

Article

Antibacterial Activity of Ethanolic Extract of Cinnamon Bark and Honey and Their Combination Effects Against Acne Causing Bacteria

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Abstract: *Propionibacterium acnes* and *Staphylococcus epidermidis* are the major skin bacteria that cause the formation of acne. The present study was conducted to investigate antibacterial activity of ethanolic extract of cinnamon bark, honey and their combination against acne bacteria. The antibacterial activity of extract of cinnamon bark and honey were investigated against *P. acnes* and *S. epidermidis* using disc diffusion method. Minimum Inhibitory Concentration (MIC) and minimal bactericidal concentration (MBC) were performed using Clinical and Laboratory Standard Institute (CLSI) methods. The interaction combination between extract of cinnamon bark and honey was determined by using a checkerboards method. The results showed that the MIC of extract of cinnamon bark and honey against *P. acnes* were 256 µg/mL and 50% v/v, respectively, while against *S. epidermidis* were 1024 µg/mL and 50% v/v, respectively. The MBC of extract of cinnamon against *P. acnes* and *S. epidermidis* were more than 2048 µg/mL, whereas the MBC for honey against *P. acnes* and *S. epidermidis* were 100%. The combination of cinnamon bark extract and honey against *P. acnes* and *S. epidermidis*, showed additive activity with the FICI value 0.625. Therefore, the combination of extract of cinnamon bark and honey has potential activity against acne causing bacteria.

Keywords: antibacterial activity, cinnamon, honey, checkerboards method, synergistic activity.

1. Introduction

Acne vulgaris is one of the most common skin disorders, mainly affects adolescents and causes severity of acne about 40% in 14-17 years old girls and 35% in boys aged 16-19 year [1]. Acne by definition is multifactorial chronic inflammatory disease of pilosebaceous units which affects the skin of the face, neck and upper trunk. Acne develops when these specialized follicles undergo pathologic alteration that results in the formation of non-inflammatory lesions (comedons) and inflammatory lesions (papules, pustules and nodules). *Staphylococcus epidermidis* and *Propionibacterium acnes* are the major skin bacteria that cause the formation of acne. Topical or systemic therapy is available for acne treatment, which includes comedolytic agents and antibiotics and various anti-inflammatory drugs and systemic therapy includes antibiotics, zinc and hormones. The excessive use of antibiotics for long periods can lead to increase resistance in acne causing bacteria. To overcome this problem of antibiotic resistance, essential oils and medicinal plant extracts is an alternative, which are safe, efficacious and multifunctional. In topical acne treatments, medicinal plants have fewer side effects than synthetic agents [2].

Cinnamon is able to kill acne causing bacteria and dry out the skin. The major constituents in cinnamon like cinnamaldehyde have anti-inflammatory properties. This could inhibit production of nitric oxide responsible for inflammatory conditions in the body. Cinnamon could also prevent production of COX-2, the pro-inflammatory agent. This proves its benefits as an antibacterial agent

and its ability to fight inflammation conditions caused by acne. It improves blood and oxygen circulation, therefore helping by unclogging and opening pores [3].

Honey works as a natural antibiotic, killing bacteria's which causes acne. Anti-inflammatory properties of honey bring down the redness of acne. Their acidic property does not allow the bacteria to grow [4]. Honey release hydrogen peroxide, which is an antibiotic that can also remove the bacteria and clear the acne [5]. Honey contains a lot of natural antioxidants which prevents damage from all of free radicals [6].

The reports on the antimicrobial activity of cinnamon extract and honey encourage this present study to investigate antibacterial activity of ethanolic extract of cinnamon bark, honey and their combination against acne causing bacteria.

2. Materials and Methods

2.1 Materials

Cinnamon bark powder and forest honey were obtained from Indonesia, reflux apparatus, and other chemicals were of reagent grade. Tests bacteria are *Propionibacterium acnes* and *Staphylococcus epidermidis* that obtained from culture collection of Microbiology Laboratory, School of Pharmacy.

