



## Royal Jelly (*Apis mellifera*) As an Antimicrobial Activity Inhibition

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**ABSTRACT:** Royal jelly (RJ) has lots of compounds such as protein, vitamins, and a variety of bioactive compounds (10-hydroxy-2-decanoic or 10-HDA acids). 10-HDA is a lipid fraction component in RJ content with antibacterial and immunomodulatory activity. RJ proteins that have antimicrobial, antifungal, anti-tumor, antidiabetic, and antihypertensive properties. The authors continue their research about the effect of RJ on the inhibitory strength of the given antibacterial activity. *L. monocytogenes*, *S. aureus*, and *E. coli* were among the microbes studied in this research. This study used an experimental method with a complete randomized design (CRD) of 5 treatments and 4 replications, if the treatment showed a noticeable effect ( $P < 0.05$ ) then Duncan's multiple distance test was continued. According to the findings of this study, the royal jelly variations given had a significant inhibitory effect on antibacterial activity. The inhibitory ability of *L. monocytogenes* proved that the second treatment had a higher inhibitory power of  $30.94 \pm 25.786^b$ , in *S. aureus*, the second treatment gave a moderate inhibitory power of  $9.013 \pm 1.841^a$ , and in *E. coli*, the second treatment gave a weak inhibitory power of  $3.24 \pm 3.748^a$ . The conclusion of studies showed royal jelly with dilution variety could be antimicrobial agent and to be strongest inhibitor in *L. monocytogenes* and lowest in *E. coli*. The suggestion of studies needs management in the isolates of bacteria available and should be estimated before we do the research about antimicrobial activity inhibitors.

**KEYWORDS:** Antimicrobial; *E. coli*; Inhibition zone; *L. monocytogenes*; Royal jelly; *S. aureus*

### INTRODUCTION

Antimicrobials are compounds that can prevent or kill the growth of potentially hazardous microbes. Antimicrobials are classified into two types based on their ability: bacteriostatic, which prevents growth of bacteria, and bactericidal, which kills microbes. Plant parts containing antimicrobial agent chemicals include rhizomes, stems, leaves, tubers, and seeds (Tharukliling *et al.*, 2021). Furthermore, antimicrobial compounds can be found in a variety of beekeeping products, including propolis, bee pollen, and royal jelly. Royal jelly has a protein level that ranges between 27 and 41%. The amino acids identified in the highest concentrations in royal jelly include proline, lysine, glutamate acid, -alanine, phenylalanine, aspartate, and serine. The concentration of free amino acids, polypeptides, sugars, fatty acids, and minerals in royal jelly (RJ) could inhibit tumor growth and prevent malignant invasion (Khoob *et al.*, 2022). In addition, royal jelly is a beekeeping product that functions as an antifungal, antitumor, antihypertensive, and antidiabetic agent. The texture and colour of royal jelly are thick and yellowish-white, with a characteristic phenol odour and an acidic pH (pH value: 3.4–4.5). The acid component of royal jelly can prevent some test microbes from growing.

There are various test bacteria usually used to evaluate microbial inhibition between *S. aureus* and *E. coli*, whereas *L. monocytogenes* test bacteria are used in some laboratories. The cell membranes of these bacteria differ. Differences in cell membranes are capable of test bacteria's susceptibility to antimicrobial treatments. *L. monocytogenes* bacteria are gram-positive, aerobic, non-spore-forming, catalase-positive, and rod-shaped (Duze *et al.*, 2021). *L. monocytogenes* had a cell size of 6-12 mm, and the genus *Listeria* include two pathogenic species, *L. monocytogenes* (Lm), *L. ivanovii*, and non-pathogenic species. *L. monocytogenes* is part of the typical microbial contamination that occurs in foods. Each individual in the Western world is expected to be infected to the microbes several times per year. (Ricci *et al.*, 2018). *L. monocytogenes* is a microbe that can survive in pH (4-9), refrigeration temperature ( $-1.5^\circ\text{C}$  to  $45^\circ\text{C}$ ), and significant salinity (a dissolved salt in water concentration of  $\geq 0.92$ ) (Townsend *et al.*, 2021; Duze *et al.*, 2021; Zhang *et al.*, 2021). *S. aureus* is another gram-positive microbe that grows quickly at  $37^\circ\text{C}$ , while pigment production is better at general temperatures of  $20-25^\circ\text{C}$ . This microbe lives in warm-blooded animal mucous membranes, ulcers, skin, and wounds, and it can cause disease due to its capacity to multiply and colony widely in tissues (Tuntun, 2016). Pigments present in *S. aureus* are generally golden



