Indonesian Journal of Veterinary Sciences
Vol. 2. No. 2 September 2021, Page. 46-52
DOI:10.22146/ijvs.v2i2.83329, ISSN 2722-421X (Online)
Available online at https://jurnal.ugm.ac.id/ijvs

Antibacterial Activities of Honey, Papaya Leaf, Basil Leaf, and Temu Ireng Extract on the Growth of *Salmonella typhimurium*

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Received: May 11, 2021, Accepted: June 22, 2021, Published: September 1, 2021

Abstract

Indonesia is the second major nation in a term of its biodiversity after Brazil. Indonesia's rich biodiversity makes Indonesian herbs a prospect of developing herbal medicines for the benefit of health and industrial products. Honey, papaya leaves, basil leaves, and temu ireng are natural and herbal that have potential as an antibacterial. *Salmonella typhimurium* is a paratyphoid-causing bacteria that can harm the poultry industry. Paratyphoid is a disease that can cause weight loss and production in chickens. This study aimed to determine the antibacterial activity of honey (Kupang honey and Sumba honey), papaya leaves, basil leaves, and temu ireng to the growth of *S. typhimurium.* Reidentification of *S. typhimurium* was carried out to confirm the colony morphology, cell morphology, and biochemical characteristics. Bacteria were then cultured in Brain Heart Infusion and incubated at 37°C for 24 hours. The bacterial culture was centrifuged at a speed of 5000 rpm for 10 minutes. The precipitate formed is resuspended with sterile Phosphate Buffer Saline. The bacterial suspension is made with a concentration of 1.5 x 10^8 CFU/mL. Antibacterial activity of honey (100%), ethanol extract (100%) and aquadest extract (33,3%) from papaya leaves, basil leaves, and temu ireng against *S. typhimurium* were carried out by modified Kirby-Bauer disk diffusion method. The results of this study show that Kupang honey, Sumba honey, ethanol extract (100%) and aquadest extract (33,3%) from papaya leaves, basil leaves, and temu ireng have low antibacterial activity (<6mm). Whereas Gunung Kidul Lanceng honey (7.37mm), Lombok black honey (6.74 mm), Lombok white honey (7.74mm) and commercial honey (7.29) have moderate antibacterial activity.

Keywords: basil leaves, honey, papaya leaves, *Salmonella typhimurium,* temu ireng

Introduction

Indonesia is a country rich in biodiversity. This country is known as the second country after Brazil which has the largest biodiversity in the world. There are 30,000 types of herbs in Indonesia out of a total of around 40,000 known types of herbs in the world (BPPP, 2017; Directorate General, 2014). Abundant biodiversity makes herbs one of Indonesia's mainstay commodities (Putri *et al.,* 2014). According to the Directorate General (2014), Indonesia has prospects for developing herbal medicines for health purposes and industrial products. Herbs are not only used in human health, but herbs have started to be used in the veterinary world. Apart from having many herbal benefits it is easy to get.

Papaya leaves, basil leaves, and Intersection ireng are medicinal plants that are easy to find in Indonesia. These three herbs contain alkaloids, saponins, and flavonoids which have potential as antibacterials (Sudarwati, 2018; Angelina *et al.,* 2015; Baharun *et al.,* 2013). Apart from herbs, honey is a traditional medicine that can be used to treat digestive tract infections. Honey has flavonoid compounds and mechanisms of acidity, osmotic pressure, and hydrogen peroxidase enzymes which are antibacterial (Mulu *et al.,* 2005; Mundo *et al.,* 2004).

Salmonella typhimurium is a bacterium that causes paratyphoid which can harm the poultry industry. Paratyphoid is a disease that can cause weight loss and production in chickens. This disease can cause mortality in young chickens.

These bacteria are bacteria that contaminate feed and can live in the environment for quite a long time (Saif *et al.,* 2008; Tabbu, 2000).

