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Syzygium alternifolium photo by Dr. N. Yesoda

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Chapter in book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta***.** 50th Annual Symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. www.molecularsystemsbiology.com. DOI:10.1038/msb.2008.24

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Survey of the bacterial diversity at two coastal beaches in Guyana

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Abstract. *Prashad L, Daniel R, Ram M. 2020. Survey of the bacterial diversity at two coastal beaches in Guyana. Biofarmasi J Nat Prod Biochem 18: 57-64.* The #63 Beach, Berbice and Marriott Beach, Kingston Seawall, Guyana, were surveyed for halophilic bacteria present in its waters. NaCl tolerance, temperature tolerance, and antimicrobial activity of isolates against *Staphylococcus aureus* and *Bacillus* sp. were tested. Samples of 100 mL were taken from the Shore, 5.0 m, and 10.0 m depth from both locations. The samples were plated and examined for the growth of bacteria of different pigmentation. A total of 4 halophilic isolates were found; 3 from Marriott Beach (Isolates A, B, and G) and 1 (Isolate M) from the #63 Beach. Isolates were yellow, light orange, and pink pigmentation, the 3 isolates from Marriott Beach were Gram-negative and cocci, while the one isolates from #63 Beach were Gram-positive and cocci. The optimum salinity tolerance for the Isolate G from Marriott Beach was 1.5M NaCl, Isolate M from #63 Beach 1.0M NaCl, Isolate B from Marriott Beach 1.0M NaCl, and Isolate A from Marriott Beach 0.5M NaCl. The optimum temperature for the growth of the isolates was 37°C. The isolates had no antimicrobial activity against *S. aureus* and *Bacillus* sp.

Keywords: Antimicrobial, bacterial diversity, coastal beaches

INTRODUCTION

Halophiles are organisms that require salt for their survival (DasSharma and Arora 2001). Halophilic organisms are categorized into 3 groups; slight halophiles require a salinity range of NaCl 0.2M-0.5M to survive, moderate halophiles require a range of 0.5M-2.5M, and extreme halophiles require 2.5M-5.5M. These organisms are both Eukaryotic and Prokaryotic. The Eukaryotic halophiles include algae, specifically the green algae which belong to the genus *Dunaliella*, protozoa, e.g., *Porodon utahensis* isolated from the Great Lake, and fungi. The Prokaryotes include cyanobacteria, anaerobic bacteria and archaea, and aerobic and facultative anaerobic Gram-negative bacteria, e.g., *Halomonas* and *Chromohalobacter* (DasSharma and Arora 2001). Halophiles adapt to the environment so that they can survive in this unique setting. They do so in two ways: The High-salt-in mechanism, which allows for all their intracellular proteins to be stable and active in the presence of different concentrations of salts (Ma et al. 2010). This method results in high potassium and sodium ions in their cytoplasm (Kunte et al. 2002). The other method is the Low salt organic solutes in; this results in the buildup and production of organic solutes that do not hinder the activities of normal enzymes (Ma et al. 2010). Halophiles are known for their pigmentation that varies, and sometimes this pigmentation results in the unique color seen in salt lakes due to the high growth density of these organisms. Colors include red, pink, yellow, orange, and in some rare cases, cream. Most of these organisms are usually Gram-negative, but recently, a few Gram-positive have also been discovered. Moderate halophilic bacteria are the most widespread of halophiles.

Halotolerance is the ability of organisms to survive in both environments with and without salt. They do not depend on salt being present in the environment for survival.

Halophiles are present in hypersaline environments, including salterns, salt lakes, coastal and deep-sea locations. The two most studied hypersaline lakes are the Great Salt Lake in the Western United States of America and the Dead Sea in the Middle East (DasSharma and Arora 2001). Apart from these areas, the Mediterranean Sea, The Solar Lake of Sina Egypt, and Antarctic hypersaline lakes were also surveyed (Jiang et al. 2006).

This study surveyed halophilic bacterial diversity at two of Guyana's marine beaches along the coast, the Number 63 beach and Marriott beach, to analyze the physical properties of the water in terms of pH, salinity level, and temperature from both locations of different depths and to test the antimicrobial properties of the isolates found.