yellow, cause coagulation, and can ferment glucose and mannitol into lactic acid in an anaerobic state, however this will prevent growth (Sulistiyowati, 2012). According to Karomah (2019), the ideal pH for microbial growth is between 7.0 and 7.5, and microbes grow well in an oxygen-rich environment (O<sub>2</sub>). Bacterial *S. aureus* will die after 60 minutes at 60°C. (Arfani, 2021). Meanwhile, gram-negative microbes have been identified in *E. coli*. intended *E. coli* can create more hemi-, mono-, and tetraterpenoids than ordinary species, whereas designed yeast strains can produce more sesqui- and di-antriterpenoid chemicals (Rinaldi *et al.*, 2022). Glucose is consumed by *E. coli* growths. A carbon source, glucose, is introduced to a minimally liquid culture medium for microbial development. (Luca *et al.*, 2022). Microbe cells from *E. coli* have a double membrane wall structure, with the plasma membrane surrounded by a permeable outer membrane. Compared to *S. aureus*, which has a single plasma membrane surrounded by peptidoglycan.

There is some of the test microbes will experience growth inhibition or will be sensitive to royal jelly as an antibacterial agent. Without any testing, royal jelly is often just used as an antimicrobial. As a result, research into the antimicrobial activity inhibitory potency of royal jelly must be innovative. The material is produced in the form of antibacterial compounds at various dilution concentrations. It could be expected that by various the concentration of royal jelly, we would be able to analyse the inhibitory activity of antimicrobial testing. The disc diffusion method (*Kirby-Bauer*) is used to test antimicrobial inhibitory activity. The disc diffusion method is frequently used to test antimicrobial resistance to antibiotics. This method uses a filter paper known as a paper disc, which works as an incubator for antimicrobial compounds. Filter paper can be placed over the top of agar that has been inoculated with test microbes and then incubated at a specific time and temperature based on the test bacteria' optimum conditions.

## MATERIALS AND METHODS

### Making Royal Jelly *Apis mellifera*

Royal jelly can be obtained by the formation of queen cells (*Queen rearing*); the manufacture of queen cells can be done using the *Doolittle* method. *Doolittle* obtained approximately 50 queen cups (artificial queen cells), this method is recommended for obtaining commercial royal jelly products. Previous research (Rompas, 2015) states that the content of royal jelly harvested in three-day-old queen-to-be larvae is as much as 200 mg for one bowl of queen. If a queen cup has 15 cells formed, then royal jelly produces as much as three grams of royal jelly per colony per period. If only 50 queen cells are formed, then 10 grams of royal jelly per colony per period are obtained. Royal jelly is produced by worker bees aged 2 – 3 days. The process of taking royal jelly (*grafting*) takes 3 x 24 hours after that, as much as ±48 grams are obtained from all queen cups.

### Royal Jelly Saving Management

The royal jelly used in this study came from the results of harvesting during the study. Royal jelly must be obtained using a clean, smooth, and strong white cloth made of nylon fabric size 100 mesh. The purpose of this filter is to ensure that there is no dirt on the royal jelly. Then packaged using a translucent glass container with a volume size of 50 grams and avoided direct sun exposure. In order to make royal jelly more durable, it can be stored in freezers at temperatures around -5°C to -7°C.