2.2 Sample Preparation

Firstly, bark of cinnamon was washed and cut into small pieces. Bark of cinnamon was dried using drying cabinet under 40- 42°C for 3-4 days. The dried plant material was grinded into powder form.

2.3 Preparation of Ethanolic Extract

As many as 300 g of powdered Cinnamon bark were weighed and placed into flask. Ethanol 96% solvent was added into flask until the plant materials are submerged by the solvent. The reflux apparatus was set up and connected to the water bath, then it was connected to the condenser. Extraction process took 2 hours after the solvent boiling, repeated for 3 times. The ethanol extract was combined and vaporated by using rotavapor to remove the solvent to obtain thick extract.

2.4 Characterization of Crude Drug and Extract

The powder of crude drug was characterized by microscopic for identification of drugs on a cellular level and continued others characterization for quality control of the crude drugs such as water content determination, ethanol soluble extract matter, water soluble extract matter and phytochemical screening. Method for characterization of crude drug and extract were adopted from *Materia Medika Indonesia* [7] and *Biological and Phytochemical Screening of Plants* [8].

2.5 Determination of Honey Quality

Sweet honey quality test was performed by Honey SNI test parameters of the National Standardization Agency 2004 included the content of hydroxymethylfurfural (HMF), diastase enzyme activity, moisture content, levels of sucrose, reducing sugars as glucose levels, ash content, water insoluble solids and metal contamination [9].

2.6 Antibiotic and Extract Preparation

Plant extract and tetracycline HCl was dissolved in 1 mL of 100% DMSO prepared as stock solution. Then 0.1 mL from the stock solution was taken and diluted with 0.9 mL of MHB media and total volume of solution was 1 mL.

2.7 Preparation of Bacterial Suspension

After the agar slant was scratched using Öse needle with the microbe of interest was kept in the incubator at $35 \pm 2^\circ\text{C}$ for 24 hours. Then the following day, the microbe that has grown on the surface of the agar slant was slowly taken using the Öse needle again and suspended in the MHB and incubated for 18-24 hours at $35 \pm 2^\circ\text{C}$. The next day, the suspended microbe was diluted to attain absorbance between 0.08-0.13 (0.5 McFarland). After it has fulfilled the absorbance requirement, the 0.5 McFarland suspensions was diluted again with to MHB to a ratio of 1:20 result of the total colony 5×10^6 CFU/mL [10].

2.8 Determination of Total Colony Forming Unit

The microbe was grown on a Petri dish by mixing homogenously 1 mL of bacterial suspension with 15-20 mL of Nutrient Agar (NA) for several dilutions. Then, the agar was incubated for 24 hours and the colony forming unit was calculated using Colony Counter equipment. The concentration of initial bacterial suspension (stock) was calculated from the Petri dish containing 30 – 300 CFU [10].

2.9 Antibacterial Screening

Antibacterial screening is generally performed by disc diffusion method. Briefly 20 mL quantities of nutrient agar were plated in Petri dish with 1 mL of bacterial suspension. The sterilized filter paper discs (6 mm in diameter) placed on solidified agar plates which filled with 10 μL of various concentrations of plant extracts, honey and tetracycline HCl. Ethanol 96% was used to dissolve the extract and distilled water used to dissolve honey. Blank filter paper disc with 10 μL of solvent ethanol 96% was used as negative control. The activity was determined after 24 h of incubation at 37°C . The zone of inhibition was measured using Vernier's caliper. Each microbe was prepared for duplo set and average inhibition zone was taken [10].

2.10 Determination of Minimum Inhibitory Concentration (MIC)

The method used to determine the MIC of the plant extract was by using microdilution method. This method used microplate 96 well which consists of 12 columns and 8 rows. Initially, all wells were added with 100 μL of MHB, except the column number 12 was used as negative control which was added 200 μL of MHB. For column number 11 was used as positive control by just adding 100 μL of bacterial suspension. For column number 1, it was added with 100 μL of extract solution. Then followed by dilution steps, pipetted 100 μL from well column number 1 was transferred into well column number 2, after that pipetted 100 μL from well column number 2 was transferred into well column number 3. The step had repeated until reached well column number 10. Then the same steps were repeated for honey and tetracycline HCl. Finally, 100 μL of bacterial suspension was added starting from the column number 1 until 10. The microdilution plate was incubated at $35 \pm 2^\circ\text{C}$ for 18-24 hours [10].

