Cell form	Gram staining	
		Positive (violet)	Negative (pink/red)
BSL1	Coccobacilli	+	
BSL2	Coccus	+	
BSL3	Rod/bacilli	+	
BSL4	Coccus		+



A B C D

Figure 2. Macroscopic morphology of bacteria isolated from the beehive on NA medium. A. BSL1, B. BSL2, C. BSL3, D. BSL4



A B C D

Figure 3. Gram staining of *Apis cerana* hive bacteria. A. BSL1, B. BSL2, C. BSL3, D. BSL4

Table 3. Biochemical properties of *Apis cerana* hive bacteria

Test	Isolate			
	BSL1	**BSL2**	**BSL3**	**BSL4**
Catalase	+	+	-	+
Motility	+	-	+	-
Indole	-	-	-	-
MR	-	-	+	-
Simmons citrate	-	+	-	+
TSIA:				
Glucose	+	+	+	+
Glucose dan sucrose	-	-	-	-
H_2S	-	-	-	-
Gas	-	-	+	-

Table 4. The inhibitory zone of bacteria isolated from *Apis cerana* hive against *Staphylococcus aureus* and *Escherichia coli*

Isolate	Mean of inhibition zone (mm)	
	S. aureus	***E. coli***
BSL1	13,8	0
BSL2	14,05	0
BSL3	0	0
BSL4	32,6	0

Biochemical test of *A. cerana* hive bacteria was carried out to identify bacteria isolated from the beehive. The biochemical test included catalase, motility, indole, Methyl Red (MR), Simmons Citrate, and TSIA test. Biochemical test of bacteria from beehive presented in Table 3.

Identification of bacteria isolated from beehive based on bacteria characteristics was carried out according to the Bergey's Manual of Determinative Bacteria (Holt et al. 1994) Isolates of BSL1 and BSL3 have the characteristics of the genus *Bacillus.* According to Holt et al. (1994), *Bacillus* is a genus of Gram-positive bacteria. *Bacillus* species showed a very broad range of colonial morphologies, affected by medium composition and incubation conditions. The colonies of the genus *Bacillus* were characteristically large (2-7 mm in diameter) and vary in shape from round to irregular. The surface is matt or granular textures, but the colonies were commonly smooth, moist, shiny, slimy, sometimes spread out and covered the entire surface. The elevation was flat, raised, and convex. The colors generally ranged from pale, gray or white, and occasionally produce black, brown, orange, pink or yellow pigment. The pigment produced by bacterial colony tends to be the characteristic of a species or subspecies.

Based on the characteristics of BSL2 isolate, it was identified as the genus *Micrococcus.* According to Cowan and Steels (1993), Colony of the genus *Micrococcus* was yellow or cream-colored, round shape, and raised colony margin. The cell was round and forming a pair, four pairs, or a non-fixed group that does not form chains, with a diameter of 0.5-2.0 μm, Gram-positive, not motile, positive catalase, negative oxidase, positive methyl red, no spore, did not produce acid, and the optimum growth temperature was from 30-37°C. *Micrococcus* sp. could be found in mammalian skin and soil, but usually isolated from food products and the air.

Based on the characteristics, BSL4 has the characteristics of the genus *Neisseria*. According to Holt et al. (1994), the genus *Neisseria* are Gram-negative bacteria in the form of cocci, generally not motile, does not produce acid and negative indole. The genus *Neisseria* has a broad range of environments; it could be found in the human body, water, soil, and air.

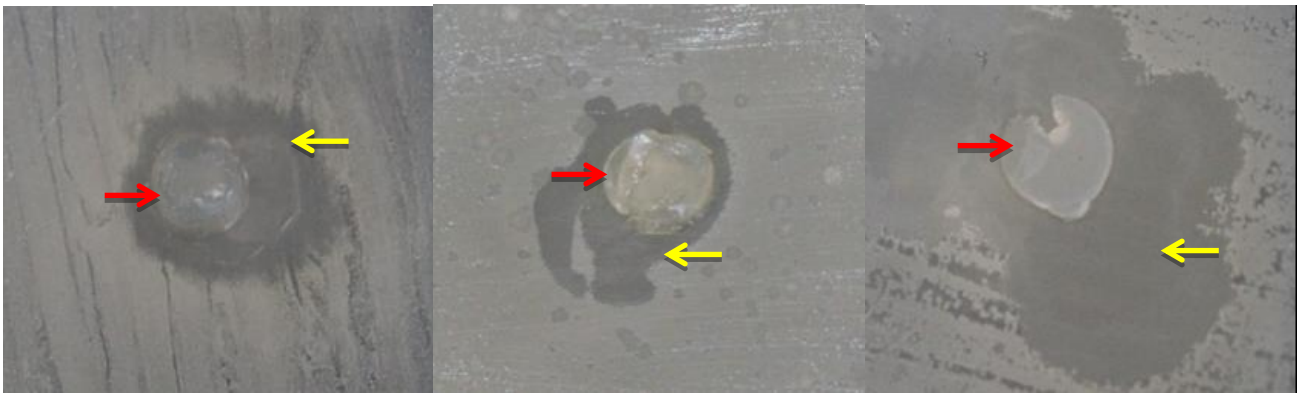
Previous studies showed that various species of the genus *Bacillus* had been isolated from a stingless beehive (Gilliam et al. (1990) and from *Apis* sp. hive (Piccini et al. 2015). Promnuan et al. (2009) reported several bacteria that commonly found in the soil were also found in a beehive. According to Piccini et al. (2015), plants around the hive were used as food sources and raw materials for making the hive. Therefore it was very likely to find various types of bacteria in *A. cerana* hive.