### Na (Nutrient Agar)

*Nutrient agar* is used for bacterial growth media in petri dishes. The NA manufacturing procedure is as follows:

- a) During research activities, make sure you have used lab coats and hand sanitizer carried out an aseptically sterile process;
- b) Prepared tools and materials that have been sterilized in advance;
- c) Weigh NA on analytical balances as needed. The study used 6.9 grams;
- d) Poured *aquadest* into Erlenmeyer, as much as 300 mL;
- e) The weighed NA was then put into the Erlenmeyer containing *the aquadest*;
- f) Placed the ingredients on a *hot plate*, and the chili sauce is stirred slowly with a stirrer until it dissolves into *the aquadest*;
- g) Turn off the *hot plate* when it is completely dissolved;
- h) Let the NA solution sit for a while, then cover the Erlenmeyer tube hole with a cotton *ball* or cotton that has been rolled up to cover the entire Erlenmeyer hole with covered using aluminum foil or cover paper. Make sure the entire top surface of the Erlenmeyer is tightly covered and put in clear plastic for sterilization;
- i) The solution sterilization process was carried out using a 121°C *autoclave* for 15 minutes;
- j) After sterilization, the solution is ready for use.



- k) NA is poured into a petri dish of  $\pm 15$  mL, and the petri dish is homogenized to form a figure eight. This is done so that the NA does not clump on one side and flatten on all parts of the cup;
- l) Waited for NA to form agar;
- m) Done the above method repeatedly on another cup.

## NB (Nutrient Broth)

*Nutrient broth* is used for the inoculation of bacteria in test tubes. The NB creation procedure is as follows:

- a) During research activities, make sure to use lab coats and gloves and carry out an aseptically sterile process;
- b) Prepared tools and materials that have been sterilized in advance;
- c) Weighted NB on analytical balances as needed. The study used 1.68 grams;
- d) Poured *aquadest* into a beaker glass, as much as 180 mL;
- e) The weighed NB is then put into a beaker glass containing *aquadest*;
- f) Gently stir NB with *aquadest* until dissolved;
- g) If it has dissolved, NB can be sterilized by closing the beaker glass hole using wrap paper or aluminum foil until completely closed;
- h) The sterilization process was carried out using a  $121^{\circ}\text{C}$  *autoclave* for 15 minutes;
- i) After sterilization, the solution is ready for use;
- j) NB solution is taken using a pipette, as much as 9 mL;
- k) The NB solution is inserted into the marked test tube;
- l) Do it repeatedly until all the necessary test tubes are filled.

## Bacterial Culture (*L. Monocytogenes*, *S. Aureus* & *E. Coli*)

Bacterial cultures are performed the day before antimicrobial testing. This happens because of the length of the incubation process, which is 1x24 hours. The procedure for making bacterial cultures is as follows:

- a) During research activities, make sure to use lab coats and gloves and carry out an aseptically sterile process;
- b) Prepared tools and materials;
- c) Sterilization of tools and materials to be used for the manufacture of bacterial cultures (*L. Monocytogenes*, *S. Aureus* & *E. coli*) using a  $121^{\circ}\text{C}$  *autoclave* for 15 minutes;
- d) While waiting for the sterilization process, an aseptic sterilization process can be carried out on *Laminar Air Flow* (LAF). The sterilization process in LAF is as follows:
  - 1) Slightly opened LAF glass so that air circulation inside the LAF can move out with the help of a fan;
  - 2) Turn on the UV lamp on the button that is available in the LAF (the function of the UV lamp in this LAF is to kill germs in the LAF) and keep the LAF sterile. The UV lamp is turned on for  $\pm 15$  minutes;
  - 3) Turned on the fan button (the fan inside the LAF) to help air circulation inside move out.
  - 4) After 15 minutes, the UV lamp and fan on the LAF can be turned off, and the LAF table can be sprayed with 70% alcohol and wiped in one direction.
- e) After the sterilization process is complete, the tools and materials in the *autoclave* can be transferred into the LAF;
- f) Taken a bacterial culture from the incubator;
- g) To keep the bunsen lamp sterile, light it with a match and keep it close to the bacterial inoculation activities;
- h) Open the lid of the test tube hole and keep the test tube cap in the palm of your hand;
- i) Performed sterile aseptically on the surface of the test tube hole so that no other bacteria enter the test tube;
- j) Dip a *blue tip* into a test tube that has contained bacterial cultures, and take bacteria as much as 1 mL;
- k) Re-closed the test tube hole cap with a *cotton ball*;
- l) Transferred bacteria that have been taken into the NB in the test tube;
- m) Do point (f) repeatedly until the test tube is filled with bacteria;
- n) Test tubes that have been given bacteria are then inserted into the incubator for incubation for 2 x 24 hours.



