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Antibacterial Activities of Papaya Leaves (*Carica papaya* L.), Basil Leaves (*Ocimum sanctum* L.), Temu Hitam Rhizomes (*Curcuma aeroginosa* Roxb.), and Honey Against *Escherichia coli*

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[1], A

Abstract

Herbs extract such as papaya leaves, basil leaves, Curcuma aeroginosa and honey contain secondary metabolites which have the antibacterial ability to treat various types of health problems. The purpose of this research is to examine the antibacterial activity of papaya leaves, basil leaves and temu hitam rhizome extracts and honey against *E. coli*. We assume that the presence of antibacterial activity of natural ingredients can potentially be used as a substitute for AGP. Re-identification of *E. coli* isolates to ensure that the isolates were pure *E. coli*. After re-identification, *E. coli* was cultured on Brain Heart Infusion (BHI) and incubated 37^{o}C for 18-24 hours. This research used 100% ethanol and 33.3% aquades of herbal extracts and 100% concentration of honey. *E. coli* culture was centrifuged at 5000rpm. The suspension of *E. coli* was spread on Mueller Hinton Agar (MHA), respectively, at the density of 1.5x10^{8} CFU/mL. Kirby-Bauer diffusion disc method was performed to examine the antibacterial activities of herbs extracts and honey against *E. coli*. The results showed a moderate inhibition zone (6-11 mm) in commercial honey and a low inhibition zone (less than 6 mm) in papaya leaves, basil leaves, and rhizomes of temu hitam.

Keyword: antibacterial; *E.coli*; herbs; honey

Introduction

Indonesia is rich in natural resources, especially herbs and honey. Herbs and honey have been widely used as medicine in this country for generations. They have several active substances as antibacterial. Their benefit to people as medicine would also be potentially applied in veterinary medicine.

Antibiotics have been used in the poultry industry for the prevention of disease and also as a food additive to induce growth in livestock (a*ntibiotic growth promoter*/AGP). The use of AGP may leave some residue that can cause various health problems in people. It can cause allergic reactions, affects microorganisms in the digestive system, affects the immune system, and cause antibiotic resistance problems (Etikaningrum and Iwantoro, 2017). Because of its negative impacts, the use of AGP has been prohibited in several countries (Arifin and Pramono, 2014).

Various herbs such as papaya leaves, basil leaves, and temu hitam rhizomes produce some active substances that can be used as antibacterials. According to Sudarwati (2018), papaya leaves often used as a traditional medicine have some active ingredients as an antibacterials, such as tannin, saponin, flavonoid, and terpenoid. Active ingredients contained in Basil leaves including alkaloids, essential oil, and phenol have bacteriostatic and bactericidal activity (Angelina *et. al.,* 2015). As mentioned by Suryani (2005), temu hitam rhizomes is a medicinal herb that has efficacy as an antibacterial as it consists of essential oil, starch, dammar, and fat. Honey is a natural product that has a lot of availability in Indonesia. There are many studies that have proved that

honey has antibacterial, antiinflammation, and antioxidation activities, and it can improve the immune system as well (Dewi *et. al.,* 2017).

Colibacillosis is an infectious disease caused by *Escherhichia coli*. It can affect chickens in all stages. The disease emerges as a second infection in chicken when it has a weak immune system or when the population of E. coli is very high (Saif *et. al.,* 2008). Furthermore, colibacillosis has affected the economic losses in the poultry industry as it caused a decrease in the productivity and weight of poultry. This disease may result in complicated digestive system diseases that are difficult to manage (Tabbu, 2000).

The ban on the use of AGP in many countries demands some alternative solutions to produce safe, effective food additions to promote the growth of poultry. Herbs like papaya leaves, basil leaves, temu hitam rhizomes, and honey contain several active ingredients which produce antibacterial activity, so they may potentially be used as a substitute for AGP. Therefore, they require more studies on the effectiveness of herbs and honey against the growth of bacteria.

The purpose of this study is to examine the abilities of papaya leaves, basil leaves, temu hitam rhizomes, Kupang honey, Lanceng Honey from Gunung Kidul, black honey/ bitter honey from Lombok, Sumba Honey, and commercial honey against *E. coli*. From this study, we expected that we may produce a substitute product that may decrease antibiotic resistance problems, secure, and effective, by use of herb extracts as a substitute for AGP.