MATERIALS AND METHODS

Study sites

The study was conducted at #63 beach in Region 6 and the Kingston sea wall strip directly opposite Marriott Hotel, Region 4as shown on the map.#63 Beach is a natural beach approximately 8.7 km in length and supports coconut reefs, swamps, and mangroves (CREP 2007). The strip of beach directly opposite the Marriott Hotel is a popular tourist and local area, but it has been altered by anthropogenic activities much more than #63 beaches.

Figure 1. Research sites in: #63 Beach, Berbice (A) and Marriott Beach, Kingston Seawall (B), Guyana

Collection of samples

Figure 1 shows the map highlighting the study sites. Three different depths (the shore, 5.0 m, and 10.0 m) were measured for #63 Beach and Marriott Beach. The jars were labeled accordingly and rinsed with the sample water just below the surface of the collection site three times. Water samples were collected in a jar and capped below the water's surface at both beaches. Three jars per depth were collected. Once all the samples were collected, they were placed in an ice cooler filled with ice for storage and transported back to the University of Guyana laboratory. The samples were stored in the refrigerator until further use.

Microbial analysis

Under aseptic conditions, 47.6g of Nutrient agar and 29.2g of NaCl were weighed using an electric scale and suspended in 1.7 liters of water in a conical flask to obtain 0.5M NaCl nutrient agar. The mixture was then boiled over a hot plate and was swirled constantly. After the contents within the conical flask were completely dissolved, it was placed into the autoclave for 1 hour at 121°C for sterilization. After sterilization, it was cooled to 47-50°C, and approximately 30 mL of liquefied NaCl nutrient agar was poured into sterile Petri plates and allowed to set. This step was done under strict aseptic conditions within the laminar airflow chamber. The plates were stored for use.

Culturing of microorganisms

The physical properties of the water samples from the different depths from both locations were tested using a water testing kit. The samples were filtered using Whatman filter paper#2. The filtered samples were then used for the culturing of the microorganisms. The work area was swabbed with 75% ethanol. Plating the samples was done by swabbing the filtered sample onto Petri plates. The swabbing was done in triplicates, and each Petri plate was labeled accordingly. A sterile cotton swab was placed into the filtered sample jar and was left to soak in the sample water for about 30 seconds. The soaked swab was then streaked onto the Petri plate to cover the entire surface. The plates were then sealed with scotch tape and stored at 37°C for 24 hours. The plates were inspected for colony growth. Colonies with distinct pigmentation were chosen and streaked onto 0.5M NaCl nutrient agar plates using the four-corner streaking technique and incubated at 37°C for 24 hours. Once pure colonies were obtained, the colony morphology of each isolate was recorded. The isolated colonies were stored for further testing.

Determining if the isolates are halophilic bacteria

Pure nutrient agar plates were prepared with 11.2g of Nutrient Agar and 400 mL of water. The laminar airflow was swabbed with 75% ethanol. The pure isolated colonies were each streaked onto the pure Nutrient Agar plates in a zig-zag pattern. The newly streaked plates were sealed with scotch tape and incubated for 24 hours at 37℃. The plates with the pure isolated colonies were also sealed with scotch tape and stored in the refrigerator for further use. The plates were inspected for colony growth after 24 hours.

Gram staining of isolated halophilic bacteria

A small drop of distilled water was placed in a clean glass slide center. A thin smear of the isolated bacterial colony was made on the slide in a circular direction. The smear was then fixed by swiftly heating through a Bunsen flame using a clothespin until it was dry. The slide was then flooded with crystal violet for 30 seconds and then washed with distilled water for a few seconds. After washing off crystal violet from the side, the slide was flooded with Grams of iodine for 1 minute and then decolorized by tilting the slide and drop by drop rinsing with 95% ethanol until ethanol runs clear. After decolorization, the slide was washed with distilled water for a few seconds. The slide was then stained with 5-6 drops of safranin (counter-staining) for 20 seconds, after which it was washed off with distilled water. After counterstaining, the slide was blotted and air-dried. It was then examined microscopically by using the oil-immersion objective to identify the type of bacteria colonies.