2.11 Determination of Minimum Bacterial Concentration (MBC)

15 mL of Muller-Hinton Agar (MHA) was poured into Petri dish and let it become solidified. Then, the bacteria were inoculated from the well that showed MIC until the higher concentration and was streaked on the surface of the agar by using Öse needle. Then, the Petri dish will be incubated at $35 \pm 2^\circ\text{C}$ for 18-24 hours. The lowest concentration which gave no growth on subculture was recorded as Minimum Bactericidal Concentration (MBC).

2.12 Determination of Fractional Inhibitory Concentration (FIC)

Antibacterial activity combination of Cinnamon extract and honey against *Propionibacterium acnes* and *Staphylococcus epidermidis* was evaluated by determining the FICI value by using microdilution checkerboard method [10]. Firstly, all the wells were added with 100 μL of MHB. Then all the first row was added 100 μL of Cinnamon extract with the concentration four times the value

of MIC. Then 100 μ L from the first row was pipetted and transferred to the second row, then 100 μ L from second row was transferred to third row, repeat this step until row number 7. Next, 100 μ L of pure honey was added into the first column. Then 100 μ L from the first column was pipetted and transferred into the second number 9. Then, 100 μ L of the bacterial suspension was added into every well. Then the microdilution plate was incubated at $35 \pm 2^\circ\text{C}$ for 18-24 hours. The concentration of individual compound in combination between extract and honey which prevent visible growth was recorded as the MIC of the individual compound in the respective combination. The Fractional inhibitory concentration index (FICI) for the combination was calculated as followed: $\text{FICI} = \text{FIC of drug A} + \text{FIC of drug B}$, where FIC A is the MIC of drug A in the combination/MIC of drug A alone, and FIC B is the MIC of drug B in the combination/MIC of drug B alone. The combination is considered synergistic when the FICI is ≤ 0.5 , indifferent/additive when the FICI is > 0.5 to < 2 , and antagonistic when the FICI is ≥ 2 [11].

3. Results and Discussions

Bark of Cinnamon was bought from one of herbal medicines stores in Bandung, Indonesia. Firstly, the bark was selected to ensure that the bark used was not damaged and free from foreign matters. Then, the selected bark was washed for about three times with using clean and fast flowing tap water to remove soil and other contaminant. The bark was then cut into small pieces to increase the surface area and to minimize the drying process period. After that, drying process was conducted at drying cabinet at temperature 40°C - 42°C for 3- 4 days. Drying process is essential to reduce the water content present in crude drug which water can promote the growth of microorganism such as yeast and moulds. After drying process completed, the crude drug was grinded into powders. The purpose is to minimize the crude drug particle size which can lead to increase the surface area contact so this will be easier for the extraction process. Then, the crude drug was stored in the well closed container and protected from light. The microscopic characterization of *Cinnamomum* sp. bark powder was showed in Figure 1.

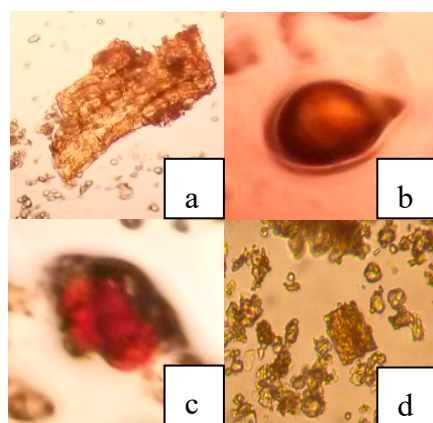


Figure 1. Microscopic characterization of *Cinnamomum* sp. bark powder: (a) parenchyma cells with starch; (b) resin cell; (c) cells with reddish brown contents; (d) crystal bearing cell.

