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Study on the Antibacterial Effect from Moonmilk Pindul Cave, Indonesia

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ABSTRACT

Background and Aim: Antibiotic resistance is the ability of microbes to survive antibiotic exposure with standard doses. Regular updating of antibiotics is one solution to overcome antibiotic resistance. One source with potential new antibiotics is moonmilk from caves because it contains various bacteria proven to have antibacterial activity. The objective of the present study is to explore the potential of moonmilk microbes from Pindul cave, Indonesia, as a source of new antibiotic compounds.

Materials and Methods: PCR and Sanger sequencing method using 16S rRNA primers was performed to identify species of isolated moonmilk bacteria. The antibiotic potency test was divided into six groups with various concentrations of isolated moonmilk bacteria (IMM) supernatant 25%, 50%, 75%, 100%, negative control (-), and positive control (+). All groups were further tested for antibiotic susceptibility using the disk diffusion (Kirby and Bauer) method to pathogenic resistant bacteria *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 on Mueller Hinton agar medium.

Results and Conclusion: The results showed that moonmilk bacteria had antibacterial activity to *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. The highest inhibition zone was observed for the bacterial extract concentration of 75% (42 mm) for *E. coli* ATCC 25922 and 100% (23 mm) for *S. aureus* ATCC 25923. The 16S rRNA sequence analysis showed that the IMM shared a 99,64% (1399 nucleotide match) similarity with *Bacillus licheniformis* strain IND706. IMM supernatant extract from Pindul cave, Indonesia, has the potential to control antibiotic-resistant bacterial pathogens.

Keywords: Antibiotics, *Bacillus licheniformis*, Moonmilk, Pindul Cave

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1 Introduction

Antibiotic resistance has become a significant problem in the medical world in recent years. Antibiotics are organic components produced by microorganisms that can kill other microorganisms or inhibit the growth of other organisms. The antibiotics mechanism inhibits cell wall synthesis, damages cell membrane structure, inhibits nucleic acids' structure and function and inhibits protein synthesis **(1)**. Antibiotic resistance has become World Health Organization (WHO) concern at every health service level and other sectors **(2)**.

Many previous studies have reported the incidence of antibiotic resistance against many pathogenic bacteria. The *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), and *Staphylococcus aureus* (*S. aureus*) were resistant to various antibiotics such as penicillin, ampicillin, tetracycline, and streptomycin **(3,4)**. Antibiotic resistance can occur due to persistent failure to develop or discover new antibiotics and non-judicial use of antibiotics are the predisposing factors associated with the emergence of antibiotic resistance **(5)**. Regular updating of available antibiotics is one solution to this problem by finding

new sources of antibiotics, such as from *Bothroponera rufipes* ants, Spongia, and moonmilk from caves **(6)**.

Moonmilk from caves is a potential source of new antibiotics. Moonmilk is a speleothem composed of CaCO3 with different colors and textures. The process of forming moonmilk is presumed to involve various microorganisms **(7)**. A lot of previous research has shown that moonmilk calcite contains many considerable bacterial that have antibacterial activity against a wide range of bacteria and fungi **(8)**. Some bacteria that can form moonmilk calcite are bacteria from the genus *Bacillus* and *Streptomyces* **(9)**. Previous studies have reported fermented *Bacillus licheniformis* (*B. licheniformis*) strain B65-1 can produce phenylacetic acid and has potential as an antimicrobial. *B. licheniformis* strain B65-1 can inhibit the growth of various Gram-positive and Gram-negative bacteria **(10)**. Pindul cave is a karst cave located in Bejiharjo Village, Karangmojo District, Gunung Kidul Regency, Special Region of Yogyakarta, Indonesia. It has a length of ± 350 m and a height of 4 m from the lowest point to the cave's roof at 12 m **(11)**. This study aimed to discover the potential of microbes from Pindul cave moonmilk as a source of new antibiotic compounds. We found that the isolated moonmilk bacteria compound could detain the growth of the pathogenic bacteria.