Antibiotic Growth Promoter (AGP) is commonly used in animal feed to prevent infection from pathogenic bacteria (Etikaningrum and Iwantoro, 2017). Control for Disease Control (CDC) estimates that around 40% of antibiotics in the world are used as AGP. Antibiotics added to animal feed range from 2.5 to 12.5 mg/kg which is a low dose (Noor and Masniari, 2005). According to a study conducted by Etikaningrum and Iwantoro (2017) many animal products such as meat and eggs were found to have antibiotic residues. The residue is caused by excessive use of antibiotics and inappropriate use. Humans indirectly consume antibiotic residues contained in animal products, so that resistance does not only occur in animals but also in humans (Barton, 2000). Therefore, the Indonesian government has made regulations regarding the prohibition of the use of AGP in livestock. This is regulated in the Regulation of the Minister of Agriculture of the Republic of Indonesia Number 14/PERMENTAN/PK.350/5/2017 concerning the classification of veterinary drugs.

The existence of regulations regarding the prohibition of the use of AGP, the availability of Indonesia's natural biodiversity that is easy to obtain, and is known to have many uses, makes herbal a subject that can be explored more deeply for its benefits. The content of antibacterial compounds contained in the six honey, papaya leaves, basil leaves and temu ireng is expected to inhibit S. typhimurium. The three herbs are expected to be used as an alternative to AGP so as to increase selling value.

Materials and methods

This study used the pathogenic bacterium *Salmonella typhimurium* from the Center for Veterinary Research (IRCVS, Bogor, Indonesia). The bacterial media used were: Xylose Lysine Deoxychocolate Agar (XLD, OxoidTM), Mueller Hinton Agar (MHA, MecrkTM), Broth Heart Infision (BHI, MerckTM), Indole, Methyl Red, Voges Proskauer, citrate, sucrose, glucose, sorbitol, lactose, mannitol, maltose, and Tripple Sugar Iron Agar (TSIA). Kovach's reagent, methyl red, alpha naptol, 40% KOH, H2O2 were used as reagents for biochemical tests. Bacterial staining using crystal violet, lugol, alcohol, and safranin. Other materials used were blank discs (OxoidTM), antibiotic discs Trimethopim (W 5µg, OxoidTM), Phosphate Buffer Saline (PBS, Sigma-aldrich®) and sterile distilled water. Papaya leaves (Carica papaya L.), basil leaves (Ocimum tenuiflorum L.), and rhizome of temu ireng (Curcuma aeruginosa Roxb.) used came from different areas. Papaya leaves were obtained from Magelang City, Central Java; basil leaves were obtained from Pakem District, Sleman, Yogyakarta; and temu ireng rhizome obtained from Kaliurang, Sleman, Yogyakarta. The three plants were identified at the Plant Systematics Laboratory, Faculty of Biology, Gadjah Mada University, with certificate number 01417/S. Tb./X/2018; 01428/ S.Tb/ XI/2018; and 01437/ S.Tb/ XII/ 2018. The honey used is honey from Kupang, Lanceng Gunung Kidul honey, Lombok black honey, Lombok white honey, Sumba honey, and Commercial honey. The tools used were petri dishes, test tubes, glass slides, microscopes, loops, Bunsen lamps, syringes, conical tubes, ependorf tubes, calipers, and tweezers.

Re-identification of *S. typhimurium* was carried out by culturing the bacteria on XLD media which is a selective and differential medium for Salmonella sp. and incubated at 37oC for 18-24 hours. Bacteria growing on XLD media were observed for colony morphology and Gram stained to confirm cell shape. Appropriate colony forms were cultured on BHI medium and incubated at 37^{o}C for 18 – 24 hours. After the bacteria grew, the bacteria were identified by biochemical tests. Salmonella thyhimurium biochemistry tests include catalase, oxidase, motility, indole, MR-VP, and citrate (IMViC) tests, urea, Triple Sugar Iron Agar, and carbohydrate fermentation tests (glucose, sucrose, lactose, sorbitol, maltose, and mannitol).

The ethanol extracts of papaya leaves, basil leaves, and temu ireng rhizomes were carried out in the extraction process at the Integrated Research and Testing Laboratory (LPPT) of Gadjah Mada University. The method used is the maceration extraction method. Extraction was carried out using 96% ethanol solvent. Aquades extract was carried out by mixing simplicia with sterile distilled water to a concentration of 33.3% (w/v).

The solution was allowed to stand for 24 hours at room temperature while stirring occasionally, then filtered aseptically. The process of making papaya leaf simplicia, basil leaves, and temu ireng rhizomes was carried out at LPPT Gadjah Mada University. The honey used is a type of honey from different origins, including Kupang honey, Gunung Kidul Lanceng honey, Lombok black honey, Lombok white honey, Sumba honey, and Commercial honey. Honey is put into the Ependorf tube.

