ORIGINAL ARTICLE

Antimicrobial Activity Screening of Bacteria Isolated from Tasik Cermin

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ABSTRACT

Introduction: With the emergence of the multi-drug resistant bacteria that threaten the human health, novel antimicrobial compounds from natural environment have been increasingly explored. Tasik Cermin is a lake completely covered by karst towers and hills and lacks any inflow or outflow of water making it an oligotrophic environment with limited nutrient availability. Increased competition among microorganisms leads to production of antimicrobial compounds that can inhibit the growth of its competitor. Thus, the aim of this study is to assess antimicrobial activity of bacterial isolates from Tasik Cermin and identify the most resistant isolate. **Methods:** The isolates were tested against five test bacteria: S*taphylococcus aureus, Bacillus subtilis, Streptococcus pneumoniae, Escherichia coli, and Proteus vulgaris* by primary screening through perpendicular streak method, secondary screening followed by MIC and MBC and the most resistant bacteria was identified by molecular identification. **Results:** The result indicates that only one isolate (Isolate TC1A) was able to show potential antimicrobial activity against *P. vulgaris* and *S. pneumoniae*. This isolate was further tested by secondary screening through agar well diffusion method and inhibition zone were observed on *P. vulgaris* (14.97 ± 0.05), *E. coli* (9.23 ± 0.25) and *S. pneumoniae* (14.93 ± 0.12). Statistical analysis by One-Way ANOVA and Tukey test method indicates that inhibition zone of *E. coli* is significantly different as compared to *S. pneumoniae* and *P. vulgaris*. Molecular identification revealed that the isolate TC1A was identified as *Achromobacter sp.* with 97.68% similarity percentage. **Conclusion:** This finding shows that bacterial isolates from under-explored areas have the potential of producing novel antimicrobial compounds.
Malaysian Journal of Medicine and Health Sciences (2023) 19(SUPP18)36-45. doi:10.47836/mjmhs19.s18.6

Keywords: Antimicrobial activity; Tasik Cermin; Oligotrophic; freshwater; *Achromobacter xylosoxidans*

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INTRODUCTION

Antimicrobial compound is very useful especially in the medical field because it can be used to produce various antibiotics or antimicrobial drugs to treat disease and infection. Antimicrobial resistance occurs when the microorganisms evolve by acquiring the gene that encodes resistance mechanism either by mutation or transfer of genetic materials that help them evade the effect of the antimicrobial substances. The inappropriate application and misuse of antibiotics such as an unfinished course of antibiotics, over-prescription of antibiotics in human and animal, unnecessary antibiotic usage in animal and fish farming, poor hygiene and infection control lead to the development of antibiotic-resistant bacteria. Major problems happen as the infection will be difficult to cure because the current antibiotic might have no effect on the bacteria or a higher dose must be administrated (1).

The growth and transmission of these antimicrobial resistance bacteria among humans may occur due to selective antibiotic pressure or spreading of these bacteria from human to human. Selective antibiotic pressure refers to promotion of one bacterial group over another due to the influence of antibiotics. When antibiotic is introduced in the human body, they will inhibit growth and kill any susceptible bacteria. However, some bacteria are naturally resistant, able to survive and proliferate even in the presence of antibiotic. Over time, most of the susceptible bacteria will be killed, leaving only the resistant bacteria to keep growing and forming new generation bacteria that has the resistance ability. Meanwhile, human to human transmission via contaminated medical equipment, fomites, aerosols

or droplets, and direct contact between patients and medical personnel (2) increase the resistance among bacteria.

Recently, these resistance pathogen has becoming a threat to humanity in both clinical and economical aspect as the infection becomes more dangerous and untreatable, with higher morbidity and mortality rates, expensive treatment costs and higher side effects if there is a possible treatment. The reported outbreak of these harmful antibiotic-resistant bacteria especially in hospitals or any healthcare facilities such as Methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant Enterococcus and *Staphylococcus aureus* (VRSA), multidrug-resistance Gram-negative bacteria, Drug-resistance *Mycobacterium tuberculosis* and *Streptococcus pneumoniae*, and many others (3).

The acuteness of these resistant bacteria can be seen as it has caused 700 000 deaths per year. By acquiring the resistance ability, it increases the mortality and morbidity rates among infected patients as the bacteria do not respond to the standard antibiotics. For example, serious infection cases such as bacteraemia due to MRSA, resistant *Streptococcus pneumoniae* strain, and Drug-resistant *Neisseria gonorrhoeae* show significantly higher treatment failure and death rates compared to infection caused by normal, antibiotic susceptible bacterial strain (4). In 2016, multidrug-resistant tuberculosis has caused 490,000 cases globally and almost 50% of the cases, which is 240,000 infected patients died of this strain as the patients show severe symptoms and the antibiotic administered does not cure or even lessen the symptoms. An increase in the severity of the infection and treatment failure has raised the cost to treat the disease caused by these antibiotic-resistant bacteria because the patients need prolonged treatment, lengthy hospital stay, and this includes the needs for intensive care, invasive equipment, excess surgery, long period and multiple antibiotics or other treatment courses (5).

