Validation methods for evaluation of ceiba honey's growth inhibitory activity against *Bacillus subtilis* ATCC 6633

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ABSTRACT

Agar diffusion and turbidimetry were commonly used to evaluation of antibacterial activity of honey. However, there is no report about which one of these two methods is better. This study attempts to validate the agar diffusion method and turbidimetry used for growth inhibitory assay of ceiba honey against *Bacillus subtilis* ATCC 6633 based on validation parameters including Limit of Detection (LOD), linearity, precision, and selectivity. The samples were ceiba honey aqueous solutions in concentration $20\% - 100\%$. It was found that the minimum inhibitory concentration (MIC) of agar diffusion and turbidimetric methods were 35% and 30% respectively, considered as LOD. In agar diffusion method, plot of inhibitory zone diameter against log of honey concentration yields linear regression equation with $r = 0.9804$ and $Vx_0 = 1.06\%$, while r and Vx_0 of linear plot from % transmittance against log concentration in the turbidimetric method were 0.9748 and 1.24% respectively. The coefficient of variation (CV) in agar diffusion method were 1.78% for repeatability and 3.13% for intermediate precision) whereas CV in turbidimetric were 3.64% and 4.05% respectively. Agar diffusion and turbidimetric methods were selective because different source of ceiba honey could give different response in term of MIC. It can be concluded that the agar diffusion and turbidimetric method were valid and suitable for growth inhibitory assay of ceiba honey against *Bacillus subtilis* ATCC 6633 and there was no significant differences between these methods. The turbidimetry was more sensitive than the agar diffusion method because of its lower LOD and it has more simple experimental technique.

Keywords: Ceiba honey, *B. subtilis*, validation method

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INTRODUCTION

Honey is a sweet liquid food, healthy and can be used as medicine (Tirtawinata, 2006). It has been used in the treatment of infectious diseases, because it contains antibacterial compounds or agents that can inhibit bacterial growth. There are three factors that play a role in the activity of bacterial growth inhibition, namely osmotic pressure, acidity and an agent called inhibine. These three factors can contribute in reducing the growth of most contaminant or dangerous microorganisms by working alone or together (Molan, 2001).

Honey is known to have inhibitory effect on bacterial growth, both against Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* or Gram-negative bacteria such as *Escherichia coli* (Mulu *et al*., 2004; Bogdanov, 2010; Sandra *et al*., 2015). The honey was also reported to have growth inhibitory effect to Methicillin-Resistant *Staphylococcus aureus* (Mama *et al*., 2019). The *Bacillus subtilis* is an aerobic bacterium which is a contaminant microbe in milk and food. It can break down proteins into amino acids and cleave the fat by the lipase enzyme cause the milk becomes acidic and slimy (Suwito, 2010).

Methods that have been used to evaluate the inhibitory activity of honey against bacterial growth include agar diffusion and dilution methods (Pimentel *et al*., 2013; Hermawati *et al*., 2016). The dilution method is performed a solution of antimicrobial agents in a serial of concentration added into the broth media that has been inoculated with a test bacteria, then dishes containing the test media inoculated by a test bacteria and the test solution are incubated at 37 °C for 24-48 (Lourenço, 2009). The dilution method may be used to determine the lowest concentration of an antibacterial agent that can inhibit the growth of test bacteria (Brooks *et al*., 2013). The presence or absence of bacterial growth in the test media can be directly observed visually or using an instrument such as in the turbidimetry method. Observation of the bacterial growth in the turbidimetric method is carried out by measuring the turbidity that occurs after treatment of the test bacteria with antibacterial substance, by a spectrophotometer at a specific wavelength (Rostinawati, 2009). Either agar diffusion method or turbidimetry have their respective advantages and disadvantages. Both methods are often applied to assay the inhibitory activity of honey against bacterial growth (Mulu *et al*., 2004; Bogdanov, 2010; Sandra *et al*., 2015). However, until now there has been no publication that state which one of those two method was better for testing the inhibitory activity of honey against bacterial growth. Diameter of the inhibitory zone is directly proportional to the antibacterial activity of the substance. The wider the diameter of inhibitory zone means the stronger antibacterial activity (Brooks *et al*., 2013; Pimentel *et al*., 2013).