Methodology

Moonmilk Sampling and Isolation

The moonmilk was collected from the dark zone of Pindul cave, Gunungkidul, Indonesia in May 2021. The moonmilk sample was carried out by a serial dilution method using Ringer's lactate sulfate solution six times to reduce the content of gram-negative bacteria. The results of the moonmilk suspension were then inoculated into starch casein agar, and the intern-ational streptomyces project two broth and nystatin solution 100,000 IU to prevent fungal growth. The moonmilk culture was then incubated for 14 days in an incubator at 27^{o}C. The Isolated moonmilk microbe (IMM) colonies were selected and continued to be observed macroscopically to see the shape, elevation, margins of moonmilk colonies, and gram staining.

Fermentation and Extraction of Moonmilk Bacterial Secondary Metabolite

IMM was fermented in international streptomyces project two broth medium for seven days at 32^{o}C. The isolate suspension was then centrifuged for 30 minutes at 8000 rpm to separate the biomass and the supernatant. The biomass and supernatant were then macerated for three days using methanol. The supernatant and biomass were separated, and the supernatant will be continued for the antibiotic potency test.

Antibacterial Activity Test

The antibacterial activity test in this study used four isolated moonmilk bacteria supernatant with variation concentrations (25%, 50%, 75%, and 100%), antibiotic streptomycin sulfate as a positive control, and distilled water as a negative control with Kirby-Bauer test based on the previous study **(12)**. A 5 mm disc was immersed in each treatment and control. The potential antibiotic test in this study used two resistant pathogenic bacteria, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. The IMM were cultured into liquid media of nutrient broth and brain heart infusion broth. The cultured test bacteria were uniformly spread into Mueller Hinton agar medium with the glass spreader. Briefly, 10 µL of the cell-free supernatant was applied to filter disks (7 mm in diameter). Inhibition zone was expressed as diameters and measured after incubation at 37°C for 24 h to see the antibiotic potential of the Pindul cave moonmilk bacteria.

Isolation of Moonmilk Microbial DNA and PCR Amplification

The IMM, which has been proven to be a potential source of new antibiotics, will be continued for molecular analysis. Isolation of IMM DNA in this study using the Wizard® Genomic DNA Purification Kit (Promega, USA). The DNA was amplified by using Go Taq® Green Master Mix (Promega, USA), primer 27F (Forward): AGA GTT TGA TCM TGG CTC A; 1525R (Reverse): AAG GAG GTG WTC CAR CC, and ddH2O. The PCR amplified program was the pre-denaturation temperature at 96°C for 5 minutes, denaturation for 30 seconds at 96°C, annealing for 30 seconds at 55°C, extension for 1 minute at 72°C, and final extension for 7 minutes at 72°C. The electrophoresis of PCR product was by using 1,5% agarose gel, red staining gel, and SMOBIO ExcelBandTM 1 KB (0.21-10 kb) DNA Ladder for 1 hour using 80 volts. The DNA sequence was identified by using Sanger sequencing.

Results and Discussion

Moonmilk Isolation and Extraction

This research uses Pindul Cave located in Gunungkidul, Daerah Istimewa Yogyakarta, Indonesia (7°55'-45.7"S 110°38'55.8"E), as the sampling location. Samples were aseptically collected in June 2021 from one moonmilk deposit. Hard moonmilk speleothem was scratched with sterile scalpels from the wall in the dark zone, near the stairs to the swallow's nest (Figure 1).

The moonmilk that we discovered has a white-colored and hard-textured Pindul cave wall. Moonmilk

is a white deposit commonly seen on the walls, ceilings, and floors of limestone caves worldwide and has a range of textures from soft to muddy **(13)**. Morphology observations were carried out on five bacterial colonies (IMM1, IMM2, IMM3, IMM4, and IMM5). All five IMM colonies had a White-colored colony, round shape, convex elevation, and entire margin. All five IMM colonies were classified as gram-positive bacteria as they showed purple color after being given gram staining (Table 1). Bacteria can live in various environments, including conditions without light, such as caves.