S. typhimurium isolates were cultured in BHI and incubated at 37oC for 24 hours. The bacterial culture was centrifuged at 5000 rpm for 10 minutes. The precipitate formed was resuspended with sterile PBS. The suspension was made at a concentration of 1.5 x 108 CFU/mL. The sensitivity test for S. typhimurium was carried out using the Kierby-bauer disk diffusion method with some modifications. Blank disks were immersed in ethanol extract with a concentration of 100%, distilled water extract with a concentration of 33.3%, and honey with a concentration of 100%, respectively. The soaked disks are placed on the surface of the MHA media which has been cultured with S. typhimurium bacteria with sufficient distance between the disks one to another (Prakasita *et al.,* 2019). Trimethoprim antibiotic disks (W 5µg, OXOIDTM) were used as positive controls and blank discs soaked in sterile distilled water were used as negative controls. The media was incubated at 37oC for 18-24 hours. The clear zone formed around the disk is measured using a caliper.

Data analysis from the test results was carried out using descriptive analysis. The results of the re-identification of bacteria were compared with the results of identification that had been carried out previously in the existing literature. Data on the test results for the antibacterial activity of honey, 100% ethanol extract and 33.3% distilled water extract from papaya leaves, basil leaves, and Intersection ireng are presented in tabular and graphical form by comparing the diameters of the inhibition zones of honey and herbs.

Results and Discussion

*Salmonella typhimurium i*s one of the pathogenic bacteria that is in the digestive tract of chickens. This bacterium is one of the causes of paratyphoid fowl (Markey *et al.,* 2013). This disease can be detrimental to chicken farmers because it causes a decrease in body weight and production of chickens (Tabbu, 2000). The use of AGP in feed additives is used to minimize infection in poultry but the use of antibiotics in the long term can cause antibiotic resistance in animals and indirectly in humans. The government has issued Regulation of the Minister of Agriculture of the Republic of Indonesia Number 14/PERMENTAN/PK.350/5/2017 concerning the classification of veterinary drugs.

The use of natural materials such as honey from different places, ethanol extract and distilled water from papaya leaves, basil leaves, and temu ireng rhizomes, in this study aims to determine antibacterial activity against the growth of S. typhimurium bacteria. The antibacterial activity was seen by the Kierby-bauer disk diffusion test of ethanol extracts of papaya leaves, basil leaves, and Intersection ireng with a concentration of 100%; distilled water extract of papaya leaves, basil leaves, and Intersection ireng with a concentration of 33.3%; and honey with a concentration of 100%.

Re-identification of *S.typhimurium* was carried out to ensure that the bacteria used were

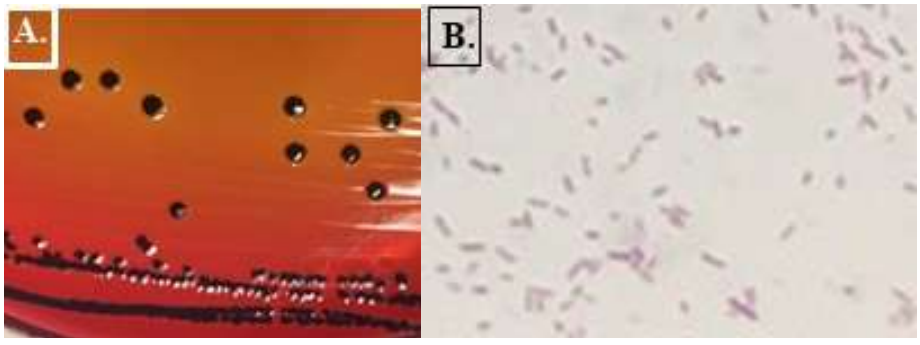

Figure 1. Morphology of S.typhimurium colonies colored red with a black dot in the middle on XLD media (A) and Morphology of short rods and red colored with Gram staining (B)

true and pure. *S.typhimurium* isolates cultured on XLD media, after incubation at 37oC for 18-24 hours, showed a round colony morphology with black dots (Figure 1). These results are in accordance with the statement of Leboffe et al. (2011) and Markey et al. (2013).