## Water Injection 100 mL (sterile)

Water injection is poured into the beaker glass and measured to as much as 100 mL, then the beaker glass is covered with wrap paper or aluminum foil for the sterilization process at the 121°C *autoclave* for 15 minutes.

## Antimicrobial agents (Royal jelly diluted with various concentrations)

- a) During research activities, make sure to use lab coats and gloves and carry out an aseptically sterile process;
- b) Prepared tools and materials;
- c) Sterilization of tools and materials to be used for making royal jelly dilution using a 121°C *autoclave* for 15 minutes;
- d) While waiting for the sterilization process, an aseptic sterilization process can be carried out on *Laminar Air Flow* (LAF). The sterilization process in LAF is as follows:
  - 1) Slightly opened LAF glass so that air circulation inside the LAF can move out with the help of a *fan*;
  - 2) Turn on the UV lamp on the button that is available in the LAF (The function of the UV lamp in this LAF is to kill germs in the LAF) and keep the LAF agar sterile. UV lamp turned on for ±15 minutes;
  - 3) Turned on the fan button (fan inside LAF) to help circulation inside move out;
  - 4) After 15 minutes, the UV lamp and *fan* on the LAF can be turned off, and the LAF table can be sprayed with 70% alcohol and wiped in one direction.
- e) After the sterilization process is complete, the tools and materials in the *autoclave* can be transferred into the LAF;
- f) Poured reaction *water injection* into each test tube as much as 100 µL;
- g) Royal jelly was taken using micropipettes with various concentrations (4x10<sup>-2</sup> µL in the first test tube, 4x10<sup>-3</sup> µL in the second test tube, 4x10<sup>-4</sup> µL in the third test tube, and 4x10<sup>-5</sup> µL in the fourth test tube) ;
- h) Homogenized royal jelly mixture with *water injection* in the first test tube using a vortex for 2-3 minutes;
- i) The bottom of the royal jelly mixture solution and *water injection* of 40 µL was taken in the first test tube, then transferred to the second test tube;
- j) Homogenized royal jelly mixture with *water injection* in the second test tube using a vortex for 2-3 minutes;
- k) The bottom of the royal jelly mixture solution and *water injection* of 40 µL was taken in the second test tube, then transferred to the third test tube;
- l) Homogenized royal jelly mixture with *water injection* in the third test tube using a vortex for 2-3 minutes;
- m) The bottom of the royal jelly mixture solution and *water injection* of 40 µL was taken in the third test tube, then transferred to the fourth test tube;
- n) Homogenized royal jelly mixture with *water injection* in the fourth test tube using a vortex for 2-3 minutes.

## Aquadest

The aquadest function in this study was used for solvents in NA and NB. Aquadest is also used for sterilization in *autoclaves*. This study used 30 liters of aquadest.

## Paper disc (Using Whattman No. 41 filter paper)

The paper disc used was Whattman filter paper No. 41; this material was chosen because it is easy to obtain and can easily absorb solutions. Paper discs were previously cut and shaped in circles using a paper punching tool; the amount of paper used was as needed. This research requires ±40 papers that have been formed into circles with a diameter of 5 mm.