The unique condition of the cave such as high humidity, low nutrients, stable temperature and high mineral diversity allows it to house a few special microorganisms, including the oligotrophs. These microorganisms help in the formation of cave structures such as stalactites and stalagmites (6). The cave is considered a special habitat that harbors many known and novel microbial species. Oligotrophs are microbes that inhabit an environment that has low or absent nutrients (7). Bacteria and archaea have a vast majority of biodiversity in limestone and ubiquitous in many cave habitats such as rock surfaces, stream waters, soils and sediments. Detection by 16S rRNA genes sequencing reveals that limestone lake is mainly constituted by several phyla of oligotrophic bacteria and archaea such as Proteobacteria, Acidobacteria, and Actinobacteria. It is also reported that *Aureimonas altamirensis, Staphylococcus aureus, Pseudomonas, Sphingomonas, Beggiatoa* species, *Thiobacillus, Desulfonema, Alcaligenes sp.* and *Inquilinus sp.* has been discovered from limestone lake sample (8). The microbial diversity can also be seen as all groups including Gram-positive, Gram-negative, and Gram-variable bacteria are managed to be discovered from the lake sample. The morphology among isolated bacterial species also shows variety from cocci to filamentous shape (9). There are also numerous fungi species found near the limestone area including Ascomycota, Basidiomycota, Bryophyta, Verrucaria, Caloplaca, Exophiala, Botryolepraria, and even highly branching mycelia (10).

Tasik Cermin or "Mirror Lake" is located in Kinta Valley, Perak and surrounded with Gunung Rapat limestone karsts and iron quarries. The geographical area is mainly dominated by closely formed caves, hills, pinnacles, notches, and mainly limestone karst towers (11). Rainwater that falls gradually on these structures will dissolve the minerals into the lake (12). Due to that reason, the lake is composed of calcium carbonate, iron, and other compounds. Located far from human activity such as agriculture, sewage and landfill management area, and animal husbandry, the ecosystem of Tasik Cermin remains to be unaltered and the lake possess a very clear water quality that is able to show reflection just like an actual mirror. Completely covered by karst towers and hills with no inflow or outflow of water, the nutrient availability is limited and make it unfavourable for some organisms to inhabit (13). Only a recent expedition by Kinta Valley Watch (KVW) found that the underwater caves at Tasik Cermin may play a role in the water discharge system (14) . The freshwater of Tasik Cermin harbors a diversity of organisms such as bacteria, fungi, and other microorganisms. The depleted resources in this kind of habitat will increase the competition among these microorganisms. Despite a shortage of energy, nutrients, and light, these microorganisms can sustain this type of ecosystem and help in the biogeochemical cycles process and regenerating nutrients. However, information on microbial diversity, their contribution to the ecosystem, and the mechanism they have to survive in this poor nutrient lake is limited as no further research is done in this area. These data are vital especially for pharmaceutical studies as most microorganisms that inhabit poor-nutrient area might show antimicrobial activity. This will become a big loss especially in the health sector as the potential of these bacteria as novel antibiotic producer to combat against emerging multidrug resistance bacteria are not fully utilized.

The objective of this study is to isolate the bacteria that inhabit the Tasik Cermin and identify their antimicrobial capability against two known Gram-negative bacteria and three known Gram-positive bacteria. Until now, the limestone karst remains under-explored and limited research regarding microorganisms in this area (9). Hence, the information regarding microbial diversity,

microbial interaction, and especially their antimicrobial activity is limited and not well-documented (15). The study increases the chances of discovering novel antimicrobial substance from natural producers that can become beneficial to humans.

MATERIALS AND METHODS

Bacterial Isolation

The samples of water were collected from Tasik Cermin (4°33′33.7″N 101°07′11.3″E), located at Gunung Rapat, Ipoh, Perak (Figure 1). The water sample was collected at the side of the lake and was collected in sterile sample collection bottles. All the bottles were labelled and carried to the laboratory for further identification and analysis. After samples were taken from two sites, the samples were stored in polyethylene bags and kept in a container that was cooled by dry ice. The water samples were collected at the side of the lake and were collected in sterile sample collection bottles. All the bottles were labelled and carried to the laboratory for further identification and analysis. The sample was stored in the refrigerator where the temperature is adjusted at 4°C until it is used for culturable study (16).