Material and Methods

E. coli BCC B2829 isolates were obtained from Balai Besar Penelitian Veteriner (Bbalivet, Bogor, Indonesia). This bacteria was isolated from the liver and spleen of the chicken. *Escherichia coli* was cultured in liquid media Brain Heart Infusion (BHI, Merck™). Agar plate media used for the disc diffusion test are Eosin Methylene Blue agar (EMB, Merck™), MacConkey Agar (MCA, Merck™) and Mueller Hinton Agar (MHA, Merck™). The stains used for staining the bacteria were crystal violet, lugol, alcohol, and safranin. Biochemical tests we used were hydrogen proxide reagent (H_2O_2) for the catalase test, oxidase paper, *Kovac* reagent for the indole test, methyl red for the methyl red test, *alpha naphthol* and KOH 40% for the *voges-proskauer* test, *Triple Sugar Iron Agar* (TSIA), citrate, *Sulfide Indol Motility* (SIM), urea agar, and carbohydrates fermentation tests of glucose, sorbitol, and maltose. Laboratory equipment which we used were petri dish, object glass, microscope, bunsen burner, ose, test tubes, forceps, digital weight scale, cotton swab, and syringe. Other materials like blank discs, aquades, and antibiotic discs of Trimethoprim (5µg, Oxoid™) as a positive control for the disc diffusion test.

Papaya leaves (*Carica papaya*) were obtained from Magelang, central java; basil leaves (*Ocimum tenuiflorum*) were obtained from Pakem, Sleman city; and temu hitam rhizomes (*Curcuma aeruginosa*) were obtained from Kaliurang street, Yogyakarta. Herbs were processed into 100% ethanol extract and 33,3% aquades extract. Honey from various regions such as Kupang honey, lanceng honey from Gunung Kidul, black honey/ bitter honey, crustal honey/white honey from Lombok, Sumba honey, and commercial honey were also tested in this study.

The Process of Herbs Exctractions

E. coli isolates were cultured on EMB agar, then incubated at 37°C for 18-24 hours. *E. coli* was observed for its colony and cell morphology using Gram staining to confirm that the morphology was matched with the literature. The separated colony was confirmed by the examination of the cells, then cultured in BHI and then incubated at 37°C for 18-24 hours. Biochemical tests for the *E. coli* were catalase test, oxidize test, indole test, methyl red test, *voges-proskauer* test, citrate test (IMViC), motility test, urease test, TSIA test, and carbohydrates fermentation tests of glucose, sucrose, lactose, sorbitol, mannitol, and maltose.

96% Ethanol Extracts. The ethanol extracts of papaya leaves, basil leaves, and temu hitam rhizomes were extracted by Laboratorium Penelitian dan Pengujian Terpadu (LPPT). The extraction used the maceration method. The concentration of 96% ethanol extracts that were used in this study was 100%.

Aquades Extracts. The aquades extracts were extracted by mixing the herbs with aquades

in 33,3% of concentration (B/V). The mixture was then vortexed and left for 24 hours while stirring occasionally. Following 24 hours of incubation, the mixture filtered aseptically.

The Disc Diffusion Test of Herbs Extract and Honey Against the Growth of *E.coli*

Bacterial suspension was prepared by cultured the identified *E. coli* isolates in the BHI media (Merck™) and then incubated at 37°C for 18-24 hours. The bacterial cultures was centrifuged at 5000 rpm and the turbidity was adjusted to 0,5 McFarland standard or equally 1,5 x 10^8 CFU/mL by resuspended the bacteria with Phosphate Buffered Saline (PBS pH 7.4, Sigma™) (Alibasyah *et. a*l., 2018). Furthermore, the suspension was inoculated to MHA media (Merck™) with cotton swab.

The inhibition of *E. coli* activities by papaya leaves, basil leaves, temu hitam rhizomes, kupang honey, lanceng honey from Gunung Kidul, black honey/bitter honey from Lombok, krustal honey/white honey from Lombok, sumba honey, and commercial honey was tested with disk diffusion by the Kirby-Bauer method. The blank paper disc was soaked in the ethanol and aquades extracts, and in the honey. The discs containing herbs and honey extracts were placed on the surface of the MHA which had been inoculated with *E. coli.* MHA was examined and measured for the inhibition zone diameter following incubation at 37°C for 18-24 hours.

Results

Bacterial inoculation isolate on EMB showed circular and metallic sheen colonies (Figure 1A), and the microscopic morphologies were red and coccobacillus (Figure 1B). Therefore, the bacteria was identified as Gram negative.

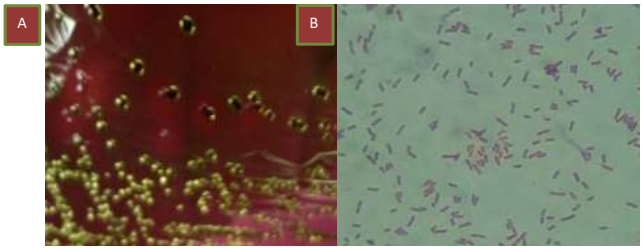

Figure 1. Morphology of E. coli isolates colonies on EMB (A); Cell morphology at 100x magnification showed red and coccobacillus bacteria (B).