Appropriate colonies were followed by biochemical tests with catalase, oxidase, IMViC, motility, urease, TSIA, and carbohydrate fermentation test. The biochemical test results of *S. typhimurium* are in accordance with the research conducted by Markey et al. (2013), these results are presented in Table 1.

Table 1. Biochemical test results for S.typhimurium

Test	Result	Reference
Katalase	+	+
Oksidase	-	-
IMViC		
Indol	-	-
Methyl Red	+	+
Voges-proskauer	-	-
Citrate	+	+
Motilitas	+	+
Urease	-	-
TSIA		
Slant	Red	Red
Butt	Yellow	Yellow
H_2S	+	+
Fermentasi Karbohidrat		
Glukosa	+	+
Sukrosa	-	-
Laktosa	-	D
Sorbitol	+	+
Manitol	+	+
Maltosa	+	+

d = 26 -75% Positive (Markey *et al.,* 2013)

The herbal activity of papaya leaves, basil leaves, and rhizome of temu ireng and honey was examined using the Kierby-bauer disk diffusion method with modifications and carried out three times. The results showed that the antibacterial activity of ethanol extract with a concentration of 100% from papaya leaves, basil leaves, and the rhizome of Intersection Ireng on the growth of *S. typhimurium* showed an inhibition zone diameter of <6mm (Figure 3a). Aquades extract with a concentration of 33.3% also showed the same antibacterial activity (Figure 3b). Three times the test was carried out on each extract showed the diameter of the inhibition zone was less than 6 mm. The average results of three repetitions are presented in Table 2.

Table 2. Diameter of inhibition zone of papaya leaf herbal extracts, basil leaves and Intersection ireng on the growth of *S.typhimurium*

Ekstract	Inhibition zone diameter (mm)		
	Papaya leaf	Basil leave	Temu ireng
Ethanol (100%)	< 6	< 6	< 6
Aquades (33,3%)	< 6	< 6	< 6

According to Prakasita et al. (2019) antimicrobial activity was divided into high (> 11 mm), moderate (6 – 11 mm), and low (< 6 mm). These results indicate that the zone of inhibition of the three herbal extracts is categorized as low, making it less effective (Figure 3). Several previous studies have been carried out using different bacteria. Research conducted by Putri et al. (2016) stated that aquades extract of papaya leaves with concentrations of 5%, 20%, 35%, 50%, 65%, and 80% could not inhibit *P. aeruginosa* bacteria. Different results were presented by the research by Tuntun (2016) and Sudarwati (2018) which stated that the ethanol extract of papaya leaves could inhibit the growth of Salmonella typhi, Escherichia coli, and Staphylococcus aureus. Antibacterial activity test of ethanol extract of basil leaves was also carried out by Solikhah et al. (2016), the results of his research stated that ethanol extract with a concentration of 100% could inhibit the growth of *Staphylococcus aureus* but could not inhibit the growth of *E.coli*. The results of this study indicate that the antibacterial activity of an herb can be determined from the type of bacteria that is inhibited. The 100% concentration of ethanol extract of basil leaves could not inhibit Gram-negative *E. coli* but could inhibit Gram-positive *S. aureus* bacteria. The theory is explained by Brooks et al. (2007) that the type of bacteria can affect the antibacterial activity of herbs. In this study, *S. typhimurium* is a Gram-negative bacterium and its growth is not inhibited by 100% ethanol extract of basil leaves. According to Rathnayaka (2013) distilled water extract from basil can inhibit *E. coli, P. aeruginosa*, and *S. typhimurium*. Research on the antibacterial activity of temu ireng extract has not been carried out much. According to Suryani (2005) Intersection blackbird can inhibit *E. coli, Shigella dysenteriae*, and *Vibrio cholerae*. Research conducted by

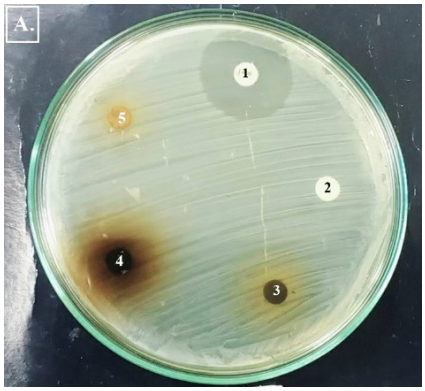

Figure 1. Growth of S. typhimurium on MHA media with trimethoprim discs (1); sterile distilled water (2); 100% concentration of papaya leaf ethanol extract (3); basil leaves (4); and meeting ireng (5) [A]

Baharun et al. (2013) stated that the essential oil of Intersection Ireng could inhibit *S. aureus* bacteria but in *B. subtilis* it had weak activity.