Survival of isolated halophilic bacteria under different physical conditions

Sodium chloride (NaCl) tolerance of isolates: 1.0M NaCl (8.4g nutrient agar, 17.52g NaCl, and 300 mL water) and 1.5M NaCl (8.4g nutrient agar, 26.28g NaCl and 300 mL water) nutrient agar plates were prepared and labeled accordingly. The 0.5 McFarland (1907) standard was prepared by mixing 1% barium chloride and 1% sulphuric acid of 0.09 mL and 9.95 mL, respectively. 4 test tubes with 10 mL of water were sterilized by UV radiation. The inoculum of the isolated halophilic bacteria was then prepared by mixing each bacterial with 10 mL of water until it was the same consistency as the 0.5 McFarland (1907) standard. A sterile cotton swab was used to swab the inoculum onto the 1.0M NaCl and the 1.5M NaCl nutrient agar plates, respectively. The swabbing of the plates of different molarity for each isolated halophilic bacteria was done in triplicates. The plates were labeled accordingly. The plates were sealed with scotch tape for 24 hours at 37℃; observations were made.

Temperature tolerance of isolated halophilic bacteria

The 0.5M NaCl plates were prepared. The 0.5 McFarland (1907) standard was prepared by mixing 1% barium chloride and 1% sulphuric acid of 0.09 mL and 9.95 mL, respectively. 4 test tubes with 10 mL of water were sterilized by UV radiation. The inoculum of the isolated halophilic bacteria was then prepared by mixing each bacterial with 10 mL of water until it was the same consistency as the 0.5 McFarland (1907) standard. A sterile cotton swab was used to streak the inoculum onto the 0.5M NaCl nutrient agar plates for incubation at 10℃, 37℃, and 45℃. The streaking was done in triplicates, and the plates were labeled accordingly. The plates were then sealed with scotch tape. The plates were inspected for colony growth after 48 hours.

Antimicrobial activity of isolated halophilic bacteria

Disc Diffusion Method: 6 mm discs were made. The 0.5 McFarland (1907) standard was prepared by mixing 1% barium chloride and 1% sulphuric acid of 0.09 mL and 9.95 mL, respectively. 75% ethanol was used to swab the working area. Seven test tubes with 10 mL of water were sterilized by UV radiation. The inoculum of then prepared by mixing each bacterial with the 10 mL of water until it was the same consistency as the 0.5 McFarland (1907) standard. *Staphylococcus aureus* and *Bacillus* sp. were streaked onto the entire surface of 0.5M NaCl nutrient agar plates; this step was done in triplicates and labeled accordingly. The discs were soaked in the inoculum of the isolated halophilic bacteria. 4 discs were then placed in each of the plates using a tweezer at 90° angles to the center of the Petri plates streaked with *S. aureus* and *Bacillus* sp. respectively. This was also done in triplicates. The plates were sealed with scotch tape and stored at 37℃ for 24 hours. The plates were observed for zones of inhibition, and observations were recorded.

RESULTS AND DISCUSSION

Halophiles are organisms that cannot survive without the presence of salt in the environment in which they reside; it is an evolutionary and adaptive mechanism that they developed over time to help cope with environmental stress. Tables 1-2 show the morphological characteristics of the 14 isolates found in the study sites. We found a total of 14 bacteria that were initially isolated based on their unique colony pigmentation. Of these 14 isolates, 4 were halophilic bacteria, while the remaining 10 were simply halotolerant. Due to the unique characteristic of halophilic bacteria to only grow in the presence of salt, 4 isolates, namely isolates A, B, G, and M, were unable to grow when they were streaked onto nutrient agar medium plates that were not supplemented with salt (in this case, Sodium Chloride (NaCl). Halophiles are usually of two taxonomic groupings; Archaea can tolerate high salt concentrations ranging from 20%-30% (3.5M-4.5M) NaCl and other salts, and halophilic bacteria can survive at salinity ranges from 2%-20% NaCl (Kanekar et al. 2012).

Table 1. Colony morphology of the isolated halophilic bacteria from Marriott Beach, Kingston Seawall, Guyana

Site: Marriott Beach	Colony	Morphology								
		Form	Surface	Texture	Pigmentation	Elevation	Margin	Gram Staining	Shape	Size
50 m	1 (G)	Circular	Shiny	Smooth	Light orange	Flat	Entire	Negative	Cocci	3 mm
100 m	1 (A)	Circular	Shiny	Smooth	Yellow	Flat	Entire	Negative	Cocci	2 mm
	2 (B)	Circular	Shiny	Smooth	Pink	Raised	Entire	Negative	Cocci	3 mm

Table 2. Colony morphology of the isolated halophile from # 63 Beach, Guyana.