Table 1. Biochemical tests result of *E. coli* culture

Test	Result	Reference
Catalase	+	+
Oxidase	-	-
Urease	-	-
Motility	motile	Motile
IMViC		
Indole	+	+
Methyl Red (MR)	+	+
Voges Proskouer (VP)	-	-
Citrate	-	-
Triple Sugar Iron Agar (TSIA)		
Slant	Yellow	Yellow
Butt	Yellow	Yellow, A
H2S	-	-
Carbohydrates Fermentation		
Glucose	+	+
Sucrose	+	D
Lactose	+	+
Sorbitol	+	+
Mannitol	+	+
Maltose	+	+

d = 26-75 % positive (Markey *et. a*l., 2013)

Table 1 showed the result of biochemical tests of *E. coli* culture. It shows catalase positive, oxidase negative, motile, indole positive, MR positive, VP negative, and citrate negative, the color of TSIA medium both in the butt and along the slant were yellow, carbohydrates fermentation of glucose, sucrose, lactose, sorbitol, and mannitol were positive. Based on these results, the biochemical tests of the bacteria matched with *E. coli* characteristics in Markey *et al.* (2013).

Antibacterial activities of 100% ethanol extracts and 33,3% aquades extracts of papaya leaves, basil leaves, temu hitam rhizomes against the growth of *E. coli* were shown in Table 2. Antibacterial activities of kupang honey, black honey from Lombok, white honey from Lombok, lanceng honey from Gunung Kidul, sumba honey, and commercial honey were shown in Table 3.

Table 2. Inhibition zone diameter of 100% ethanol extracts and 33,3% aquades extracts of papaya leaves, basil leaves, and temu hitam rhizomes against the growth of E. coli.

The concentration of 100% ethanol extracts and 33,3% aquades extracts	Inhibition zone diameter		
	Papaya Leaves	Basil Leaves	Temu Hitam Rhizomes
	<6mm	<6mm	<6mm

Table 3. Inhibition zone diameter of kupang honey(1), lanceng honey from Gunung Kidul (2), black honey from Lombok (3), sumba honey (4), commercial honey (5), and white honey from Lombok (6) against the growth of *E. coli*

Honey	Inhibition zone diameter					
	1	2	3	4	5	6
100%	<6mm ±.00	<6mm ±.00	<6mm ±.00	<6mm ±.00	7,135mm ±.0.28	<6mm ±.00

Around 100% ethanol extracts (Figure 2) and 33,3% aquades extracts (Figure 3) of papaya leaves, basil leaves, and temu hitam rhizomes have a low diameter zone of inhibition against *E. coli* (<6mm). Commercial honey has the highest antibacterial effect (7,135 mm ± 0,28 mm) compared to other honey inhibition zone diameters (Figure 4). Kupang honey, lanceng honey from Gunung Kidul, black honey from Lombok, sumba honey, and white honey from Lombok have less than 6 mm inhibition zone diameters.

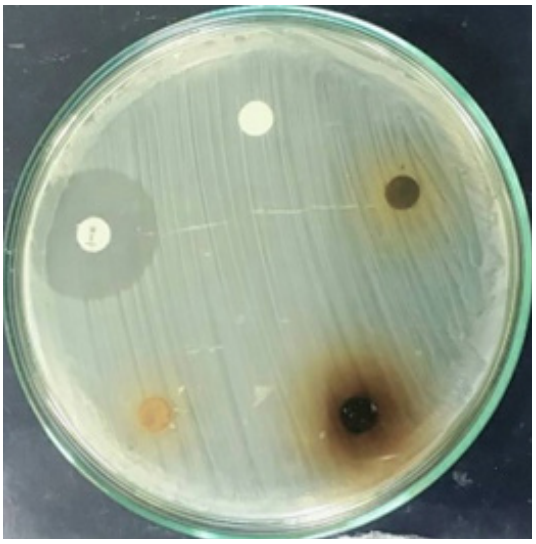

Figure 2. The evaluation of antibacterial activities against *E. coli* on MHA medium of 100% ethanol extracts of papaya leaves (1), basil leaves (2), and temu hitam rhizomes (3). Trimethoprim (4) as a positive control and aquades disc (5) as a negative control.