The powdered crude drug undergone extraction process by using reflux method. About 300 g of crude drug was dissolved in 1 L of ethanol 96% as the solvent system. The extraction process was repeated for three times to obtain maximum yield. The solvent was then removed using a rotavapor to obtain the concentrated extract. Then, characterization of the extract and crude drug was conducted by determining water content, density, total ash, water and ethanol extractable matter and phytochemical screening. Standardization of characterization extract and crude drug are important to ensure the consistency of the plant extract in terms of quality, safety and efficacy. The result of characterization of crude drug and extract are shown in Table 1 and Table 2.

Table 1. Characterization of Cinnamon Bark Extract

Test	Result
Water content (v/w)	7.0%
Total ash (w/w)	6.5%
Ethanol extractable matter (w/w)	16.0%
Water extractable matter (w/w)	11.3%
Loss on drying (w/w)	6.98%
Total yield % (w/w)	15.93%

Based on the result of phytochemical screening of the crude drug and ethanolic extract of Cinnamon (Table 2) contained alkaloid, flavonoid, steroid/triterpenoid, tannin, and quinone and absence of saponin. It was similar with the previous study showed that *Cinnamomum cassia* bark extract contained phenols, alkaloids, steroids and tannins, while saponins and glycosides were not detected in the test extract [12].

Table 2. Phytochemical Screening of Crude Drug and Extract of Cinnamon bark

Compounds	Crude drug	Extract
Alkaloid	+	+
Flavonoid	+	+
Steroid/ Triterpenoid	+	+
Tannin	+	+
Saponin	-	-
Quinone	+	+

Note : (+) Detected; (-) Not detected

Furthermore, there have several methods for determination the characteristic of plant extracts. Firstly, the water content was determined by method of azeotropic distillation. This method was selected to determine the water content of the Cinnamon crude drug because of volatile compound which can lead to show inaccurate result when determining the water content by gravimetric method. Based on the evaluation, the water of Cinnamon crude drug powder was 9.0% (v/w) and the water content of ethanolic Cinnamon bark extract was 7.0% (v/w).

The total 1 kg of Cinnamon bark after grinding was 950 g so the percentage of yield was 95% (w/w). Besides, the total 300 g of Cinnamon bark powder undergoes reflux process and concentrated using rotavapor, as final weight of extract was 47.67 g, so the percentage yield of extract was 15.93% (w/w).

The characterization quality test of honey for the parameters such as enzyme diastase activity, water content, hydroxyl methyl furfural value, reducing sugars, acidity value, ash content, metal impurities include lead and copper fulfilled the requirement based on Standard Nasional Indonesia.

In this experiment, two tested bacteria strains were chosen which were *Propionibacterium acnes* and *Staphylococcus epidermidis* obtained from Microbiology Laboratory, School of Pharmacy. The bacteria cultures were streak on solidified slanted nutrient agar and incubated for 24 hours at 37°C. After 24 hours growth, the bacteria will be inoculated into Nutrient Broth (NB) and incubated again for 24 hours. Then prepared bacteria suspension at absorbance ranges between 0.08 - 0.13 by using

UV-Vis Spectrophotometer at wavelength of 625 nm. Based on determination of total Colony Forming Unit, followed by the serial dilution procedure for each bacteria to 10^7 and 10^8 . The result for *P. acnes* at absorbance of 0.093 was 154×10^7 and 146×10^8 , whereas for *S. epidermidis* at absorbance of 0.083 was 271×10^7 and 177×10^8 which both the bacteria strains fulfilled the CFU normal ranges from 30-300.

