

Anti-Helicobacter pylori Effects of Propolis Ethanol Extract on Clarithromycin and Metronidazole Resistant Strains

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Anti-Helicobacter pylori Effects of Propolis Ethanol Extract on Clarithromycin and Metronidazole Resistant Strains

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ABSTRACT

Antimicrobial resistance for *Helicobacter pylori* infection still become problem in Indonesia especially for metronidazole and clarithromycin resistance that is commonly used in the national regimen therapy. Finding a new antimicrobial agent against this bacterium is still a challenge to face. Propolis extract is well known substance which has antimicrobial effect against some gram-negative bacteria. However, the effect against *H. pylori* remain unknown. We analysed the antimicrobial effect of propolis extract against *H. pylori* in clarithromycin and metronidazole Resistant Strains. Anti-*H. pylori* effect of ethanol propolis extract was tested on 10 types of *H. pylori* strains isolated from dyspeptic patients in Indonesia. Ethanol propolis extracts (PRO) show inhibitory activity at concentrations of 5% and 10% on all strains *H. pylori*. Determination minimum inhibitory concentration (MIC) value of propolis extract used microdilution method, the MIC values were in the range of 1024-8192 µg/mL. Determination of the minimum bactericidal concentration (MBC) value is performed by scratch method based on the results of the microdilution test and produces an MBC value in the range

of 1024 - 16348 µg/mL. The required concentration of propolis extract for the bactericidal effect against *H. pylori* was the same or eight-fold higher than the corresponding value. The combination of propolis extract and metronidazole based through paper tape method showed an additive effect, as well as the combination of propolis extract and clarithromycin. This extract might be can become alternative treatment against *H. pylori* infection that resistance for metronidazole and clarithromycin treatment

Keywords: *Helicobacter pylori*, ethanol propolis extract, clarithromycin, metronidazole

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INTRODUCTION

Helicobacter pylori is a gram-negative pathogenic bacteria that lives in the human stomach and the major cause of gastrointestinal diseases such as peptic ulcer diseases and chronic gastritis. This bacterium also responsible for most cases gastric cancer around the world (1). In general, the geographical distribution of *H. pylori* infection is related to the economic development. Infection rate decreases along with socioeconomic development. This relationship shows that lifestyle alteration affecting the presence of bacteria (2, 3). *H. pylori* infection in Indonesia was reported as low prevalence. However, a very diverse rate of infection was reported. The prevalence of Java Ethnic was 2.4% while Papuans, Bataks and Bugis had prevalence of 42.9%, 20% and

36.7% respectively (4). Hence, eradication of *H. pylori* in Indonesia requires a serious concern in several ethnics.

Eradication therapy for *H. pylori* infection uses a combination of therapies such as clarithromycin- or levofloxacin-triple therapy with quadruple therapy as second line(5). However, research in several countries shows that the prevalence of *H. pylori* resistance in metronidazole is 85% with clarithromycin and levofloxacin are find to cross the threshold of 15%, making the expected efficacy dropped to insufficient levels, in the great number of WHO regions countries respectively(6-8). This resistance problem also occurs in Asian countries which reached a rate of up to 67%(9). The antibacterial resistances become challenge to

find an alternative antibacterial agent to improve the eradication outcome.

Indonesia, a part of Southeast Asia countries, has a total population of ~260 million in 2017 compounds from many ethnics makes this country become the fourth most populous country in the world. Unfortunately, the resistance rates to some antibiotics treatment in Indonesia are high. Specific for *H. pylori* treatments, some antibiotics in the national regiment are not achieve the standard of the Maastricht consensus. For example, clarithromycin and metronidazole resistance rate showed resistance exceeds 15% and 40% (46.8% and 21.4% respectively) (10-13). Therefore it is necessary to find a natural substance as an adjuvant therapy for *H. pylori*, especially in case of clarithromycin and metronidazole resistant strains.