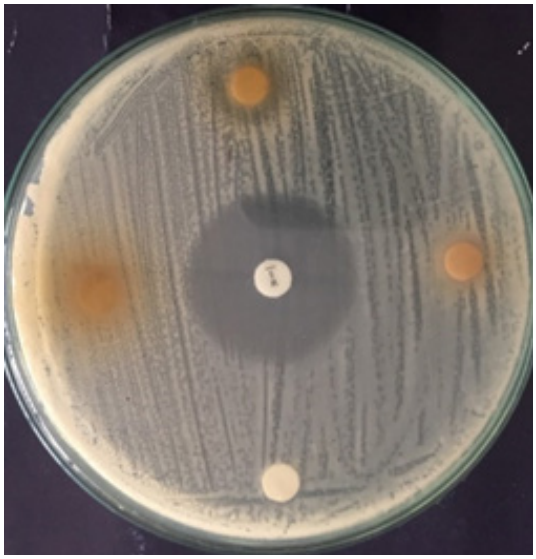

Figure 3. The evaluation of antibacterial activities against *E. coli* on MHA medium of 33,3% aquades extracts of papaya leaves (1), basil leaves (2), and temu hitam rhizomes (3). Trimethoprim (4) as a positive control and aquades disc (5) as a negative control.

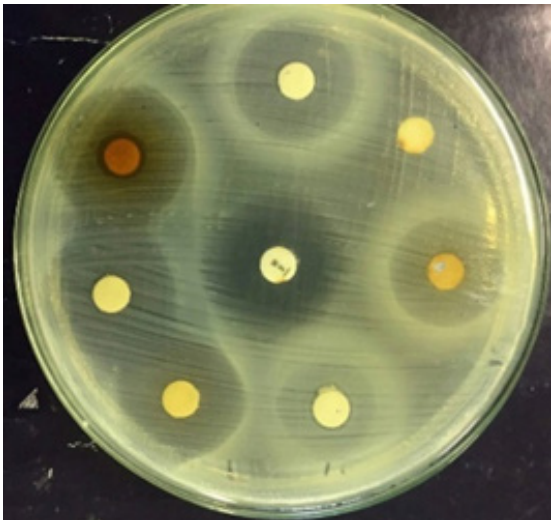

Figure 4. The evaluation of antibacterial activities against *E. coli* on MHA medium of kupang honey (1), lanceng honey from Gunung Kidul (2), black honey from Lombok (3), sumba honey (4), commercial honey (5), and white honey from Lombok (6). Aquades disc (7) as a negative control and trimethoprim (8) as a positive control.

Discussion

Ethanol and aquades extracts of papaya leaves, basil leaves, and temu hitam rhizomes, and honey from several regions were natural ingredients we observed to examine their antibacterial activities against pathogenic bacteria. Herb extracts and honey were expected to be able to minimize the infection of poultry, and can be used as substitute for antibiotics growth promoter. *E. coli* was the bacteria we evaluated for the study. These cause infectious disease in poultry called colibacillosis. The disease has affected the economic losses in the poultry industry as it caused a decrease in the productivity and weight of poultry (Tabbu, 2000).

Antibacterial activities of ethanol and aquades extracts of papaya leaves, basil leaves, and temu hitam rhizomes, and honey from several regions against *E. coli* tested with the *Kirby-bauer* disc diffusion method. The concentration of ethanol

extracts of papaya leaves, basil leaves, temu hitam rhizomes, kupang honey, lanceng honey from Gunung Kidul, black/bitter honey from Lombok, krustal/white honey from Lombok, sumba honey, and commercial honey used in this study were 100%. The concentration of aquades extracts of papaya leaves, basil leaves, and temu hitam rhizomes were 33,3%.

Re-identification to confirm the bacteria was *E. coli* was done by identifying tthe morphology of the bacterial colony on EMB and cells with Gram staining, and identifying several biochemical tests. Biochemical tests we used for identifying *E. coli* were catalase test, oxidase test, IMViC tests, motility test, urease test, TSIA test, and carbohydrates fermentation tests of glucose, sucrose, lactose, sorbitol, mannitol, and maltose.

According to Leboffe and Pierce (2011), metallic sheen colony is a characteristic of *E. coli,* and this can be use to distinguish *E. coli* from other bacteria in the *Enterobacteriaceae* families. Microscopically, it showed gram negative and short bacillus (coccobacillus) bacteria. Biochemical tests of the bacteria showed positive reactions in the catalase test, indole test, MR test, carbohydrates fermentation tests of glucose, sucrose, lactose, sorbitol, mannitol, and maltose. The bacteria showed negative reactions in the oxidase test, urease test, VP test, and citrate test. These results were matched with the characteristics of *E. coli* in Markey *et al*. (2013).