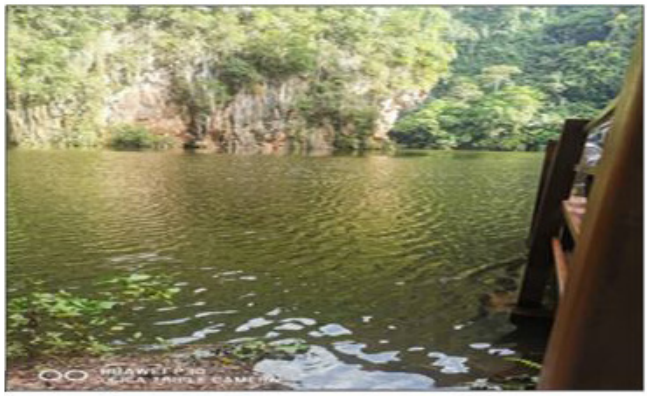


Figure 1: The location of sampling in Tasik Cermin

Primary screening and secondary screening of antimicrobial activity

Antimicrobial activity of the bacterial isolates were tested against three Gram-positive bacteria, which are *Staphylococcus aureus* (ATCC63300), *Bacillus subtilis* and *Streptococcus pneumoniae*, and two Gram-negative bacteria, which are *Escherichia coli* (ATCC25922), and *Proteus vulgaris* (ATCC11437). Test bacteria that do not have ATCC number was taken from samples long maintained in the laboratory and their species verified through sequencing.

During the primary screening, the antagonistic activity of bacterial isolates was screened against the five tested bacteria by using perpendicular streak method. A single streak of pure isolate was inoculated on the Mueller–Hinton agar in the middle of the plate. The plates were incubated for 4 days at 28°C to 30°C. Then, the test bacteria from the broth stock solution were streaked perpendicular to the isolate at a 90° angle to the initial isolates streak. The plates were further incubated at 28°C for 1 to 2 days, and the antimicrobial activity was determined based on the distance of the inhibition zone (17).

Based on the result of primary screening, the bacterial isolate that potentially showed antimicrobial activity was subjected to submerged state fermentation method to produce a crude extract that contains the antimicrobial assay. The crude extract produced was tested by using the agar well diffusion method. Overnight test bacteria that have been adjusted to 0.5 McFarland standard (cell count of 1.5×10^{-8}) was swabbed uniformly on the agar surface using a cotton swab. The wells with 6 mm diameter were made on the Mueller–Hinton agar plate by using cork borers. The bottom of each well was covered by adding 15 µL of Mueller-Hinton agar solution in order to avoid the added solution to overly diffuse into the agar. After the added agar had solidified, 50 µL of crude extract was poured into the wells. An amount of 50 µL of 1 mg/mL ampicillin and gentamycin solution were used as a positive control and 50 µL of ethyl acetate was used as a negative control. The plates were left for 2 hours to allow diffusion of the compound across the agar and incubated at 37°C for 24 hours. The zone of inhibition was then measured, and the steps were repeated three times to obtain accurate results (18).

MIC, MBC and statistical analysis

The MIC method was performed by using the crude extract in a 96-well microtiter plate. An amount of 80 µL of nutrient broth was placed into well 1-10 and 160 µL into well 12 (negative control). An amount of 80 µL of the crude extract solution was added into well 1 and 2, and the well was gently mixed by suspending using micropipette. The crude extract was serially diluted by transferring 80 µL of mixed solution from well 2 to well 3 and continued serially up to well 10. An amount of 80 µL Ampicillin solution (1 mg/ml) was added to well 11 for positive control. An amount of 10 µL of standardized test bacteria, was added into test tubes 1-12 and incubated at 37 °C overnight. The growth of the bacteria in each test tube was observed, and MIC value was determined (19 and 20). Minimum bactericidal concentration (MBC) was used for determination of the least amount of antimicrobial agent needed to kill the bacteria (18). This determination was done by inoculating the subculture from MIC on the Mueller Hinton agar (MHA) agar using the plate streak method. Then, the plates were incubated overnight, and the MBC was observed. The medium that showed no growth with the lowest concentration of antimicrobial agent was observed as MBC.

The data of inhibition zone obtained from the secondary screening process was statistically analyzed using One-Way ANOVA and Tukey method test. The One-Way ANOVA test is used to determine any significant differences and to compare the level of significance within the bacterial isolates. However, because the test

does not indicate where the differences are, a Tukey test was used to identify the location of the differences. The tests were conducted by using IBM Statistical Product and Service Solutions (SPSS) version 16 to interpret the result (18).

Microscopic identification

For the microscopic characterization, the bacterial isolates from every sample were analyzed under the microscope to observe its cellular morphology. In Gram staining method, red or pink-coloured stain indicate Gram-negative bacteria while purple-coloured stain indicate Gram-positive bacteria.