Antibacterial activity test

Antibacterial activity test was carried out by observing and measuring the diameter of the inhibitory zone of bacteria isolated from *A. cerana* hive against *Staphylococcus aureus* and *Escherichia coli*. The results were presented in Table 4.

BSL1, BSL2, and BSL4 isolates were able to inhibit the growth of *S.aureus* with the diameter of the inhibitory zone were 13.8 mm, 14.05 mm, and 32.6 mm, respectively. Gupte (2010) stated that that the diameter of the inhibitory zone indicated bacteria sensitivity to the antibacterial agents. BSL1 and BSL2 have strong antibacterial activity, whereas BSL4 has very strong antibacterial activity based on the antibacterial categories by Davis and Stout (1971). According to Davis and Stout (1971) the antibacterial potency categorized based on the diameter of the inhibitory zone as follows: very strong (>20 mm), strong (sensitive) (10-20 mm), intermediates (5-10 mm), and weak (resistant) (<5 mm). Dharmawan et al. (2009) stated that the inhibitory zone was varied for different species because every species produces different secondary metabolites and different concentrations of secondary metabolites.

BSL1, BSL2, and BSL4 were able to inhibit the growth of Gram-positive bacteria (*S. aureus*) but were unable to inhibit the growth of Gram-negative bacteria (*E. coli*). It was assumed due to the structural differences of the bacterial cell wall. Bacteria cell walls determined the characteristics and primary function to protect the inside of the cell. The cell wall of Gram-negative bacteria consists of several layers, while Gram-positive bacteria have a relatively simpler cell wall structure than Gram-negative bacteria. The gram-positive cell wall is composed of relatively thick peptidoglycan layer, surrounded by teichoic acid and in some species has polysaccharide. The gram-negative cell wall has a relatively thin layer of peptidoglycan, surrounded by a lipoprotein, lipopolysaccharide, phospholipid, and some proteins (Hardy 2002).



A **B** **C**

Figure 4. Inhibitory zone of *Apis cerana* hive bacteria against *Staphylococcus aureus.* A. BSL1; B. BSL2; C. BSL4. Note: → : Bacteria isolate; → : Inhibitory zone

Staphylococcus aureus is a Gram-positive bacteria with thick cell walls consist of peptidoglycan. According to Brooks et al. (2013), peptidoglycan is a component that determines cell wall flexibility at Gram-positive and plays a role in Gram-positive integrity. The antibacterial activity is more susceptible to Gram-positive bacteria because the outer membrane of the cell wall is only protected by peptidoglycan so that more accessible to antibiotic molecules. Gram-negative bacteria contain lipopoly-saccharide on the outer membrane result in the bacteria to be more resistant to certain types of antibiotics, while Gram-positive bacteria are more susceptible to antibiotics.

Toxicity test of *Apis cerana* hive bacteria: Brine Shrimp Lethality Test (BSLT)

Toxicity tests were carried out on beehive extract, and bacteria extract using Brine Shrimp Lethality Test (BSLT). Beehive and beehive bacteria were extracted using ethanol because it has high polarity so that they can extract more chemical compounds compared to other organic solvents. Ethanol also has a low boiling point, tends to be safe or non-toxic (Gamse 2002). According to Meyer et al. (1982), BSLT was carried out by observing the mortality rate of brine shrimp larvae after treating with the extract. Toxic compounds cause high mortality. Mardany et al. (2016) stated that mortality in *Artemia salina* larvae caused by toxic compounds that enter the body, resulting in changes in concentration inside and outside the cell. After these toxic compounds enter orally and dermally, and absorbed into the tissues and attack cells, followed by functional and metabolic damage. The effect occurs quickly within 24 hours because *A. salina* had a very thin skin membrane so that it allows diffusion of substances from the environment. Furthermore, Mardany et al. (2016) stated that the growth phase of shrimp larvae used in BSLT was the nauplius phase as being the most active phase.

The results showed that the mortality rate of *A. salina* larvae treated with beehive and bacteria isolated from beehive after 24 hours in various concentrations was shown in Table 5.

Table 5. The mortality rate of *Artemia salina* larvae treated with beehive and bacteria isolated from beehive extract

Concentration	% Mortality	
	Beehive ethanol extract	Bsl4 ethanol extract
0	0	0
32	15	10
64	55	50
126	75	60
250	95	85
500	95	95
1000	100	100

The extracts of beehive and bacteria isolated from beehive result in brine shrimp larval death, increasing concentration of extract results in an increased mortality rate. The highest mortality occurred in extracts with a concentration of 1000 ppm, while the lowest mortality percentage was at 32 ppm, negative control treatment/ without extract did not result in larval death. Based on the probit analysis by SPSS it showed that the LC_{50} value of *A. cerana* hive extract was 67,744 ppm and LC_{50} value of *A. cerana* hive bacteria extract was 86,985 ppm. It showed that bioactive compounds in beehive were more toxic than bioactive compounds produced by BSL4. A study by Thamrin et al. (2016) showed that the LC_{50} value of *Trigona incisa* hive ethanol extract was 355.46 ppm. Secondary metabolites of extracts affected LC_{50} value.

Meyer et al. (1982) stated that the higher the concentration of toxic secondary metabolites, the smaller the LC_{50} value. Toxicity level of extract categorized as follows: $LC_{50} \leq 30$ ppm = Very toxic; 31 ppm $\leq LC_{50} \leq$ 1000 ppm = Toxic, $LC_{50} >$ 1000 ppm = Not toxic. It showed that ethanol *A. cerana* hive extract of and *A. cerana* hive bacteria extracts were categorized as toxic.

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