Honey is viscous liquid that can be yellow to brown in color, depending on substances in the original plant whose nectar is sucked by honey-producing bees. A turbid and/or colored substance will affect percentage of transmittance in the turbidimetry method, therefore its effect to the result of inhibitory growth assay shoul be evaluated. Whereas in diffusion method, it is easier to conduct the range of concentration of antibacterial agents, but sometimes the narrow range of concentration cannot show the differences in the zone inhibitory diameter result from treatment with tested solution. Therefore it was necessary to study which method was more suitable for testing the inhibitory activity of honey against bacterial growth by comparing the agar diffusion method and dilution using turbidimetry. The two method was evaluated based on the value of validation parameters including LOD (Limit of Detection), linearity, precision, and selectivity. It is expected that this research can provide recommendations on what method can be used to assay the inhibitory activity of honey against bacterial growth in terms of which methods are better according to the requirements of validation parameters.

MATERIALS AND METHOD

Materials

The materials used include: *Bacillus subtilis* ATCC 6633 obtained from the Microbiology Laboratory-Faculty of Pharmacy University Airlangga, Nutrient Agar media and Nutrient Broth media (Oxoid), honey X-brand obtained from beekeeping in the Pati-Central Java, honey Y-brand obtained from beekeeping in Pasuruan-East Java, NaCl p.a (Merck), Kanamycin (Meiji), distilled water, and 0.22 μm membrane filters (Whattman).

Preparation of ceiba honey solution

Honey solution in concentration of 100% w/v was prepared by weighing 100 grams of the honey sample in sterile distilled water until a volume of 100 ml was obtained, then the solution was sterilized by membrane filtration using filter that have pore size of 0.22 μm. From the 100% w/v honey solution, we prepared some tested samples by dilution using distilled water until concentrations of 20% b, 30, 40, 50%, 60%, 70%, 80%, 90% were obtained.

Assay methods

Agar diffusion method

Base layer was made from 30 ml sterilized liquid Nutrient agar which was allowed to solidify in sterile petri dishes. Inoculum of *B. subtilis* ATCC 6633 in 25% transmitance as much as 5 μL was homogeneous with 20 mL of liquified Nutrient agar, then the sterile liquid was then poured on the solid base layer, and became the seed layer (Lourenço, 2009). A number of reservoir were made on the solid media by molding the holes using a cylindrical ring, each consisting of 5 honey solutions with different concentrations, each of 3 replications, one positive control, and one negative control. 75 μL of each solution was put into the reservoir, then the media in the petri dish was incubated at 37 °C. After 24 hours, the inhibitory zones formed around the reservoir were observed and its diameters were measured.

Turbidimetric dilution method

Sterile Nutrient Broth 9 mL in several sterile test tubes was added by 100 μL Bacillus subtilis ATCC 6633 in 25% transmittance, then shaken until homogenous with a vortex. Sample of one concentration of honey solution was added to each tube, then incubated at 37 °C for 24 hours. After incubation, the transmittance was measured with a spectrophotometer at wavelength of 530 nm.

RESULTS AND DISCUSSION

Characterization of ceiba honey solution

Characteristic of heney are largely determined by habitat and flowers of the plant as a source of honey. Therefore monoflore and polyflore honey are different in character. The honey from ceiba plant used in this study came from a honeybee farm in Pati, Central Java. The physicochemical properties of honey samples were determined before their antibacterial activities were tested to ensure that honey samples are suitable for use in this study. The result of physical performance test, pH, viscosity, specific gravity and specific rotation of honey samples (Table I) showed that the honey physical characteristic met the SNI standard.

