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Characteristics of Indonesian wild honey and cultured honey and their antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*

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Introduction

Honey is a natural product that has been widely used by people. In addition to its sweet flavour, honey also known to have antibacterial property. Various studies have been conducted over years to investigate the antibacterial activity of honey by determining its minimun inhibitory concentration (MIC), such as the forest honey from Australia and Manuka honey from New Zealand (Sindi et al., 2019). The antibacterial activity of honey is attributed to its active compounds, hydrogen peroxide, high osmolarity, and low pH.

Honey has been used for several kinds of wound treatments. Manuka honey from New Zealand has been used as standardized medical honey in various researches, yet Manuka honey is costly and not easy to be found in Indonesia. Therefore, it is necessary to conduct research about Indonesian local honey characteristic.

There are various kinds of Indonesian local honey originated from Sabang to Merauke. The diversity of local Indonesian honey could be affected from the different origins, harvest seasons, bees species, nectar source plants, bees way of life (cultivated or wild), harvest methods and honey processing methods after harvest. The different nectar sources would produce the different kinds of honey. The variety of honey could be observed physically by the difference in colour, scents and tastes. The dark-colored honey indicated that the honey is ripe and it contents less water. The darker-colored honey varieties contain higher amounts of antioxidants (Fatma et al., 2017).

Staphylococcus aureus and *Escherichia coli* are the most common bacteria species found in sepsis and infected wounds. The bacterial culture sensitivity test in a research discovered that the various bacteria showed the multidrug resitance characteristic in infected wounds and sepsis (Ayub, 2015). The efforts to reduce resitance and microbial production rate are slower than the growth of antibiotic resistance level (WHO, 2014). Therefore, a novel strategy is needed to treat the infections. Honey could be used as complementary medication to reduce the microbial resitance.

Until recenty, the local honey researches were limited to the honey quality assay, thus, the characteristic of cultivated and wild local honey were mostly unidentified. Therefore, this research aims for studying the local Indonesian honey (from cultivated and wild bees) characteristics and the antibacterial activity againts *staphylococcus aureus* and *Escherichia coli.*

Methods

This research was conducted from April 2021 to July 2021 in Biochemistry laboratory, Biochemistry section, UWKS Medical Faculty, Surabaya. Honey samples characterization were conducted in Assessment Service Unit, Faculty of Pharmacy, Airlangga University, Surabaya. Antibacterial assay was conducted in Gastroentritis and Salmonellosis laboratory, ITD, UNAIR Surabaya.

Materials

Carisa honey sample from wild bees [Wild Klanceng (WK) and Wild Cerana(WC)] and cultivated (Cultivated Cerana (CC) and Cultivated Malifera (CM)], Zinc Acetate ($Zn(CH_3COO)_2.2H_2O$), potassium ferrocyanide ($K_4Fe(CN)_6.3H_2O$), glucose, alcohol, sodium bisulfite ($NaHSO_3$ 0,1%), 3,5-Dinitro Salicylate reagent, potassium sodium-Tartrate Tetrahydrate, sodium hydroxide (NaOH), Mueller Hinton Agar (MHA), Mueller Hinton Broth (MHB), *Escherichia coli* (*E. coli)* and *Staphylococcus aureus* (*S. aureus*) were used in this research. The equipments used for this research were analytical balance, spectrophotometer, autoclave, moisture balance, calipers, petri dish, glass bottle, volumetric flask, Beaker glass and stirrer.

Procedure

A. Honey characterization

Honey characterization consisted of ash content, free acidity, Hydroxy Methyl Furfural, Diastase enzyme, glucose and water content analysis were determined using standard SNI 8664-2018 methods.

B. Antibacterial Assay

Bacterial Isolate Rejuvenation

E. coli and *S. aureus* bacterial isolates were purchased from Gastroentristic and Salmonellosis laboratory, ITD, UNAIR Surabaya. The isolates were cultured in NA medium using streak method and incubated for 24 h in the temperature of 37ºC.