Site: #63 Beach	Colony	Morphology								
		Form	Surface	Texture	Pigmentation	Elevation	Margin	Gram Staining	Shape	Size
100 m	1 (M)	Circular	Shiny	Smooth	Light Pink	Raised	Entire	Positive	Cocci	3 mm

Table 3. The growth density of halophilic bacteria when subjected to different temperatures. (SCALE: 0 (no growth) 1 (slight growth) 2 (medium growth) 3 (high growth) to 4 (very high growth).)

Colony	Temperature °C and growth intensity		
	10°C	37°C	45°C
A	No growth	3	1
B		4	3
G		4	1
M		4	3

Table 4. The tolerance ranges of halophilic bacteria at different NaCl concentrations. (SCALE: 0 (no growth) 1 (slight growth) 2 (medium growth) 3 (high growth) to 4 (very high growth)

Colony	NaCl range/ density of growth		Classification
	1.0 M	1.5 M	
A	0	0	Slight halophilic bacteria
B	1	0	Moderate halophilic bacteria
G	4	4	Moderate halophilic bacteria
M	2	0	Moderate halophilic bacteria

Table 5. The antimicrobial properties of halobacteria against two pathogens

Colony	Zone of Inhibition	
	Staphylococcus aureus	*Bacillus* sp.
A	No zone of inhibition	No zone of inhibition
B	No zone of inhibition	No zone of inhibition
G	No zone of inhibition	No zone of inhibition
M	No zone of inhibition	No zone of inhibition

Since the 4 isolates showed maximum activity within the NaCl concentration of 0.5M-1.5M, these isolates are identified as moderate halophilic bacteria, as shown in Table 4. The Moderate bacteria halophiles fall under the Kingdom of Eubacteria; organisms in this Kingdom are more commonly found than archaeal-type organisms and are producers of antibiotics (Eubacteria, n.d.). There was no access to 16S rRNA to identify the genus of the four isolates; these were classified as belonging to the Kingdom Eubacteria and within the Family Halomonadaceae. The Family Halomonadaceae is divided into two broadly categorized genera; the Gram-positive and Gram-negative genera (Kanekar et al. 2012).

Pigmentation

It was seen that of the 4 halophilic bacteria were found; halophilic Bacterial Isolate A was yellow-pigmented and halophilic bacterial isolate G was light orange pigmented and flat in terms of elevation, and halophilic bacterial isolate B was pink pigmented; these were isolated from Marriott Beach and halophilic bacterial isolate M, from #63 Beach, was light pink pigmented and raised in terms of elevation. The colony morphology of these halophilic isolates was circular, surfaces were shiny, the texture of the colonies was smooth, and all had entire margins, as shown in (Tables 1-2) Halophilic isolates B, G, and M were 3 mm in size while halophilic isolate A was 2 mm. The Gram stain test revealed that halophilic isolates A, B, and G, were Gram-negative and cocci shaped while halophilic isolate M was Gram-positive and cocci shaped (Figures 2-9). These findings are similar to that of Azhar et al. (2014).

Salinity tolerance

The optimum salinity range was found to be 0.5M for all the halophilic bacteria found. Halophilic bacteria A showed no growth activity at 1.0M and 1.5M NaCl (Figure 11). this is due to its intercellular enzymes being unable to survive at such concentrated salinity. Halophilic bacteria G can thrive in 0.5M, 1.0M, and 1.5M NaCl; this shows that the enzymes of this organism are able to remain stable and active at such a high concentration of salinity. Halophilic bacteria B and M had medium growth at 1.0M but no growth at 1.5M NaCl; this shows the range for their enzyme's salinity concentration tolerance.