Table I. Physicochemical properties of ceiba honey sample

Physicochemical properties			SNI (2013)
Organoleptic	Texture	Viscous liquid	Viscous liquid
	Odor	Specific	Normal/ specific
	Taste	Normal	Normal/ specific
	Color	Yellowish brown	Not stated
pH*		3.45 ± 0.01	Not stated
Viscosity (cps)*		18.459 ± 0.606	Not stated
Specific gravity (g/ml)*		1.263 ± 0.001	Not stated
Specific rotation (° ml/g dm)*		-40.0 ± 4.16	Not stated

*observation in triplicate

The analysis results showed that the activity of diastase enzyme in ceiba honey was 12.50 DN, the hydroxymethyl furfural level was 7.00 mg/kg, and the water content was 18.371% w/w. The results of the physicochemical evaluation of ceiba honey were in accordance with the criteria of honey stated in SNI 2009. Specific rotation of the honey sample that has negative value indicates that the ceiba honey used was natural honey, because the sugar composition contained in the majority of honey was fructose which rotate the plane-polarized light to the left (Wibowo *et al*., 2016).

Validation parameters of agar diffusion and turbidimetric methods

Agar diffusion and turbidimetric dilution are methods widely used to assay the inhibitory activity of honey against bacterial growth (Mulu *et al*., 2004). Both methods are usefulness for daily practice to evaluate honey quality. Barberies *et al*. (2018) have compared disk diffusion and agar dillution methods for determining in vitro susceptibility of antimicrobial agents against *Corynebacterium* spp. by using 20 antimicrobial agents containing disk. For daily test purposes, the disk diffusion is simpler, but very expensive. The low correlation coefficient obtained for some antimicrobial agents such as vancomycin, minocycline and trimethoprim/ sulfamethoxazole did not allow establishment of breakpoints for the disk diffusion method.

This study was performed to find a simple and rapid test with low cost method for evaluation of the honey quality. Validation parameters obtained from the agar diffusion and turbidimetric dilution methods are very important for evaluation of the quality based on the growth inhibitory activity of ceiba honey against *B. subtilis* as a test bacteria. Validation parameters evaluated including LOD, linearity, precision, and selectivity. The LOD values were determined from the minimum inhibitory concentration (MIC) of the honey samples against *Bacillus subtilis* ATCC 6633 using each method. The MIC must be found first as a reference in choosing the concentration of honey samples that will be used in determining the validation parameters for comparing the methods.

Limit of detection

The MIC value, which was the lowest concentration of honey solution that still inhibit the bacterial growth ranging from the concentration of 100%, 50%, 40%, 35%, 30%, 25%, and 20%. Based on the honey characteristic, the range of concentration is varies greatly. Mama *et al*. (2019) used 25-100% concentration for evaluation antibacterial activity of honey against MRSA with MIC of 9.38-37.5%. The MIC of ceiba honey solution against *Bacillus subtilis* ATCC 6633 in the agar
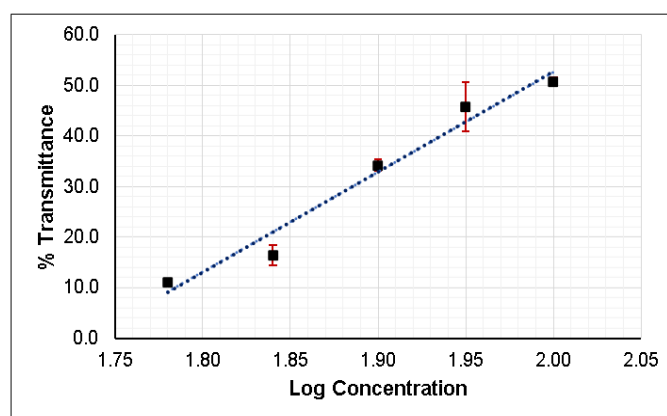
diffusion and turbidimetric dilution methods were 35% and 30% respectively. Besides the honey characteristic, the MIC really depends on the strain of the test bacteria. The MIC value in this study in the concentration rang of *Mama et al.* (2009). Furthermore, the concentration of honey solutions above 35% and 30% was used for the comparison study of the agar diffusion and turbidimetric dilution method respectively.