Mueller Hinton Agar (MHA) Preparation

A 19 g of MHA powder was weighed and dilluted to 500 ml using distilled water in an Erlenmeyer flask. The suspension was homogenized by heating in a hot plate. Then, the medium suspension were sterilized using autoclave (121°C, 15 min). The sterilized medium were poured in a petri dish (±25 ml) and allowed to solidify at room temperature.

Mueller Hinton Broth (MHB) Preparation

A 21 g of MHB powder (2 g Beef infusion, 17.5 g Casein hydrolysate, and 1.5 g Starch) were dilluted to 1L of distilled water. The medium was heated on a hot plate, and stirred using magnetic stirrer. The final homogenized medium colour was clear yellow. The medium was sterilized using autoclave (121°C, 15 min), and poured in a sterile microtube aseptically in LAF.

Bacteria Inoculum Preparation

E.coli **and** ***S.aureus*** **inoculation were prepared by picking one single colony from NA medium to the Muller Hinton Broth (MHB) tube and were incubated overnight in 37°C. The bacterial cultures were then centrifugated (5000rpm; 5min). The supernatant was separated from the bacterial pellet (precipitate) from each tube. The pellet was resuspended with saline water and adjusted to OD490=0.5 to be used for inoculum/suspension in every treatment.**

Antibacterial Assay

The 0.1 ml of bacterial suspensions were inoculated in MHA using spread method. The 20µL of honey samples were dropped to the test discs along with the blank solution (control solution) and placed on the MHA medium aseptically. The medium were then incubated in 37°C with reverse position.

Data Analysis

The clear zones around the test discs were measured for diameter using calipers. The data obtained were tabulated and analyzed statistically.

Results

A. Honey Characterization

Carisa Honey WK, WC, CC and CM were tested for ash content, free acidity, Hydroxy Methyl Furfural, Diastase enzyme, glucose and water content. The characterization results were demonstrated in Table 1.

Table 1. Honey characteistic

	Wild Cerana Honey (WC)	Wild Klanceng Honey (WK)	Cerana Honey (Cultivated)
HMF (mm/kg)	4,01 ± 0,06	6,92 ± 0,18	5,32 ± 0,11
Diastase Enzyme (DN)	5,11 ± 0,09	4,2 ± 0,12	5,38 ± 0,08
Water Content %(w/w)	20,1 ± 0,14	>25	18,3 % ± 0,07
Ash Content %(w/w)	0,46 ± 0,01	1,3 ± 0,01	0,10 ± 0,00
Acidity (ml eq/kg)	75,7±0,37	418,9ml± 8,55	61,8 ml ± 0,11
Glucose (%)	18,690±0,319	29,240 ± 0,061	9,286 ±{1,733

B. Antibacterial Assay against ***E. coli*** **and** ***S. aureus***

The antibacterial assay were performed using agar diffusion method. The assay used 6 treatment groups and 4 honey samples concentration of 40%, 60%, 80% and 100%. The positive control (chloramphenicol) and negative control (distilled water) were used in this assay. Table 2 and Table 3 demonstrated the inhibition ability of honey samples against ***E. coli*** **and** ***S. aureus*** **growth.**

Table 2. The average inhibition zone of honey against *S. aureus* growth

Honey samples	The average inhibition zone against *S. aureus* (mm)				C +	C -
	40%	60%	80%	100%		
CC	0^{a}	0^{a}	0^{a}	$1,75\pm0,96^{b}$	$21,5\pm0,58^{c}$	0^{a}
WC	0^{a}	0^{a}	$2\pm0,82^{b}$	$4,25\pm1,89^{c}$	$23,5\pm1,73^{d}$	0^{a}
WK	$9,18\pm0,85^{b}$	$9,55\pm1,17^{b}$	$10,10,71^{b}$	$12,33\pm1,69^{c}$	$25,93\pm1,42^{d}$	0^{a}

Notes: abc superscript consists of different alphabets on the same row implies the significant difference (P<0,05) according to Anova test and Post Hoc LSD

Discussion

Cultivated honey and wild honey are two different types of honey. The cultivated honey are mostly produced from a single area with limited or even single plant variations. The single plant cultivation are frequently called monofloral cultivation. The forest honey cultivation could be produced naturally or intentionally from a wide range species of plants, called multifloral cultivation. In this research, Cerana Cultivated honey (CC) were produced from Avocado plant nectare.