(Photo : (a) ; (b) Diyah Novi Sekarini; (c) ; (d) Astri Amelia Suma)

Table 1. Comparison of Colony Characters of Desired Isolates in SCA and Gram Staining Identification

Colony number	Selection from dilution tube	Isolation technique	Culture media for isolation	Colony characters of desired isolates in SCA	Gram Staining identification
1	10-5	Spread plate method	SCA, ISP 2 Broth	White-colored colony, round shape, convex elevation, and entire margin	+
2	10-5				+
3	10-5				+
4	10-5				+
5	10-5				+

The microorganisms that can cooperate to form the moonmilk structure depend on the state of the moonmilk. The bacteria found in the wet moonmilk texture, water, and air sample are dominated by gram-negative bacteria such as *Chloroflexi*, *Proteobacteria*, and *Acidobacteria*. Gram-positive bacteria were founded in dry moonmilk texture, sediment, and cave rocks, such as bacteria from the phyla of

Actinobacteria and *Firmicutes* **(10)**. The microorganism that cooperates in forming moonmilk is highly influenced by environmental factors such as sunlight, Waterflow, cave sediment, cave wall surfaces, biofilm, pH, nutrition, trace elements, temperature, humidity, and cave depth **(14)**. Previous studies have reported the influence of microorganisms on moonmilk formation using various methods. However, it remains unclear how microbial communities' composition diversity depends on the type of moonmilk **(15)**.

Antibacterial Activity Test

The antibacterial activity test of IMM1, IMM2, IMM3, IMM4, and IMM5 supernatant extract against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 was shown with various concentrations of 25%, 50%, 75%, and 100% (Figure 2). However, only IMM5 displayed inhibition zone results against E. coli ATCC 25922 and S. aureus ATCC 25923. The inhibition zone of IMM5 supernatant extract results was very strong with a strong susceptibility. Moonmilk bacterial supernatant inhibited E. coli ATCC 25922 bacteria in a maximum inhibition zone of 42 mm (75 %) and S. aureus ATCC 25923 bacteria at 23 mm (100 %).. However, no antibacterial activity was observed on isolated moonmilk bacteria supernatant 50% against S. aureus ATCC 25923 (Table 2)..

Table 2. Antibacterial Activity of Isolated Moonmilk Microbe Supernatant Extract Against E. coli and S. aureus

Treatment	Inhibition zone diameter (mm)			
	E. *coli*	**Susceptible**	**S. *aureus***	**Susceptible**
Streptomycin (Positive Control)	25 mm	Very Strong	35 mm	Very Strong
Waterone (Negative Control)	0 mm	No Response	0 mm	No Response
Supernatant 25%	22 mm	Strong	17 mm	Strong
Supernatant 50%	13 mm	Strong	0 mm	No Response
Supernatant 75%	42 mm	Very Strong	10 mm	Strong
Supernatant 100%	10 mm	Strong	23 mm	Very Strong