Molecular identification

Bacterial isolate with the strongest antimicrobial activity based on the result of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) was determined its identity by using molecular identification. Bacterial isolate with the strongest antimicrobial activity based on the result of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) was determined its identity by using molecular identification. The methods that were involved in this molecular identification are DNA extraction, gel electrophoresis of the extracted DNA, determination of DNA purity and DNA concentration, 16S rRNA gene amplification by polymerase chain reaction (PCR), PCR product Clean up, gel electrophoresis of the PCR clean up product, and sequencing of the 16S rRNA gene.

The DNA of Bacterial isolates with the strongest antimicrobial activity was extracted using PrimeWay Genomic DNA Extraction Kit. The DNA integrity of the extracted DNA was analysed by gel electrophoresis with 0.8%(w/v) agarose gel. An amount of 5 µL extracted DNA was mixed with 2 µL of 6X DNA loading dye and it was placed inside the gel electrophoresis wells. An amount of 6 µL Lambda-HindIII DNA Marker was used as DNA ladder.

Purity and concentration of the extracted DNA was determined by measuring the absorbance of the extracted DNA using a spectrophotometer. DNA concentration was determined by measuring the extracted DNA in the eluate by its absorbance at 260 nm and for the DNA purity determination, the absorbance of the extracted DNA was measured again using absorbance at 280nm. The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) will approximately determine the purity of DNA with respect to contaminants that absorb UV, such as protein and RNA. Pure DNA has a value of A_{260}/A_{280} ratio in between 1.8 - 2.0.

Amplification of the 16S ribosomal DNA sequence from the isolated bacterial DNA was carried out using 27F (5′- AGAGTTTGATCMTGGCTCAG -3′) and 1492R(5′-TACGGYTACCTTGTTACGACTT -3′) primers targeting the V1-V9 region. An amount of 4 µL Forward and reverse primer, 1 µL of extracted DNA, 7 µL of sterile distilled water, and 4 µL of 5X FIREPoL® Master Mix (Consist of FIREPoL® DNApolymerase, 1 mM dNTPs, 12.5 mM $MgCl_2$, and 5X reaction buffer B (0.4 MTris-HCl, 0.1 M $(NH_4)2SO_4$, and 0.1 %(w/v) Tween-20)) was used as the reagent in the 0.2 mL PCR tube. For positive control of the PCR reaction, 1 µL of lambda DNA, 1 µL of primer mix solution, 4 µL of 5X FIREPoL® Master Mix and 14 µL of sterile distilled water was added in the 0.2 mL PCR tube. For negative control of the PCR reaction, 4 µL of both forward and reverse primer, 4 µL of 5X FIREPoL® Master Mix, and 8 µL of sterile distilled water was added in the 0.2 mL PCR tube. Both control tubes were briefly centrifuged at 13000 rpm. Then, the PCR product was cleaned by removing the primers, nucleotides, dyes, enzymes, agarose, salts and other impurities using QIAquick® PCR Purification Kit. Gel electrophoresis was conducted again on PCR clean up product with 1.0 %(w/v) agarose gel.

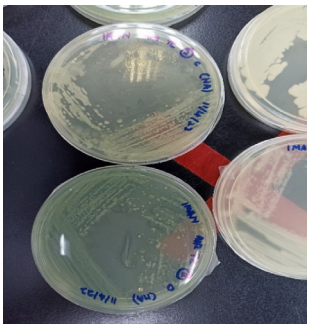
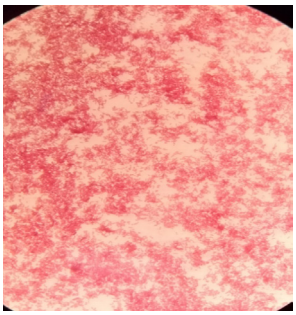
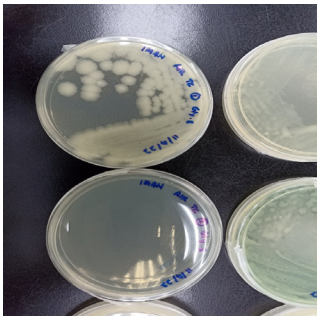
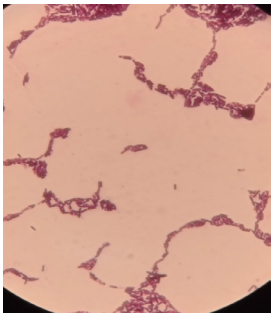
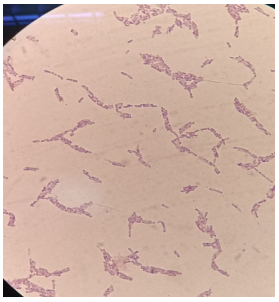
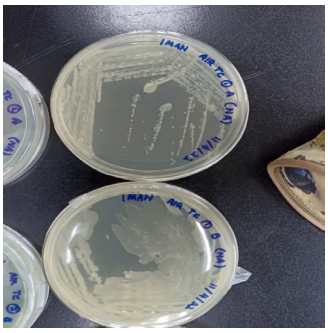
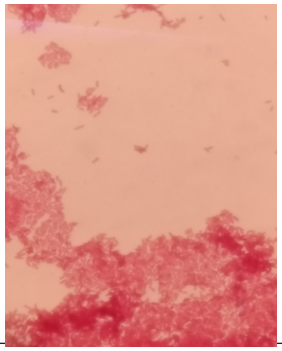
The product of PCR that contains 16S rRNA gene was sent for sequencing in order to determine the identity of the bacterial isolates that possess the strongest antimicrobial activity. From the result of sequencing, the identity of the bacterial isolate was determined by using BLAST.