Wild Cerana (WC) and Wild Klanceng (WK) honey showed higher water content, ash content, acidity and glucose from Cerana Cultivated (CC) honey as demonstrated in Table 1. This results are in accordance with a research in Greece, which stated that Wild/forest multifloral honey possesed a higher acidity to inhibit the growth of microbes in honey and loaded with minerals, such as calcium (Karabagias et al., 2018).

The antibacterial properties of honey samples were tested using clear zone measurement around the the discs from the diffusion of the antibacterial compounds in solid medium to inhibits the growth of bacteria and were referred as inhibition zone (Perdana, 2016). The inhibition zones were formed due to the potential of honey samples as antibacterial agent. According to the series of data in Table 2 and 3, in 100% concentration, WC and WK samples were able to inhibit both Gram positive (+) bacteria *S. aureus* and Gram negative (-) bacteria *E. coli*, meanwhile CC sample only inhibited Gram positive (+) bacteria *S. aureus.* It could be concluded that the antibacterial activities of WK > WC > CC consecutively. The potential of honey as antibacterial agent is attributed to its osmolarity, acidity, pH, high glucose, hydrogen peroxide, and non hydrogen peroxide compounds such as phenolic acid and flavonoids (Aggad and Guemour, 2014).

Klanceng Wild honey (WK) showed the highest antibacterial activity compared to the other samples, due to the highest acidity in WK sample. The acidity in honey is caused by the presence of gluconic acid , that is formed by the reaction of glucose oxidase and glucose (Bittmann et al., 2010). The higher the glucose level is, the higher the acidity. Honey provides an acid environment that is unfavorable for bacteria to grow and also inhibits most of microorganisms activities (Brudzynski et al., 2011). With the higher acidity level in honey, the hydrogen ion concentration is increased. The enhancement in hydrogen ion concentration could interfere the proton transmembrane gradient fom bacterial cells (Hariyati, 2010).

Cerana Cutivated (CC) honey showed weak activity against Gram positive bacteria (+) *S. aureus*. The bioactive compounds of CC honey, phenol and flavonoids, are suspected to be in a lower level than the wild honey. Ahmed (2013) stated that total phenolic and flavonoids compounds of common cultivated honey were lower compared to the wild honey, that the cultivated honey was innefective against Gram negative bacteria. A phytochemical research in Indonesia also stated that wild honey contains more flavonoids and saponins than the forest and cultivated honey (Yelin and Kuntadi, 2019). The mechanism of phenol as antibacteria is by poisoning the protoplasm, breaking and invading the cell wall, then precipitating the microbe cell protein (Hariyati, 2010).

Table 2 and 3 demostrated that Gram (-) bacteria *E.coli* was insensitive against antribacterial compounds from honey samples, where *E. coli* inhibition zones were narrower than *S. aureus.* It could be caused by *E. coli* as Gram negative bacteria is equipped with complex cell wall structure, consists of peptidoglican, lipopolysaccharide and periplasmic space. The periplasmic space has more ability to hold the plasma membrane firmly. Meanwhile, *S. aureus* has thick cell wall consists of peptidoglican only, therefore the antibacterial agent might work effectively to inhibit the bacterial growth (Nur *et al*., 2019).

Conclusion

Indonesian wild honey showed relatively weak against *S. aureus* bacteria. Cultivated honey possesed minimum antibacterial effect. Both wild honey and cultivated honey showed insignificant antibacterial activity against *E. coli.* The characteristic of honey that might contribute to the antibacterial effect was acidity level.

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