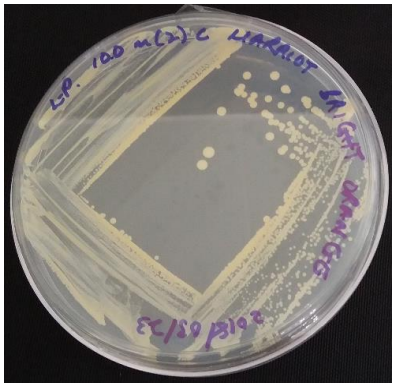

Figure 2. The halophilic bacterial isolate A**.**

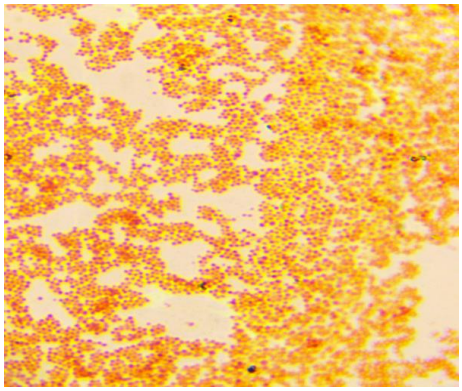

Figure 3. Gram stain of halophilic isolate A

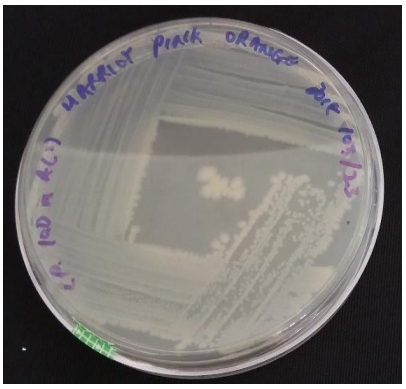

Figure 4. Halophilic bacterial isolate B

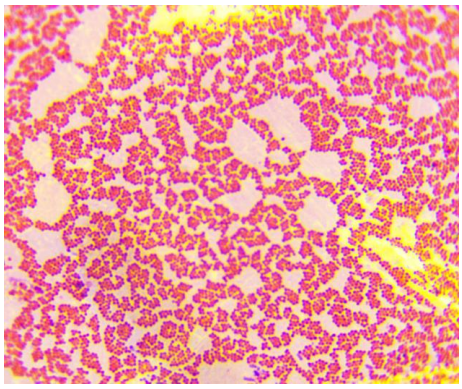

Figure 5. Gram stain of halophilic bacterial isolate B

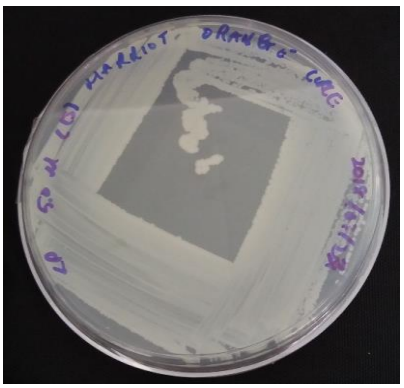

Figure 6. Halophilic bacterial isolate G.

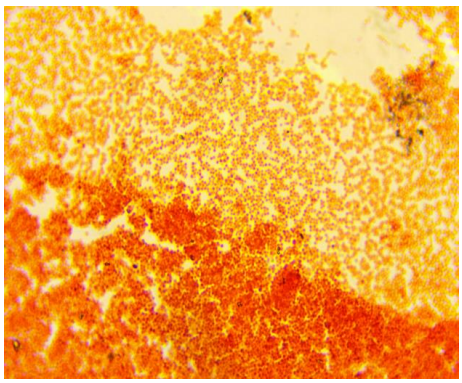

Figure 7. Gram stain of halophilic bacterial isolate G

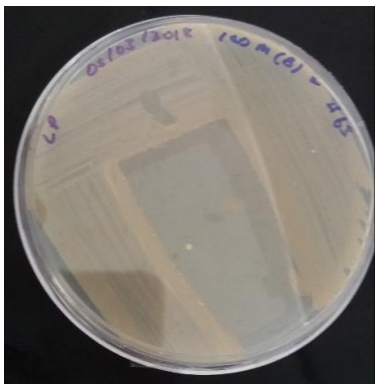

Figure 8. Halophilic bacterial isolate M.

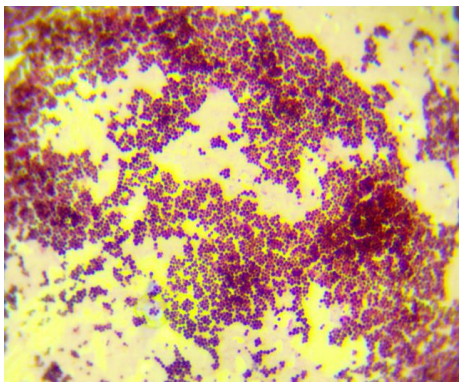

Figure 9. Gram stain of halophilic bacterial M.