RESULT

Microscopic identification

For the microscopic characterization, the bacterial isolates from every sample were analyzed under the microscope to observe its cellular morphology. Microscopic characterization shows that isolates TC2D and TC1A are Gram-negative bacteria, while isolates TC1B and TC1C are Gram-positive bacteria. Based on the cellular shape, all isolates exhibit rod-like shape or bacillus as seen in Table I.

Table I: Characterization through colony morphology and microscopic observation

Isolates origin	Microscopic Characterization (Observed using 100X Magnification)	Macroscopic Observation	Microscopic Characterization (Observed using 100X Magnification)	Microscopic Observation
Tasik Cermin Water Sample 2D (TC2D)		Curled margin Green Pigmentation Punctiform shape		Gram-negative Rod-shaped cell (Bacilli)
Tasik Cermin Water Sample 1B (TC1B)		White / Opaque Colour Irregular Shape Undulate margin		Gram-positive Rod-shaped cell (Bacilli)
Tasik Cermin Water Sample 1C (TC1C)		Smooth & Curled Shape Milky Coloured Colony		Gram-positive Rod-shaped cell (Bacilli)
Tasik Cermin Water Sample 1A (TC1A)		Smooth & Round shape Greyish white / Milky colour		Gram-negative Rod-shaped cell (Bacilli)

Primary screening and secondary screening of antimicrobial activity and the statistical analysis

Among four chosen bacterial isolates, only one isolate which is isolate TC1A able to shows antimicrobial activity against two out of five tested bacteria. The results revealed that the isolates TC1A showed potential antimicrobial activity against *S. pneumoniae* and *P. vulgaris*. The isolate TC1A managed to inhibit the growth of *S. pneumoniae* and *P. vulgaris* as 10 mm and 5 mm of inhibition zone. The overall results of perpendicular streak method are described in Table II.

Table II: Measured inhibition zone (mm) of the isolates tested against five test bacteria using perpendicular streakmethod

Isolates	Test Bacteria (Gram-Negative)		Test Bacteria (Gram-Positive)		
	E. coli	*P. vulgaris*	*S. aureus*	*B. subtilis*	*S. pneumoniae*
TC1A	-	5 mm	-	-	10 mm
TC2D	-	-	-	-	-
TC1C	-	-	-	-	4 mm
TC1B	-	3 mm	-	-	-

The inhibition zone is measured in mm and the symbol "- " represent no zone of inhibition

Although isolate TC1C is able to produce 4 mm inhibition zone on S. pneumoniae growth plate while Isolate TC1B able to produce 3 mm inhibition zone on *P. vulgaris* growth plate, the diameter of inhibition zone produced is too small. This indicates that the isolate TC1B and TC1C antimicrobial activity is too weak and not significant.

Based on the primary screening result, only isolates TC1A shows the potential to produce antimicrobial compounds that able to inhibit the growth of other bacteria. So, crude extract produced from isolates TC1A was subjected to secondary screening by agar well diffusion method. The fermentation of isolate TC1A in yeast-malt extract broth medium was done to allow the production and diffusion of antimicrobial substance into the medium. Yeast malt extract broth medium (ISP2) is a nutrient-rich media that is used to support the growth and maintenance of yeasts, molds, and other aciduric microorganisms. This type of medium is usually used to characterize *Streptomyces spp.* and for the extraction and purification of bioactive compounds produced by microorganisms.

Based on the outcome, the crude extract from isolate TC1A showed promising antimicrobial activity as it capable to inhibit the growth of three test bacteria, which are *E. coli, P. vulgaris*, and *S. pneumoniae*. The inhibition zone of 9.23 ± 0.25 mm on *E. coli,* 14.97 ± 0.05 mm on *P. vulgaris,* and 14.93 ± 0.12 mm on *S. pneumoniae* growth plate managed to be observed (Table III). No zone of inhibition managed to be recorded on the *S. aureus* and *B. subtilis* growth plate. The results obtained in secondary screening can be seen having a slight difference with the result in primary screening in terms of the antimicrobial activity of isolate TC1A. This difference is due to the insufficient incubation time of bacterial isolate on the agar plate during the primary screening that will lead to low production and diffusion of antimicrobial substance in the agar. Plus, one of the limitations of perpendicular steak method used in primary screening is the difficulty in reading the result since the inhibition zone produced is usually fuzzy and unclear.