Many factors can affect the antibacterial activity of herbs. The manufacture of extracts greatly influences the secondary metabolites contained in herbs which are influenced by the environment in which the plants grow (Verpoorte and Alfermann, 2000). The content of secondary metabolites found in herbs is influenced by environmental factors such as climate, weather, altitude, and soil fertility (Widaryanto and Nur, 2018). The age of the plant and the part of the plant extracted also affects its antibacterial activity. The papaya leaves used are fresh old papaya leaves (Tuntun, 2016) while the basil leaves used are basil leaves and stems (Solikhah *et al.,* 2016).

The honey used comes from different places and/or different types. The honey used includes Kupang honey, Lanceng Gunung Kidul honey, Lombok black honey, Lombok white honey, Sumba honey, and commercial honey. The same test was carried out to see the activity of the six honeys with a concentration of 100% on the growth of *S. typhimurium*. The inhibition zone (clear zone) formed around the disk shows the activity of honey on bacterial growth (Figure 4). The average results of three repetitions of the honey diffusion test against *S. typhimurium* are presented in Table 3.

Lombok white honey has the largest diameter of the inhibition zone (7.74 mm) compared to other honeys. Kupang honey and Sumba honey do not have low inhibition (<6mm). Gunung Kidul

Table 3. Diameter of honey inhibition zone on the growth of S.typhimurium

Honey	Inhibition Zone Diameter (mm)					
	1	2	3	4	5	6
100%	< 6 ±.00	7.37 ±0.52	6.74 ±0.42	< 6 ±.00	7.29 ±.60	7.74 ±.16

Description: Kupang honey (1); honey Lanceng Gunung Kidul (2); Lombok black honey (3); Sumba honey (4); Super Archipelago honey (5); Lombok white honey (6)

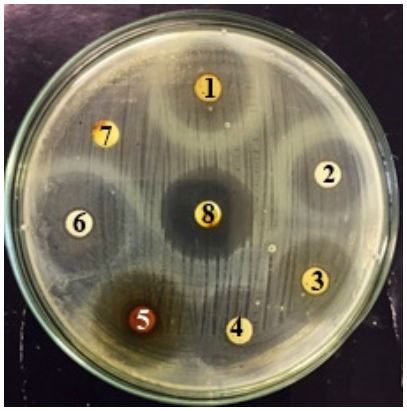

Figure 3. Growth of S. typhimurium on MHA medium with Kupang honey disc (1); honey Lanceng Gunung Kidul (2); Lombok black honey (3); Sumba honey (4); honey (5); Lombok white honey (6); sterile distilled water (7); trimethoprim (8)

Lanceng honey, Lombok black honey, commercial honey, and Lombok white honey have moderate antibacterial activity (6 – 11 mm) while Kupang honey and Sumba honey have low antibacterial activity. Other research says that honey has antibacterial activity against *P. fluorescens and P. putida* (Andriani *et al.,* 2012). According to Yuliati (2017) honey can inhibit the growth of *S. aureus* and *P. aeruginosa*. Honey is antibacterial because it has acidity, osmolarity, hydrogen peroxidase enzymes, aromatic acids, and phenols (Mundo *et al.,* 2004). The antibacterial activity of honey is influenced by the type of honey produced by bees. The source of the nectar will affect the color, taste, and components of honey (Andriani *et al.,* 2012). The results of this study prove that honey from different places has different antibacterial activities.

Conclusion

Gunung Kidul Lanceng honey, Lombok black honey, Lombok white honey, and commercial honey with a concentration of 100% had moderate antibacterial activity while Kupang honey and

Sumba honey had low antibacterial activity against the growth of *S. typhimurium*. 100% ethanol extract and 33.3% distilled water from papaya leaves, basil leaves, and temu ireng rhizomes have antibacterial activity against the low growth of *S. typhimurium*.

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