Propolis is well known traditional medical product in Indonesia with many benefits in health including antibacterial effect against some bacteria (14). Propolis or bee glue is a mixture of natural resins produced by honey bees from saliva, beeswax and substances collected from plant parts, buds and exudates. In general, propolis consists of resin (50%), wax (30%), essential oils (10%), pollen (5%) and other organic compounds (5%). The substances in propolis depend on the place and time of collection. The substances are polyphenol compounds, benzoic acid derivatives, cinnamic alcohols, cinnamic acids and their derivatives, sesquiterpenic hydrocarbons, triterpenes, benzaldehydes and their derivatives, alcohols, ketones, heteroaromatics, hydrocarbons, minerals, sterols, sugar and amino acids (15). Previous research has proven that propolis extract from Sulawesi province, Indonesia has the best anti-ulcer activity compared to propolis extract from Banten and North Sumatra of Indonesia. The high flavonoid and phenol content in propolis is strongly suspected to be responsible for anti-ulcer activity (16). Even though propolis extract is well known substance which has antimicrobial effect against some bacteria including gram negative bacteria, but the effect against *H. pylori* remain unknown. It is possible to make Propolis become adjuvant therapy beside the regiment to cure *H. pylori* infection, especially for antimicrobial resistant strains. This study was conducted to investigate the antibacterial effect of Propolis against *H. pylori* clarithromycin and metronidazole resistant strains.

MATERIALS AND METHODS

Preparation of Propolis Extract

Raw propolis from bee *Trigona* sp. was obtained from South Sulawesi. Raw propolis stored in the freezer was cut into smaller pieces. Propolis was extracted by reflux method using 70% ethanol solvent in the ratio (1: 5/400 gram: 2000 ml) for 2 hours and repeated 3 times. The extracted substance then was filtered using a glass funnel and filter paper 2 times. The filtrate was concentrated with a rotavapor at 50°C and then rises to 65°C until the volume of the solvent remains low and can still be poured into the vaporizer cup. Extracted substances then were heated on a water bath while stirring until a thick extract is obtained with a fixed weight. The thick extract is put into a jar that has been coated in aluminum foil. The simplicia obtained from the screening process is

extracted again using the reflux method as above instructions for 3 repetitions.

Culture of *H. pylori*

H. pylori bacterial isolates were obtained from dyspeptic patients through endoscopy examinations in 6 cities on the 5 largest islands of Indonesia including SBY 151, SBY 131 on Surabaya, Java Island, KPG 22, KPG 30 on Timor Island, PTK 20 on Kalimantan Island, MKS 47, MKS 52 and MND 20 on Sulawesi Island, and SMS 20, SMS15 on South Sumatra Island. Hence stratified sampling were applied to get the samples from each island that consisted of resistant and sensitive strain to either clarithromycin or metronidazole. Bacterial isolates were identified as *H. pylori* through observations of colony morphology, gram staining, positive reactions for oxidase, catalase and urease, then classified as strong, moderate or low based on virulence factor VacA as shown in table 1. *H. pylori* isolates were stored in the -80°C freezer in Brucella broth media supplemented with horse serum (10% v/v) and glycerol (10% v/v).

The media used was blood agar consisting of Mueller-Hinton Agar and horse blood (10% v/v). Horse blood is mixed aseptically on a warm sterilized agar medium at 56°C. Frozen *H. pylori* stock is inoculated in blood agar media and incubated for 3-5 days at 37°C under microaerophilic conditions in 10% CO₂ incubator. Bacterial suspension solution is made using serum broth consists of Mueller-Hinton Broth and horse serum (10% v/v) which is mixed aseptically after the sterilization process. Bacterial culture from the blood agar media is suspended in serum broth then incubated for 24 hours in 10% CO₂ incubator at 37°C. The bacterial suspension is diluted with serum broth until the turbidity is equivalent to a standard solution of 0.5 McFarland (10⁸ CFU/mL). In microdilution and turbidimetry testing, the suspension was diluted again 1:20 to produce a colony count of 5x10⁶ CFU / ml(17).