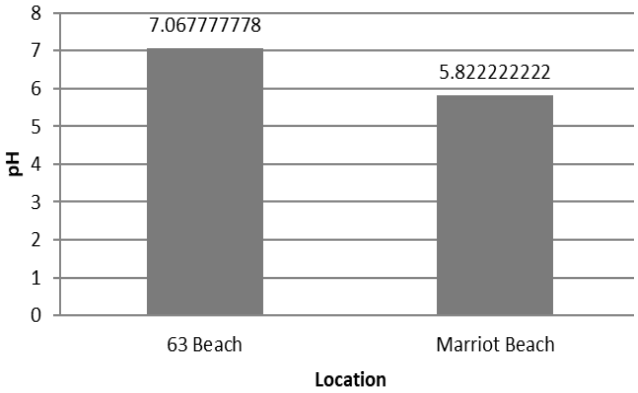

Figure 10. The average pH between the two sites. #63 Beach had a higher pH value overall when compared to Marriott Beach. Using the ANOVA testing, it was found that the p-value is 2.61E-11 which shows that there is a significant difference between the pH generally for both locations since the p-value found is lesser than the alpha value of 0.05.

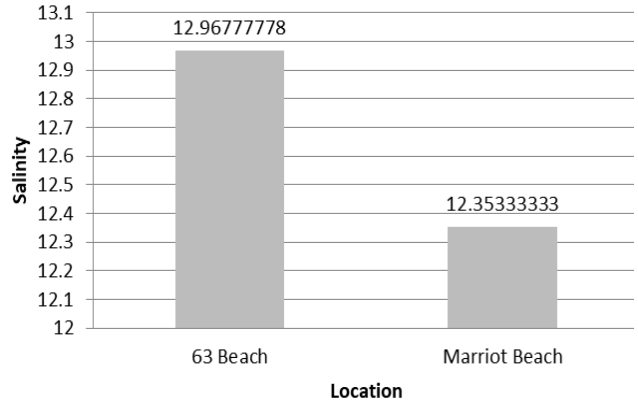

Figure 11. Average salinity between the two locations. Overall, #63 Beach had a higher salinity level than Marriott Beach. The ANOVA test shows a p-value of 0.5, greater than the alpha value of 0.05, which signifies there is no significant difference between the salinity of both locations.

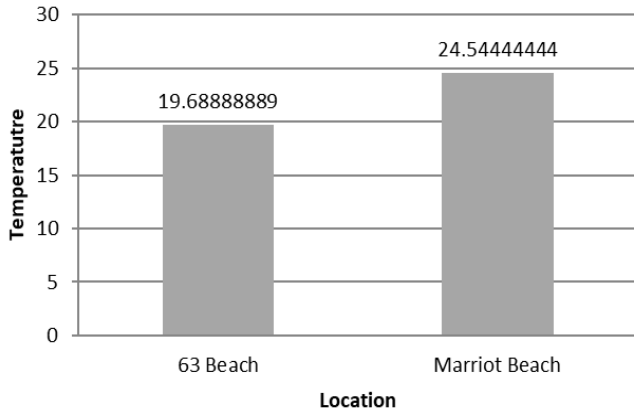

Figure 12. The average temperature of both locations. Marriott Beach has a higher average temperature for all depths than #63 Beach. From the ANOVA test, a p-value of 0.0004 was found. This shows there is great variation between the temperatures from both locations since the p-value is lower than the alpha value of 0.05.

The ability of halophiles to survive in different salinity levels compared to depends on the method they employ to adapt to their environment. Members of the Family Halomonadaceae use salt in a method that requires that the "proteins should maintain their proper conformation and activity at a near-saturating salt concentration" (Oren 2008). Therefore, the saturation level of halophilic bacteria A was 0.5M NaCl. At the same time, the saturation point for halophilic bacteria B and G was 1.0M NaCl. Halophilic Bacteria G can be further classified as a borderline extreme halophile based on the defined range by Donn Koshner. He stated that borderline halophiles thrive in the range of 1.5M to 4M salt (Oren 2008).