Table III Inhibition zone produced by TC1A isolate and ther substances based on agar well diffusion method

Isolates	Test Bacteria (Gram-Negative)		Test Bacteria (Gram-Positive)		
	E. coli	*P. vulgaris*	*S. aureus*	*B. subtilis*	*S. pneumoniae*
TC1A	9.23 ± 0.25	14.97 ± 0.05	-	-	14.93 ± 0.12
Positive Control 1 (Ampicilin)	25.20 ± 0.20	18.93 ± 0.21	16.73 ± 0.64	24.50 ± 0.56	23.77 ± 0.40
Positive Control 2 (Gentamicin)	28.03 ± 0.57	20.53 ± 0.50	23.13 ± 0.23	25.27 ± 0.65	24.60 ± 0.36
Negative Control (Ethyl Acetate)	-	-	-	-	-

The inhibition zone is measured in mm and the data are expressed as the average of three reading measured ± standard deviation.

The symbol "- " represent no zone of inhibition

However, the isolates TC1A has low effectiveness of antimicrobial activity as all the inhibition zone produced in each test bacteria were smaller compared to other commercial antibiotics. By ignoring the concentration of isolates TC1A extract, this indicates that the antimicrobial compound produced by TC1A is not suitable to be used as antibiotic because of its poor ability to inhibit bacterial growth. Based on this result, the antimicrobial substance managed to inhibit the growth of all Gram-negative test bacteria and one Gram-positive test bacteria.

The One-Way ANOVA test that was calculated using IBM SPSS statistical software shows that there is significant difference between the inhibition zone produced by the TC1A extract against the tested Gram-positive and Gram-negative bacteria. Further test by using Tukey test method reveal that inhibition zone produced by TC1A extract against *E. coli* is significantly different compared to *S. pneumoniae* and *P. vulgaris.* However, the difference between inhibition zone of *S. pneumoniae* and *P. vulgaris* is not significant as seen in Figure 2. This indicates that the antimicrobial activity of the TC1A extract has a specific mode of action in inhibiting bacteria and does not show any specificity against neither Gram-negative nor Gram-

positive bacteria. The major difference between Gram-positive and Gram-negative bacteria is the structure of their cell walls which greatly affects how susceptible they are to various antimicrobial compounds. Because antimicrobial compound produced by isolate TC1A able to inhibit both Gram-positive and Gram-negative bacteria, it is hypothesized that the TC1A antimicrobial compound might be a bacterial protein or nucleic acid synthesis inhibitor based on study by (21). Due to that reason, the effectiveness of the antimicrobial compound does not affect by the bacterial cell wall structure as it directly modifies the bacterial biomolecule synthesis that led to cell death.

InhibitionZone

			Subset for alpha = 0.05	
	TestBacteria	N	1	2
Tukey HSD[a]	E.coli	3	9.2333	
	S.pneumoniae	3		14.9333
	P.vulgaris	3		14.9667
	Sig.		1.000	.966

Figure 2: Tukey test method for analyzing significant different using IBM SPSS statistical software

MIC and MBC

All of the test bacteria were treated with crude extract at different concentrations, where the concentration is serially diluted by reducing the potential of antimicrobial substance by half. Among five tested bacteria, the isolate TC1A crude extract shows strong antimicrobial activity against *S. pneumoniae* and *P. vulgaris* as it is able to inhibit these test bacteria at the lowest MIC, which is at 1/8 concentration as seen in Table IV. Moderate antimicrobial activity managed to be recorded as the extract capable of inhibiting *E. coli* at 1/4 concentration. High MIC of TC1A extract reflect that the extract has low antimicrobial activity when it is tested against *S. aureus* and *B. subtilis*. The MBC ranges between 1 to 1/4 extract concentration against *P. vulgaris* and *S. pneumoniae* while the MBC range between 1 to 1/2 extract concentration against *S. aureus* and *E. coli*. However, the crude extract is unable to show bactericidal effect towards *B. subtilis* as no MBC is recorded.