H. pylori Growth Inhibition by Disk Diffusion

We performed several test to check the ability of propolis to inhibit the *H. pylori* growth. First, growth inhibition of *H. pylori* was assayed by the disc diffusion method (DDM). A total of 50 µl of bacterial suspension was pipetted into a petri dish then mixed with a serum medium of 15 mL consisting of Mueller-Hinton Agar supplemented with 10% horse serum and homogenized. The media should be left to solidify then prepared 5 sterile paper (d = 6.7 mm).

Disc paper is dipped in each of these solutions then placed on the surface of the media. Petri dishes are stored in a 10% CO₂ incubator at 37°C for 3 days. Then the diameter of the clear zone was observed using a digital calipers(18).

Determination of Minimum Inhibitory Concentration (MIC)

Second, MIC values were determined through the broth microdilution method using several concentration of propolis extract (0-8192 µg / ml) in serum broth that contained bacterial suspensions. Then the microdilution plate was incubated at 37°C for 3 days in a 10% CO₂ incubator. The MIC value is shown from the smallest concentration that successfully inhibits bacteria so that there is no precipitation or turbidity is founded in the plate well (18).

Determination of Minimum Bactericidal Concentration (MBC)

Determination of the MBC value was performed by scratch method based on the results of the microdilution test. The Ose needle is dipped into the well with a clear solution then inoculated on the blood agar media that has been prepared. Petri dishes were incubated for 24 hours in a 10% CO₂ incubator at 37°C. The lowest concentration of the test solution that showed no bacterial growth on solid media was determined as the MBC (18).

Combination Effects Determination

Combination effects were determined using the paper tape method. A total of 50 µl of bacterial suspension was pipetted into a petri dish then mixed with a serum medium of 15 mL consisting of Mueller-Hinton Agar and horse serum (10% v/v) and homogenized. The media to be left to solidify then prepare sterile Whatman paper with a length of 4 - 5 cm and a width of 0.5 - 0.9 cm. Each filter paper is dipped in an extract solution (8192µg/mL) and an antibiotic solution (clarithromycin: 0.5 µg/mL and metronidazole: 100 µg/mL) then placed on the media to form an angle of 90°. Then the petri dish was incubated in a 10% CO₂ incubator at 37°C for 18-24 hours. The combination properties of the extract solution and the antibiotic solution are interpreted based on the pattern of clear zone that appears around the paper tape. Additive properties are characterized if the clear zone on stacker paper tape do not increase in width, synergistic properties are characterized if the clear zone on stacker paper tape is widened, while antagonistic properties are characterized if the clear zone on stacker paper tape is narrow down.

Data Analysis

Discrete variables were tested by using the chi-square test; continuous variables were tested with the Mann-Whitney U. The SPSS statistical software package version 18.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

RESULTS

H. pylori Growth Inhibition Analysis

For the first screening, the growth inhibition was assayed by disc diffusion method (DDM) with three different concentrations, 10 mg/mL, 50 mg/mL and 100 mg/mL as shown in Table 1. The result showed that the propolis concentration at 10 mg/mL did not show any inhibition, except for SMS20 strain. However, we observed inhibition of growth after the increase of concentration from 10 mg/mL to 50 mg/mL with significantly different (P Value < 0.001) with the average inhibition 0.71mm for concentration 10 mg/mL and 7.95 mm for concentration 100 µg/mL. The further increase of concentration to 100 µg/mL also significantly improve the inhibition with the average inhibition 8.89 mm (P < 0.001).

In attempt to confirm the inhibitory concentration of the propolis, we also determined the MIC propolis. The propolis MIC was ranged from 1024 to 8192 (Table 2). However, according to the previous study (19), the MIC that ranged from 1024 to 8192 was assigned as non-effective inhibitory

effect. We also analyzed the MIC of clarithromycin and metronidazole for each strain. As expected, the MIC of propolis was significantly higher compared to the MIC of metronidazole and clarithromycin.