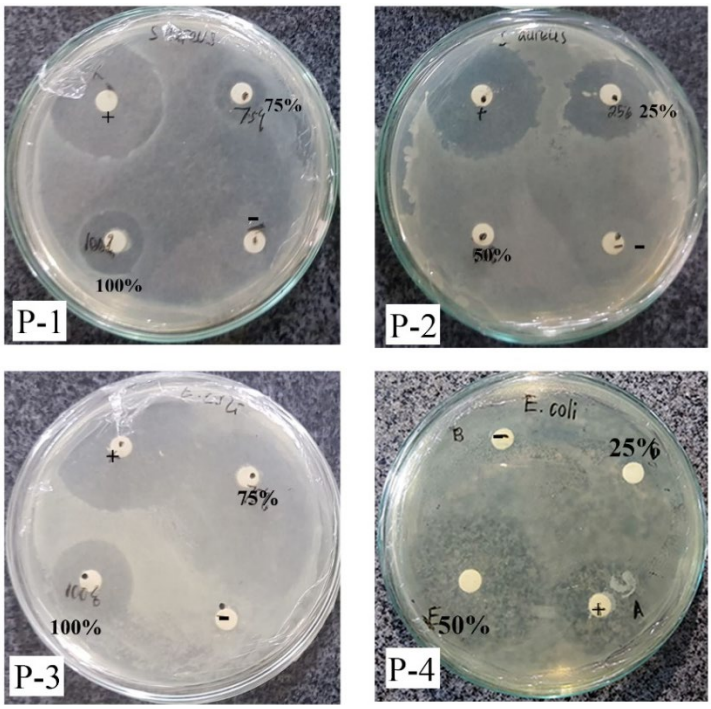

Figure 2. Isolated moonmilk bacteria supernatant antibacterial activity test against bacteria *S. aureus* ATCC 25923 (P-1, P-2) and *E. coli* ATCC 25922 (P-3, P-4). Isolated moonmilk bacteria supernatant antibacterial activity test using streptomycin disk as a positive control (+), Aquadest as a negative control (-), variations concentration of moonmilk bacteria supernatant 25%, 50%, 75% and100%.

In this result, we speculate that secondary metabolites extract of IMM5 showed wider results than several previous studies that tested various sources of new antibiotics against bacteria E. coli ATCC

25922 and S. aureus ATCC 25923. Zone of inhibition formed using Streptomyces isolated from marine sediment in India named *Streptomyces* spp. VITBRK2 has an inhibition zone of 21 mm against *S. aureus* ATCC 25923, and this result showed that secondary metabolites produced by *Streptomyces* spp. VITBRK2 could be used as a lead to control drug-resistant bacterial pathogens **(16)**. B. licheniformis of infant milk formulated also showed antimicrobial activity against S. aureus with all of the inhibition zones formed was under 20 mm and exhibited no antimicrobial activity against *E. coli*.

Bacillus licheniformis could produce a non-ribosomal peptide called subpeptin and bacitracin antibiotics. Bacitracin is an antibiotic produced by *B. subtilis* and *B. licheniformis*, which is formed by five polypeptides and consists of bacitracin A, B, and C. Bacitracin is used topically and orally to treat infections against Gram-positive bacteria such as *Staphylococcus* but not against Gram-negative bacteria. Lichenicidin is a putative lantibiotic mersacidin precursor that could inhibit the growth of *Listeria monocytogenes*, methicillin-resistant *S. aureus*, and vancomycin-resistant enterococci strains **(17)**. In this study, we found that the supernatant extract from isolated moonmilk bacteria has a very strong effect as an antibacterial and potential as the new antibiotic source.

Identification Moonmilk Bacteria Isolate

The results of PCR amplification on moonmilk bacterial isolates using 16S rRNA primer showed bands in 1,500 base pairs (Figure 3). The Sanger sequencing was performed to the identified sequence of PCR products. Moonmilk bacteria were identified and classified by comparing the moonmilk bacterial isolate genome with the all known species genome. Data on GenBank (https://www.ncbi.nlm.nih.gov-/genbank/) shows that moonmilk microbes were related to *B. licheniformis* sequence (strain IND706 16S ribosomal RNA gene) with 100% Query Coverage and 98.72% Percent Identity.