Table IV:TC1A Crude Extract MIC tested against several test bacteria

TC1A Extract Concentration Test Bacteria	1									
S. aureus	-	-	+	+	+	+	+	+	+	+
S. pneumoniae	-	-	-	-	+	+	+	+	+	+
P. vulgaris	-	-	-	-	+	+	+	+	+	+
B. subtilis	-	-	+	+	+	+	+	+	+	+
E. coli	-	-	-	+	+	+	+	+	+	+

The symbol " - " represents no bacterial growth while " + " represents the presence of bacterial growth

Bactericidal effect can be observed when the bacteria was unable to re-grow after it was treated with the antimicrobial compound and showed no growth during the MIC. Based on the MIC and MBC result in Table IV, the antimicrobial compound from isolates TC1A has the bactericidal effect on *S. aureus, P. vulgaris, E. coli,* and *S. pneumoniae* although it only managed to inhibit the bacteria at high concentration, as the test bacteria was unable to re-grow when it was streaked on the nutrient agar. As for *B. subtilis*, the antimicrobial compound from isolates TC1A only has bacteriostatic effect as the bacteria managed to grow after it is treated with antimicrobial compound of TC1A. These MIC and MBC results are tallied with the primary and secondary screening as both showed that isolates TC1A to has the strongest antimicrobial activity against *P. vulgaris*, and *S. pneumonia*.

Molecular identification

For the molecular identification, the DNA of isolate TC1A managed to be extracted and the analysis of extracted DNA integrity by gel electrophoresis indicate positive results as a high molecular weight and a smear of DNA band managed to be observed. Then, analysis by spectrophotometer shows that the concentration of DNA that managed to be extracted is 3.3 ng/µL and the DNA purity based on the value of 260/280 absorbance reading ratio is 2.08. As the value of 260/280 absorbance reading ratio recorded was higher compared to this range, this indicates a slight RNA contamination in the extracted DNA sample.

After PCR for 16s rDNA amplification, the purified DNA band on the gel electrophoresis has a length of 2.0 kb as seen in Figure 3. This indicates moderate quality of purified PCR product as the expected size is approximately around 1.5 kb. Size shift after purified process from 1.5 kb to 2.0 kb might be due to the RNA contamination that binds together with the PCR product during the purification process and lead to increase in band size. Another reason that causing this size shift is due to the bacterial intergenic region located in the 16S rRNA gene. This intergenic region tends to be amplified together with the 16S rRNA gene during PCR process, causing the increase in size after the purification process. After the sequencing process, the identity of the bacterial isolate TC1A is determined by using BLAST (Basic Local Alignment Search Tool) by comparing the nucleotide sequence with the database. The significant alignment by BLAST shows that the isolate TC1A is classified under the genus Achromobacter and has 97.68% similarity percentage with the A*chromobacter xylosoxidans* species as seen in Table V.

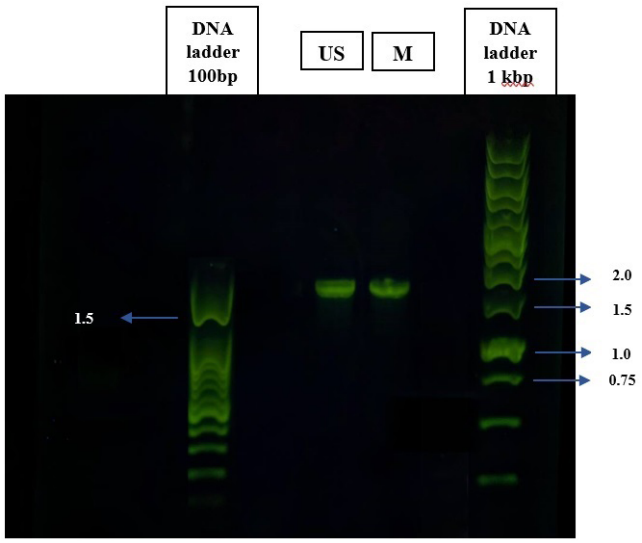


Figure 3 Electrophoresis of DNA purification of two isolates (M and US) on agarose gel (1.0%). Two ladder were used; right lane is 1 kb DNA ladder and left lane is 100 bp DNA ladder

Table V: Bacteria TC1A identity based on BLAST nucleotide sequence

Species Name	Strain	Percent Identity (%)	Accession Length
Achromobacter xylosoxidans	NBCR 15126	97.68	1456
Achromobacter pulmonis	R-16442	97.61	1489
Achromobacter xylosoxidans	Hugh 2838	97.46	1495
Achromobacter insuavis	LMG 26845	97.39	1483
Achromobacter insolitus	LMG 6003	97.17	1473

DISCUSSION

Nowadays, the dramatic rise on the issue of antimicrobial resistant microorganisms has become a major concern for the public health worldwide as these microorganisms are able to cause more serious threat especially to human health. As they can compromise the current treatment and medicine, a rising number of infectious and untreatable diseases was recorded. Due to this reason, there is an urgent need to establish and developing new drugs formulated from novel antimicrobial compound that can combat with these resistant microbes. Isolation of antimicrobial compounds from freshwater lake located near limestone cave environment can be one of the solutions towards this problem as previous studies show that numerous antibiotics producers and novel antimicrobial compounds managed to be isolated from the freshwater environment (22).