The bactericidal effect of the propolis was similar or higher than MIC in all strains. Antibacterial agent was usually regarded as bactericidal if the MBC values were not more than four times of MIC. The average MBC value was 2.3 times higher than MIC values which has range from 1-8 times (Table 3). Only one strains had MBC value 8 times higher than the MIC. Hence, this propolis had potential bactericidal effect; even though the concentration very high.

Combination Effect of Propolis and Antibiotics

We observed the usage of propolis combined with either clarithromycin or metronidazole resulted in additive effect for some strains as shown in table 4. The additive effect of the propolis to clarithromycin was observed in 60% (6/10) of the samples. The same percentage of additive effect was also observed in the metronidazole. The results also showed that the additive effect still presented in 42.9% (3/7) of the metronidazole-resistant strain. In addition, the additive effect also presents in 67% (2/3) of clarithromycin resistant strain. However, the propolis could not give any effect to the strains that has extremely high MIC (>256 µg/mL) either in metronidazole or clarithromycin.

DISCUSSION

This study represents the first propolis antimicrobial effect investigation against resistant strain of H. pylori in Indonesia. Our study showed an antibacterial effect of propolis against H. pylori, even in the clarithromycin and metronidazole resistance strain. This antibacterial effect of propolis was hypothesized due to its antimicrobial bioactive substance and urease inhibitory effect (20). Other previous study also reveal several compound in the Brazilian propolis had antibacterial effect on the H. pylori (21). Thus, we attempted to confirm the antimicrobial property of Indonesian propolis on the clinical isolates of H. pylori.

In the diffusion test, the range of inhibitory diameter of propolis extract with different concentration against H. pylori resistant strains was at 7.05 ± 0.15 mm to 10.85 ± 1.48 mm. H. pylori considered as sensitive if an antibacterial is able to provide a resistance diameter >12 mm (22) while the results of the inhibition diameter test of propolis extract shows a diameter size of <12 mm that indicate it has low inhibitory effect. Previous study showed that propolis has many compounds, i.e. caffeic acid phenetyl ester (CAPE) (23), that had an effective antimicrobial effect against sensitive H. pylori strain (21). However, the propolis concentration to create a diameter inhibition among the reports were not standardized. In this study, we used 1%, 5% and 10% concentration. Hence, we performed other tests to confirm the antimicrobial activities of the propolis.

Determination MIC value of propolis extract used microdilution method, the MIC values were in the range of 1024 - 8192 µg/mL. Tamakou et al. (2017) mentioned that a plant extract can be categorized become four level of antimicrobial activity. The extract has very good antibacterial

⁸ activity if it has MIC <100 µg / mL; significantly active if it has 100 ≤ MIC ≤ 512 µg / mL; quite active when 512 ≤ MIC ≤ 2048 µg / mL; and has weak antibacterial effect if the MIC is >2048 µg / mL (19). According to this criteria on the 60% of *H. pylori* strains, propolis extract showed a quite active antibacterial activity while the remaining strains indicated weak inhibitory activity of propolis. It should be noted that most of the weak inhibitory activity was observed in the resistant strain with extremely high MIC. This result was in concordant with the previous study, that showed a good MIC and MBC for some *H. pylori* strains, but the effect was decreased in resistant strains (20). This result can be explained based on previous study that at inhibitory concentrations, after exposure to individual polyphenol compounds, propolis shown to make the formation of vesicles and cell lysis (24). Interestingly, the MBC value of the propolis was less than four-times compared to the MIC in 90% of the samples. This may suggest a potential bactericidal activity of propolis which support the utilization for the alternative antimicrobial against *H. pylori*.

The propolis extract also showed an interesting result of additive effect against *H. pylori* using with clarithromycin or metronidazole. The fact that propolis has urease inhibition (23) and HpPDF (*Helicobacter pylori* peptide deformylase) inhibition (20), might be the result of additive effect with anti *H. pylori* regimen. Moreover, the additive effect was still observed in the resistant strains.