Linearity

Determination of linearity was carried out using honey solutions of 60% to 100% with three replications. It was found that linearity was obtained on relationship between logarithmic of honey concentrations and diameter of the inhibitory zone in the agar diffusion methode (Figure 1), with the correlation coefficient (r)= 0.9804 and Vx_0 = 1.06%. On the otherhands, a linear relationship between logarithmic of honey concentrations and % transmittance in the turbidimetric dilution (Figure 2) obtained lower correlation coefficient compared to the diffusion method (0.9748) with Vx_0 = 1. 24%. Physically, the influence of the color of honey solution needs to be considered to minimize the occurrence of measurement errors, although it has been anticipated by the use of negative controls and blank solutions.
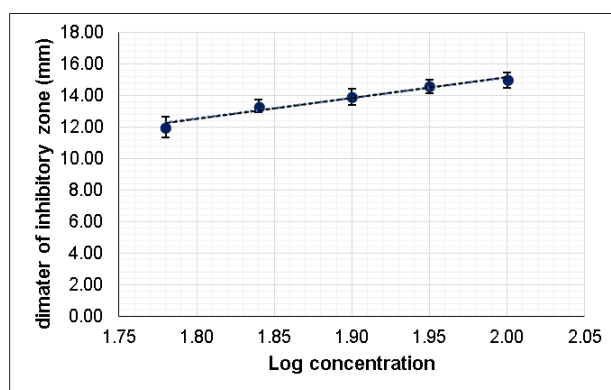
Figure 1. Linear relationship between logarithmic concentration of ceiba honey solutions and average of inhibitory zone diameter (mm) in the agar diffusion method

Figure 2. Linear relationship between logarithmic concentration of ceibahoney solutions and the average% transmittance in the turbidimetric dilution method

These results indicate that the r and Vx_0 values from both methods meet the requirements, namely r-calculated was greater than r-table (0.8783) at a significance level of 0.05 and degree of freedom 3. The value of Vx_0 obtained from the relationship curve was smaller than the requirement of 5% (Yuwono and Indrayanto, 2005; Susanti *et al*., 2009). According to the results, the agar diffusion

and the turbidimetric dilution methods both met the linearity requirements. It can be concluded that there was linear correlation between logarithmic concentration of honey from 60% to 100% with inhibition zone diameter (mm) in agar diffusion method and between logarithmic concentration of honey and % transmittance in the turbidimetric dilution method.

Precision

The determined precision parameters were repeatability (intra-day assay) and intermediate precision (inter-day assay), which were tested using honey concentrations of 60%, 80%, and 100% each with 3 replications. Calculation the data of the diameter of inhibitory zone in the agar diffusion method and the% transmittance in the turbidimetric dilution method generated the coefficient of variance (CV) values. The CV values in the agar diffusion method were 1.78% for repeatability and 3.13% for intermediate precision, while the CV values in the turbidimetric dilution method were 3.64% (intra-day) and 4.05% (inter-day). These results indicated that the CV values of the precision in the agar diffusion and turbidimetric dilution methods met the validation requirements, i.e the CV value was less than 5%.

The data resulted from repeatability examination in the agar diffusion method produce an average CV values of 1.78% ± 0.92 and in the turbidimetric dilution method produces average CV values of 3.64% ± 2.26. The average CV value of the turbidimetric dilution method was higher than the agar diffusion method. This mean that in the turbidimetric dilution method there was a greater variation of replication compared to the agar diffusion method, which might occured because the turbidimetry method was more sensitive than the agar diffusion method so that a slight difference in the concentration of honey solution in each replications could produce a distinct difference response in % transmitance. Nevertheless, the average CV values in the repeatability test of the both methods met the requirements, which did not exceed 5% (Susanti *et al*., 2009). The average CV values resulting from the intermediate precision in agar diffusion method and in turbidimetric dilution method were 3.13% ± 1.52 and 4.05% ± 2.75.

Not much different from the results of repeatability test, the average CV values of the turbidimetric dilution method in determination intermediate precision was also greater than the average CV values of agar diffusion method. However, the average CV values from intermediate precision of the two methods were greater than their average CV values resulted from each of their repeatability. This fact might occured because the time interval between days of determination was quite long, which was 3 days, so that there could be changes in properties of honey sample during storage. Therefore, the time interval between the days of determination should not be too long so that the sample conditions did not change much during storage. However, the average CV values resulted from the intermediate precision of the two methods both met the requirements, which did not exceed 5% (Susanti *et al*., 2009).