## Data Analysis

Microbial inhibitory zone analysis using measurements with digital calipers. The inhibitory zone formed on the cup was drawn using a black marker. Next, measurements will be measured horizontally and vertically in the zone formed. The data obtained will be inputted in Microsoft Excel and presented in the form of tables and diagrams. The data was statistically analyzed by using analysis of variance (ANOVA) from a Completely Randomized Design (CRD) with four treatments (1 x 10<sup>1</sup> µL, 2 x 10<sup>2</sup> µL, 4 x 10<sup>3</sup> µL, and 6 x 10<sup>4</sup> µL) and four replications. If a significant difference (P<0.05) is obtained, Duncan's Multiple Range Test is used (Sudarwati *et al.*, 2019). The following is formula applied for statistical analysis:

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$$



Information:

$Y_{ij}$  = observation on treatment  $i$  and replication  $j$

$\mu$  = general average

$\tau_i$  = the effect of the  $i$ - $j$  treatment level

$\epsilon_{ij}$  = random effect on treatment  $i$  and replication  $j$

## RESULTS AND DISCUSSION

### Result

Concentration of royal jelly revealed a highly significant difference ( $P < 0.05$ ) against the test microbes, notably *L. monocytogenes*, *S. aureus*, and *E. coli*. Antimicrobial inhibitory actions have distinct effects on the three microbes in all. In a dilution concentration of  $2 \times 10^2 \mu\text{L}$ , *E. coli* microbes were more resistant to royal jelly at P2. While *E. coli* bacteria show that plasma membrane cells are double, so that inhibitory zone is feeble. Figure (1) illustrates the diameter category of antimicrobial inhibitory zones as follows:

Diameter	Kekuatan Daya Hambat
$\leq 5$ mm	Lemah ( <i>weak</i> )
6–10 mm	Sedang ( <i>moderate</i> )
11–20 mm	Kuat ( <i>strong</i> )
$\geq 21$ mm	Sangat kuat ( <i>very strong</i> )

**Figure 1.** The Category of Inhibition Zone Diameter

Source: Surjowardojo *et al.*, 2015

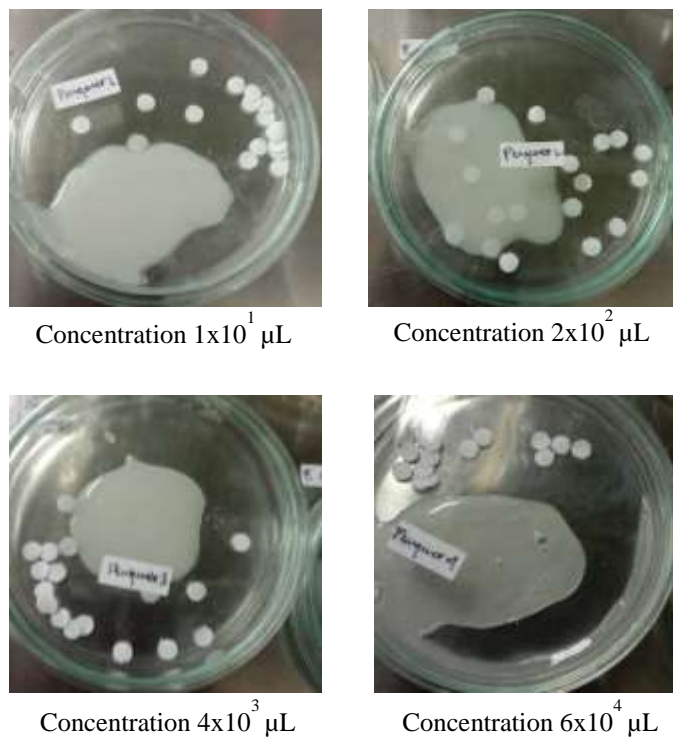
Figure (1) explains that the diameter of the inhibitory zone measuring  $\leq 5$  mm has weak resistance strength, medium inhibitory zone strength is between 6 – 10 mm in diameter, the strong inhibitory zone has a diameter of 11 – 20 mm, and the diameter of the inhibitory zone  $\geq 21$  mm can be said to be very strong. While the average test results of the inhibitory zone in each microbe will be seen in Table (1), they are as follows:

Name of Microbial	Microbial Inhibition Zone (mm)			
	Average $\pm$ SD			
	P1 ( $1 \times 10^1 \mu\text{L}$ )	P2 ( $2 \times 10^2 \mu\text{L}$ )	P3 ( $4 \times 10^3 \mu\text{L}$ )	P4 ( $6 \times 10^4 \mu\text{L}$ )
<i>L. monocytogenes</i>	$20.76 \pm 8.446^{ab}$	$30.94 \pm 25.786^b$	$7.91 \pm 2.014^a$	$10.58 \pm 3.902^{ab}$
<i>S. aureus</i>	$8.01 \pm 3.578^a$	$9.01 \pm 1.841^a$	$8.05 \pm 1.035^a$	$7.79 \pm 3.165^a$
<i>E. coli</i>	$7.11 \pm 0.920^{ab}$	$3.24 \pm 3.748^a$	$8.76 \pm 5.131^b$	$3.64 \pm 1.659^{ab}$

Note: <sup>a, b</sup> = Different superscripts on the same line showed that variations in the concentration and dilution of royal jelly exerted a marked effect ( $P < 0.05$ ) on the antimicrobial inhibitory power test.

Antimicrobial activity can be seen in Figure (2). While the antimicrobial agent used is a variation in the concentration of royal jelly. Royal jelly concentrations of  $1 \times 10^1 \mu\text{L}$ ,  $2 \times 10^2 \mu\text{L}$ ,  $4 \times 10^3 \mu\text{L}$ ,  $6 \times 10^4 \mu\text{L}$  can be seen in Figure (3).





**Figure 2.** Variation of Concentration

### Discussion

The presence of several substances contained in royal jelly that entered cell membranes, such as 10-hydroxy-2-decanoic acid (10-HDA), caused statistically significant differences in the antimicrobial activity of royal jelly against the three test bacteria, namely *L. monocytogenes*, *S. aureus*, and *E. coli*. 10-HDA is a lipid fraction of royal jelly that contains antimicrobial and immunomodulatory activities. Furthermore, multiple royal jelly proteins, such as *Major Royal Jelly Proteins* (MRJPs) and royalisin, can inhibit the microbes such as *L. monocytogenes*, *S. aureus*, and *E. coli*. *L. monocytogenes* has been resistant to royal jelly, such as in Table (1). This is because *L. monocytogenes* essentially becomes resistant to antimicrobial agents through the acquisition of cellular genetic elements such as plasmids, transposons, and integrons. Environmental stresses such as osmotic, acidic, oxidative, and cold contribute to increased antimicrobial resistance in *L. monocytogenes*. The royal jelly used contains acids with a pH of 3.4–4.3. While the optimum pH for *live L. monocytogenes* is 4–9.6. Acidic environmental conditions will inhibit *L. monocytogenes*' growth and cause it to die. P2 treatment with as much as  $2 \times 10^2 \mu\text{L}$  shows that the inhibitory power produced by it is very significant. It turns out that *E. coli* microbes have a feeble inhibitory power when given this treatment. This is due to *E. coli*'s double-system cell wall, in which the cell membrane is surrounded by a permeable outer membrane. *S. aureus* and *E. coli* are both gram-positive pathogens, but *S. aureus* possesses a single-system cell wall. Furthermore, *S. aureus* can survive in alkaline pH surroundings ranging from 7 to 7.6. If the antimicrobial substance used is acidic, *S. aureus* microbes will be susceptible to or will die under acidic conditions.

### CONCLUSION

Antimicrobial agents given in a form of royal jelly have an effect on the inhibitory capacity of microbial activity. The more acidic the environment in which the antimicrobial agent is given, the greater potential of bacterial resistance, which results in microbes that are unable to survive or die off. Not only that, but the structure of the test microbial cell membrane influences the level of antibacterial inhibition. The thicker the cell wall of the test bacteria, less probable the antimicrobial agent would penetrate the test microbial cell.



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