In this study, four isolates managed to be cultured and grown from Tasik Cermin water sample and only one of the isolate; isolate TC1A showed potential antimicrobial activity during the screening test. Molecular identification by PCR amplification of 16S rRNA gene shows that the identity of TC1A is *Achromobacter sp.* with 97.68% of the sequence match with the BLAST nucleotide sequence database. The identity of isolate TC1A also is also further validated based on the macroscopic and microscopic characterization on isolate TC1A, where all of the colony and cellular morphology match with the *Achromobacter xylosoxidans* characteristics. *Achromobacter xylosoxidan*s is a Gram-negative, rod-shaped bacteria that can be naturally found in freshwater and soil environment (23).

Data obtained during both primary and secondary screening of antimicrobial activity against five test bacteria shows that *Achromobacter sp.* able to inhibit the growth of all Gram-negative bacteria (*E. coli* and *P. vulgaris*) and one Gram-positive bacteria species (*S.pneumoniae*).The same result was also reported earlier by (24), where *Achromobacter xylosoxidans* isolated from mangrove environment was able to shows its antibacterial activity by inhibiting the growth of several Gram-positive and Gram-negative bacteria. This shows that this bacterium has the potential to produce antimicrobial compound. However, the result obtained in this experiment shows slight difference as compared to findings by (24) as they reported that *Achromobacter xylosoxidans* was unable to inhibit *E. coli* growth.

The MIC and MBC results indicate that the extract of *Achromobacter sp* shows strongest antimicrobial activity against *P. vulgaris* and *S. pneumoniae*. MIC and MBC results also show that the extract is able to inhibit the growth of all Gram-negative test bacteria, but high MIC and MBC is recorded for *E. coli*. Similar results were also reported by (25), where the antibacterial activity of ethyl acetate extract of an *Achromobacter sp*. against several pathogenic bacteria such as *S. pneumoniae, S. aureus,* and *S. typhi*. Therefore, the crude extract of bacteria isolated from lake near limestone-rich area might become a potent source of antimicrobial substance production.

Achromobacter xylosoxidans are found naturally in soil and more commonly in aquatic habitats. This isolate is more frequently found as a pathogen as compared to Achromobacter denitrificans, another close subspecies of this isolate. The first pneumonial case caused by this isolate was by (26) that was successfully treated by meropenem for two weeks. In 2017, Awadh et al. reported numerous reports of respiratory infections reported with this isolate as the causative agent. Urbanization, agricultural and wastewater management and water resources development might lead to emergence of waterborne infectious agent in aquatic habitats (27). Nevertheless, even though Tasik Cermin is still categorized as under-exploited, the existence of pathogenic species is possible.

Further studies need to be conducted in order to

analyze the antimicrobial substance within the crude extract of *Achromobacter xylosoxidans.* In line with the study by (25), reveal that Cyclic dipeptides or CDPs (diketopiperazines or 2,5-Diketopiperazines or 2,5-DKP) might be one of the potential antimicrobial substances contained in the ethyl acetate extract of an *Achromobacter sp.* Cyclic dipeptides is the smallest cyclic peptides that can be synthesized by 90% of Gram-negative bacteria and known for its antitumor, antiviral, antifungal, antibacterial, anti-prion, antihyperglycemic, and glycosidase inhibitor agents (28).

CONCLUSION

The bacteria isolated from Tasik Cermin water sample that shows the most promising antimicrobial activity is identified and confirmed as *Achromobacter xylosoxidans* Antimicrobial screening test result indicate that *Achromobacter xylosoxidans* able to inhibit most of the tested Gram-negative bacteria (*P. vulgaris* and E*. coli*) and one of the tested Gram-positive bacteria, which is *S. pneumoniae*. The strongest antimicrobial effect by the isolates TC1A was recorded against *P. vulgaris* and *S. pneumoniae*. TC1A has the bactericidal effect on *S. aureus, P. vulgaris, E. coli,* and *S. pneumoniae*. As for *B. subtilis*, the antimicrobial compound from isolates TC1A has bacteriostatic effect. This evidence shows that *Achromobacter xylosoxidans* is one of the bacterial species that can be potentially used as antibacterial producer, thus adding to alternative to the problem of multidrug resistant microorganisms. However, further research can be done to observe its antimicrobial efficacy against other bacteria or fungi. This also proves that under-exploited area has the possibility to harbor many novel microorganisms that can be beneficial to human, especially in the healthcare sector.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to the Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM) Shah Alam Selangor Malaysia for their assistance, as well as ReNeU UiTM for the publication incentive provided through Program MEE 2.0 and Tasik Cermin Ipoh Sdn Bhd for the permission granted for sampling purposes.

Portions of this study were presented previously in oral and abstract form: Rasol, R., Ahmad Iman Ammer Azman and Norashirene, M. J. Antimicrobial Activity Screening of Bacteria Isolated From Tasik Cermin, 9th International Conference on Science and Social Research (CSSR2022), HW 6, p. 33, 2022.

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