Selectivity

Selectivity was examined using honey sample obtained from two different farms, each was prepared as solutions in concentrations of 60%, and blank media which was treated with negative control. The ceiba honey samples used were purchased from beefarms in Pati, Central Java and the beefarms in Pasuruan, East Java. The agar diffusion and turbidimetric dilution methods capable to distinguish the response of tested samples (ceiba honey) from different sources, as well as with blanks. The two methods could provided significantly different responses from the two types of ceiba honey and the blank so that it was concluded that both were selective methods (ICH, 2005).

Comparison of validation parameters between agar diffusion and turbidimetric dilution methods

The validation parameters such as LOD, linearity, precision, and selectivity of the agar diffusion and turbidimetric dilution method are summarized in Table II.

Table II. Comparison of validation parameters of agar diffusion and turbidimetric dilution methods

Validation Parameters		Requirement*	Agar Diffusion	Turbidimetric Dilution
LOD	MIC	-	35%	30%
Linearity	r	r calc $\geq r$ table (0.8783)	0.9804 ± 0.00	0.9748 ± 0.03
	Vx_0	$Vx_0 \leq 5\%$	$1.06\% \pm 0.00$	$1.24\% \pm 0.81$
Repeatability	CV	CV $\leq 5\%$	$1.78\% \pm 0.92$	$3.64\% \pm 2.26$
Intermediate precision			$3.13\% \pm 1.52$	$4.05\% \pm 2.75$
Selectivity	-	Selective	Selective	Selective

Notes: *according to ICH (2005), Yuwono dan Indrayanto (2005), Susanti *et al*. (2009)

The agar diffusion method and the turbidimetric dilution method met the requirements of LOD, linearity, precision, and selectivity. The values of validation parameter obtained from the two methods were compared using statistical analysis independent t-test. Significant value (2-tailed) was obtained from the analysis data of the validation parameters, namely P> 0.05 so that it could be stated that there was no significant differences between the validation parameters of the agar diffusion method and the turbidimetric dilution method.

Overall results of the validation parameters determined from the agar diffusion method were better because the greater r value and the Vx_0 value,and the smaller CV value, compared to the turbidimetric dilution method. The turbidimetric and agar diffusion methods were methods that can be used to determine the bacterial growth inhibition of antibacterial substances (Mulu *et al*., 2004; Bogdanov, 2010; Sandra *et al*., 2015). In addition, it was known that the turbidimetric dilution method was more sensitive than the agar diffusion method. This result was implied from the MIC of ceiba honey against *B. subtilis* ATCC 6633 which was determined by the turbidimetric dilution method was lower than MIC which was assayed by agar diffusion method.

In the diffusion method, it is necessary to have the skill and accuracy of researchers in measuring the diameter of inhibitory zone which was done visually while in the turbidimetric dilution method, measuring % transmitance was carried out using more sensitive spectrophotometer (Hashim, 2014). The agar diffusion method was also influenced by several critical factors such as agar thickness and uniformity of the agar components. This happens because of differences in thickness of agar media and uniformity of agar influenced the diffusion process of the test substance so that it could affected the diameter of inhibitory zone produced as well (Bonev *et al*., 2008). Nevertheless, the results of statistical analysis using the independent t-test in the SPSS program showed that there was no significant difference between the agar diffusion and the turbidimetric dilution method for determining inhibitory activity of ceiba honey against the growth of *B. subtilis* ATCC 6633.

When evaluated from technical point of view, the turbidimetric dilution method was more simple when compared to the agar diffusion method. The sensitivity of the turbidimetric dilution method was also higher than the agar diffusion method because the turbidimetric dilution method produces lower LOD value than agar diffusion method. Based on these considerations, the

turbidimetric dilution method was more recommended for evaluation of the inhibitory effect of ceiba honey against the growth of *Bacillus subtilis* ATCC 6633, but the test should be done in a short time so that the sample used did not experience many physical or chemical changes during storage.

CONCLUSION

The agar diffusion method and the turbidimetric dilution method met the requirements of validation parameters for the inhibitory activity test of ceiba honey against the growth of *Bacillus subtilis* ATCC 6633. Both methods didn't have significant differences based on the validation parameters generated, but the turbidimetric dilution method is more recommended for evaluation of this inhibitory activity. This method was simpler technically and more sensitive with lower LOD value than the agar diffusion method.

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