Antibacterial activities of papaya leaves, basil leaves, temu hitam rhizomes, and honey extracts on *E. coli* were evaluated by examining the inhibition zone around paper discs. According to Muharni *et al.* (2017), these are the following classification of antibacterial characteristics on bacteria inhibition: inhibition zone diameter less than 6 mm was categorized as low antibacterial activity, inhibition zone diameter 6-11 mm categorized as moderate to high, and inhibition zone diameter more than 11 mm was categorized as high. Therefore, 100% ethanol extracts (Figure 2) and 33,3% aquades extracts (Figure 3) of papaya leaves, basil leaves, and temu hitam rhizomes were not effective in inhibiting the growth of *E.coli* as the zone was less than 6 mm.

Based on the result of the study by Syafriana *et al*. (2016), it showed no inhibition zone was formed in the concentration of 12,5-100% ethanol extracts of papaya leaves. However, the result of the study by Tutun (2016) was different. This study used papaya leaves ethanol extracts with a concentration of 20-100%. It showed antibacterial activities with mean of inhibition zone diameters 6,5-9,1 mm. Furthermore, active compounds, phenol and alkaloid carpaine, contained in papaya leaves create the inhibition zone to hinder bacterial growth. Phenol can destroy the membrane cells of bacteria, while alkaloid carpaine has a base chain that can react with bacterial DNA, which subsequently damage the nucleus. [1, A]

The result of the study on ethanol extract inhibition zone in basil leaves differs from the result conducted by Angelina *et al.* (2015), which ended up with conclusion that the ethanol extract in basil leaves with 20-100% of concentration can hinder the *E.coli* growth rate. It also noted that the basil leaves contain secondary metabolites such as tannin, flavonoid, and essential oil. Those secondary metabolite substances can produce the antibacterial effect.

According to Rahmawati *et al.* (2014), the aquades extract in temu hitam rhizomes showed an inhibition zone with diameter less than 5 mm. In contrast, Baharun (2013) showed a different result, that is essential oil of temu hitam rhizomes with concentration of 25-100% can inhibit the bacterial growth.

The difference in bacterial inhibition zone diameters of herb extracts can be associated with many factors. According to Tutun (2013), the difference in bacterial inhibition zone is caused by a variety of secondary metabolites and the extraction selection method. Secondary metabolites of herbs are influenced by the environment of herbs grown (Verpoorte dan Alfermann, 2000). According to Wirdayanto and Nur (2018), environmental factors which influence secondary metabolites in herb extract are climate, weather, height, and soil fertility. The selection of a solvent for herb extraction is one of the key factors in order to obtain several active compounds. The type of solvent affects the amount of active compounds that are contained in the extract. According to the concept of like dissolves like, which polar compounds will dissolve in polar solvents and non-polar compounds will dissolve in non-polar

solvents (Arifianti *et. a*l., 2014). This is also in accordance with the study resulted by Prakasita *et al*. (2019). The study has proven the ethanol extract has higher antibacterial activity compared to the aquades extract. The age of herb selection for extraction also affects the rate of secondary metabolites. According to the study by Anwar *et al.* (2017) comparing the rate of active compounds based on the age of *Aquilaria beccariana* plant, it said the mature leaf was more capable of synthesizing secondary metabolites, while the older leaf would synthesize less. Baharun *et al.* (2013) was used the 10 months old of age of temu hitam rhizome in his study. According to the study, the essential oil of temu hitam rhizome is mostly produced when the rhizome hasn't budded.

The commercial honey has the highest antibacterial effect on *E. coli* compared to other honey. It has a 7,135 mm inhibition zone diameter (Figure 4). The antibacterial activity of honey is by producing hydrogen peroxide compound (Sakri, 2012). Hydrogen peroxide destroys functional groups of biomolecules in bacterial cells such as protein, nucleic acid, and cell wall (Huda, 2013). Kupang honey, lanceng honey from Gunung Kidul, black honey from Lombok, sumba honey, and white honey from Lombok developed less than 6 mm of inhibition zone diameter. According to Jaya (2017), it was difficult to expect the same quality of honey, due to the influence of topography, climate, agricultural patterns, the length of storage of analyzed samples, different method of testing samples, and different types of nectar-producing bees.

Conclusion

100% ethanol extracts and 33,3% aquades extracts of papaya leaves, basil leaves, and temu hitam rhizomes, and 100% concentrations of kupang honey, black honey from Lombok, white honey from Lombok, Lanceng honey from Gunung Kidul, and sumba honey showed low antibacterial activities on *E. coli.* Commercial honey showed moderate antibacterial activity on *E. coli.*

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