The high value of MIC makes it a drawback for propolis. Hence it is less likely to use this for the single therapy or complete substitution of *H. pylori* antimicrobial agent. However, potential function of propolis was supported by the bactericidal properties and additive effect of propolis, even in the resistant strains (with mild MIC). Further studies are necessary to investigate antimicrobial effect of propolis as the adjuvant therapy against *H. pylori* resistance strains, either molecular mechanism or clinical trial study.

CONCLUSION

Ethanol propolis extract has activity against some *H. pylori* clarithromycin and metronidazole resistant strains. Further clinical trials are needed to give more information and validate the effectivity of Propolis extract against *H. pylori* infection especially in resistant strains.

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CONFLICT OF INTEREST

All authors have no conflict of interest to report.

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Table 1. Inhibitory Effects of Propolis Extract on *Helicobacter pylori*

Strain Name	Clear Zone (mm)		
	10 mg/mL Propolis	50 mg/mL Propolis *	100 mg/mL Propolis *
SMS 20	7.05 ± 0.15	8.35 ± 0.25	9.15 ± 0.05
KPG 22	0	8.15 ± 0.35	10.85 ± 148
PTK 20	0	7.60 ± 0	8.45 ± 0.35
SBY 151	0	8.50 ± 0.20	9.6 ± 0.30
MKS 52	0	7.60 ± 0	8.7 ± 0.10
MKS 47	0	7.70 ± 0.28	8.85 ± 0.35
MND 20	0	8.20 ± 0.30	8.70 ± 0.10
SMS 15	0	8.05 ± 0.35	8.10 ± 0.57
KPG 30	0	8.20 ± 0.30	8.25 ± 0.45
SBY 137	0	7.15 ± 0.07	8.25 ± 0.63

*P <0.001

Table 2. MIC result of Propolis in comparison with metronidazole and mlarithromycin

Strain Name	MIC metronidazole (µg/mL) (Resistant/Susceptible)	MIC clarithromycin (µg/mL) (Resistant/Susceptible)	MIC Propolis (µg/mL)
SMS 20	64 (R)	0.032 (S)	4096
KPG 22	>256 (R)	0.125 (S)	2048
PTK 20	>256 (R)	0.250 (S)	8192
SBY 151	1,5 (S)	0.016 (S)	4096
MKS 52	64 (R)	0.125 (S)	2048
MKS 47	8 (S)	0.032 (S)	2048
MND 20	8 (S)	64 (R)	2048
SMS 15	86 (R)	0.500 (R)	4096
KPG 30	96 (R)	0.032 (S)	2048
SBY 137	>256 (R)	>256 (R)	1024

Table 3. The Results of MBC Measurement

Strain Name	MIC Propolis (µg/mL)	MBC Propolis (µg/mL)	MBC/MIC rate
SMS 20	4096	4096	1
KPG 22	2048	4069	2
PTK 20	8192	16348	2
SBY 151	4096	4096	1
MKS 52	2048	2048	1
MKS 47	2048	2048	1
MND 20	2048	2048	1
SMS 15	4096	16384	4
KPG 30	2048	4096	2
SBY 137	1024	8192	8

Table 4. The combinatory properties of propolis to metronidazole and clarithromycin

Strain Name	Properties (Additive/Synergistic/Antagonistic)		MIC µg/mL (Resistant / Susceptible)	
	Metronidazole	Clarithromycin	Metronidazole	Clarithromycin
	SMS 20	Additive	Additive	64 (R)
KPG 22	No effect	No effect	>256 (R)	0,125 (S)
PTK 20	No effect	No effect	>256 (R)	0,250 (S)
SBY 151	Additive	Additive	1,5 (S)	0,016 (S)
MKS 52	No effect	No effect	64 (R)	0,125 (S)
MKS 47	Additive	Additive	8 (S)	0,032 (S)
MND 20	Additive	Additive	8 (S)	64 (R)
SMS 15	Additive	Additive	86 (R)	0,500 (R)
KPG 30	Additive	Additive	96 (R)	0,032 (S)
SBY 137	No effect	No effect	>256 (R)	>256 (R)

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