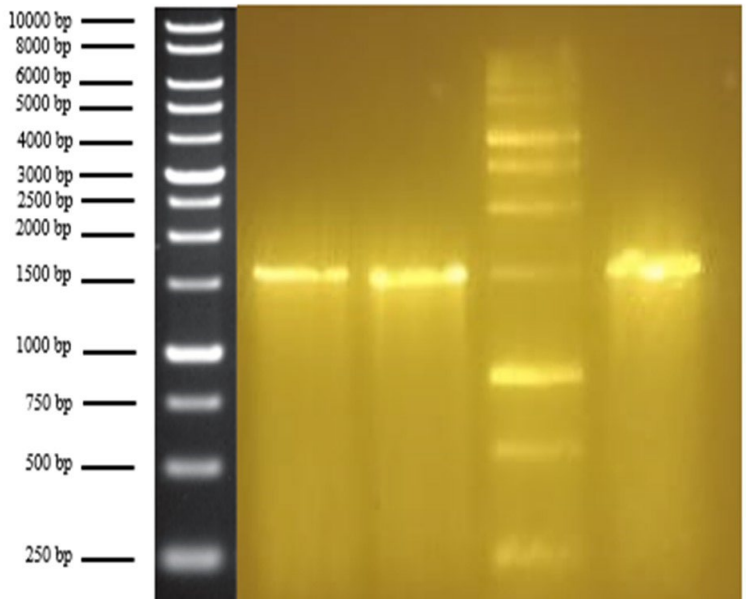

Figure 3. Isolated Moonmilk Microbe-stained perpendicular DGGE separation patttern of 3 PCR samples using 16S rRNA primer and 1 Kb DNA Ladder showed that Isolated Moonmilk Microbe DNA had bands of 1,500 base pairs.

Bacillus licheniformis is a Gram-positive bacteria found mainly in the soil and can form moonmilk calcite. *B. licheniformis* has been reported to have anti-obesity, anti-diabetic, reduced accumulation of β-amyloid in the hippocampus, and can produce antibiotic called lichen-forming **(18,19)**. *B. licheniformis* also can be used for large-scale industrial fermentation to produce amylase **(20)**.

The phylogenetic tree presented the moonmilk microbe is closely related to the species *B. licheniformis*. The bootstrap value between the two sequences is 100%, with an identity value of 99.64%. The phylogenetic tree was created based on the match of moonmilk microbe with the bacterial gene in the GenBank database. The alignment of the 16S rRNA gene sequences showed a high similarity between strains (Figure 4).

Figure 4. NCBI BLAST search of the assembled sequence of Isolated *Moonmilk* Microbe (IMM5). Alignment result of the assembled consensus sequence of *Bacillus licheniformis* sequence (strain IND706 16S ribosomal RNA gene). The two sequences matched with 100% Query Coverage and 98,72% identity.

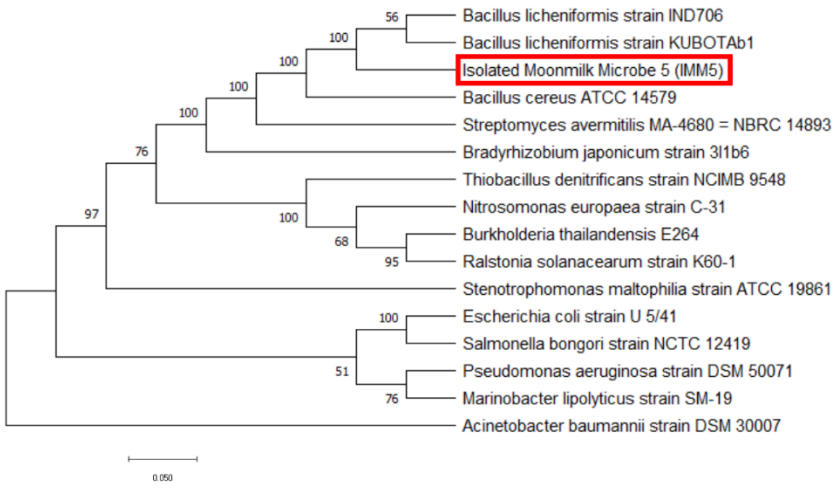

Figure 5. The joined neighboring phylogenetic tree of the 16S rRNA sequence shows the relationship between *Bacillus licheniformis* strain IND706, and related species of the genus *Bacillus* with Bar, 0.050 substitutions per nucleotide position

5. Conclusion

Bacillus licheniformis supernatant extract compound from Pindul cave, Indonesia, has potency as a new antibiotic source and could be used as a lead to control drug-resistant bacterial pathogens.

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Author's Contributions

ADP, DNS, AAS, and RM designed this study and took responsibility for the data's integrity. ADP, DNS, and AAS performed the research. RM supervised and provided expertise. ADP, DNS, AAS, and RM contributed substantially to the study design, data analysis, and data interpretation. ADP and RM contributed substantially to writing the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

All authors declared no conflict of interest.

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