The antibacterial screening performed by disc diffusion method to determine the zone of inhibition of the plant extract, honey, tetracycline HCl and the solvent of ethanol 96% against two bacteria strains. From antibacterial screening of Cinnamon bark extract, honey and tetracycline HCl 250 µg/mL presented in Table 3. The highest activity of plant extract was 17.2 mm diameter of zone inhibition found against *P. acnes* followed by 16.8 mm diameter of zone inhibition against *S. epidermidis* whereas the control disc used for solvent (ethanol 96%) had no zone of inhibition. Furthermore, the activity of honey was 16.2 mm diameter of zone inhibition observed against *P. acnes* and against *S. epidermidis* was 16.7 mm. While the reference standard tetracycline 250 mg/mL, had the diameter of zone inhibition against *P. acnes* was 18.9 mm and against *S. epidermidis* 24.8 mm. Based on diameter zone of inhibition of extract and honey against both bacteria more than 16 mm, it can be concluded that Cinnamon bark extract and honey can be categorized as susceptible.

Table 3. Antibacterial screening of ethanolic extract of cinnamon bark, honey and tetracycline HCl

Strains	Diameter of zone of inhibition (mm)		
	Cinnamon bark extract 90% (w/w)	Honey 100% (v/v)	Tetracycline HCl 250 ppm (mg/mL)
<i>P. acnes</i>	17.2	16.2	18.9
<i>S. epidermidis</i>	16.8	16.7	24.8

The result of MIC showed that Cinnamon bark extract gave better inhibitory effect against *P. acnes* with the smallest MIC which was 256 µg/mL, followed by *S. epidermidis* with MIC of 1024 µg/mL. Meanwhile, the honey demonstrated same MIC against *P. acnes* and *S. epidermidis* which were 50% (v/v).

Table 4. Result of MIC and MBC of Cinnamon bark extract, Honey and Tetracycline HCl

Strains	Cinnamon bark extract (µg/mL)		Honey (%) (v/v)		Tetracycline HCl (µg/mL)	
	MIC	MBC	MIC	MBC	MIC	MBC
<i>P. acnes</i>	256	>2048	50	100	8	8
<i>S. epidermidis</i>	1024	>2048	50	100	2	2

The MBC was obtained after determining the MIC of the extract. MBC is the least amount of drug required to kill 99.9% of bacteria. The MBC of extract was determined by inoculating the bacteria strains from the well that showed MIC and streaked by using Öse needle to the MHA agar plate. This step was continued in order to determine the MBC of the extract until the concentration of extract in the well at 2048 µg/mL. From the evaluation, the Cinnamon bark extract showed growth at the agar plate until the concentration of extract at 2048 µg/mL. Therefore, the MBC cannot be obtained and it was assumed that the MBC might be greater than 2048 µg/mL (see Table 4).

Antimicrobial combinations were then tested in the hope of better interaction between extract of cinnamon and honey against those bacteria. When two drugs are given simultaneously, there a

possibly to occur interaction in the body that cause pharmacological effect medication such as synergistic, addictive or antagonist effect. Combination of extract and honey was evaluated from the FICI values for each combination using microdilution checkerboard method.

The concentration of individual compound in combination between extract and honey which prevent visible growth was recorded as the MIC of the individual compound in the respective combination. The extract and honey combination against *P. acnes* and *S. epidermidis* which displayed additive effect with the FICI value 0.625 (see Table 5). This means that the combined effect of cinnamon extract and honey is equal to the sum of the effect of each agent given alone. The combination of ethanolic extract of cinnamon bark and honey has potential activity against acne causing bacteria. In addition, since both of agents have a good flavor and texture therefore is a promising prospect to develop them as topical dosage form against acne causing bacteria.

Table 5. Result of FICI Between Tested Extract and Honey Against Tested Bacteria

Strains	Combination Extract + Honey	MIC ($\mu\text{g/mL}$)		FIC		Outcome
		Alone	Combination	FIC	FICI	
<i>P. acnes</i>	Cinnamon extract	256	32	1/8	0.625	additive
	Honey	50%	25%	1/2		
<i>S. epidermidis</i>	Cinnamon extract	1024	128	1/8	0.625	additive
	Honey	50%	25%	1/2		

Supplementary Materials: Figure S1: Result of microdilution checkerboard for combination of extract and honey against *P. acnes*, Figure S2 Result of microdilution checkerboard for combination of extract and honey against *S. epidermidis*

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Conflicts of Interest: The authors declare no conflict of interest

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