pH and temperature

Since 3 of the halophilic isolates found were from Marriott Beach, the null hypothesis that #63 beach will have a higher number of halophilic bacteria was rejected, and the alternative was accepted. The possible reason for this is that even though the salinity between the two locations did not have a significant difference with a p-value of 0.5, the pH varied for the depths of both locations; and was significantly different based on the ANOVA analysis; since only one halophilic bacterium was found from #63 beach of 10.0 m depth, when the pH was compared to the salinity, it was found that the pH of the 100m depth was low compared to the pH of the shore and 5.0m depth which were within the neutral range (Figure 10). Two halophilic bacteria were found in the 10.0 m depth of Marriott beach, which had low pH of 5.8 and a medium salinity level. There was a significant difference between this depth for both locations and one halophilic bacteria from the 5.0 m depth of pH 5.8 and high salinity level. A trend here is seen that most of these organisms were found where the pH was low and the salinity level high. This can be because there is a high level of carbon dioxide in the waters as it gets deeper. The presence of sodium chloride increases as the water becomes deeper because carbon dioxide reacts with water to form carbonic acid (Rose et al., 2016). Sodium chloride dissociates the carbonic acid and thereby frees the hydrogen ions, which results in a lower pH for the water (Reddi 2013). This also explains why the dissolved oxygen content for Marriott beach was lower than that of #63 Beach as the depth increased.

Generally, there was a significant difference between the temperature and the pH of Marriott beach and #63 Beach (Figure 12). The ANOVA analysis found that the temperature between the two locations had a variance p-value of 0.0004. Marriott Beach has a higher general temperature compared to #63 beach. This is due to two variables; the first one being that Marriott Beach is more exposed to pollution and carbon emissions and waste disposal as it is closer to the city compared to #63 Beach, which is more located in the countryside where carbon emissions and waste disposal is not that prevalent in the water; the second being the time which the samples were taken, the #63 Beach samples were collected earlier in January when temperatures are generally much lower while the Marriott samples were taken in March as the

temperature became higher. The temperatures showed significant variance for both locations' 5.0m and 10.0m.

The halophilic bacteria were tested for their temperature tolerance range by subjecting them to grow under 10°C, 37°C, and 45°C (Tables 3-4). The optimum temperature for growth of the 4 halophilic bacteria found was 37°C. Halophilic bacteria B and M had high growth at 45°C as well. Therefore, these halophilic bacteria are mesophilic as they survive within the range of 35°C to 45°C. Mesophiles are the most common type of bacteria found; they are able to thrive in a temperature range of 20°C to 45°C (Eddleman 1998). At 45°C, Halophilic bacteria A and G had less growth. This is due to the enzymes present in these organisms being denatured; the optimum temperature for enzymatic activities is 37.5°C; this finding corroborates with Schneegurt (2012), where it was found that the temperature range of 35°C-45°C was best for growing halophilic bacteria. For Halophilic bacteria B and M, the enzymes they produced were able to tolerate temperature at the extreme end of the mesophilic scale and were not easily denatured; the increase in temperature resulted in an increase in metabolic activities, which increased metabolic activity in the rapid growth of their cells. The halophilic bacteria showed no growth at 10°C because, at lower temperatures, the movement of molecules within the cells becomes slower and enzymatic reactions can no longer be carried out accurately. Eventually, cellular activities cease (Blamire 2000).

Antimicrobial properties

Staphylococcus aureus is a Gram-positive, cocci, pathogen found in the environment and even on the human skin. This bacterium causes a series of potentially fatal infections if it enters the human bloodstream (Taylor and Unakal 2017). *Bacillus* sp. is also Gram-positive, but it is rod-shaped. This bacterium is also a pathogen that leads to numerous infections when it enters the human body (Turnbull 1996). The halophilic bacterial isolates from Marriott and #63 Beach showed no antimicrobial activity against these pathogens (Table 5). Therefore, the alternative hypothesis is accepted and the null rejected. The finding here correlates with the findings of Irshad et al. (2013), where 5 of the halophilic isolates that were found in his study had no antimicrobial effects on pathogens; this can be due to the release of chemical toxins induced by deleterious microorganisms and mechanical stress (Velho-Pereira et al. 2012). Halophilic bacteria that thrive at very high salinity are most commonly known to inhibit the growth of pathogens. Sometimes, they are known only to release chemo toxins if it feels threatened by invading species (Ventosa et al. 1998).

In conclusion, Marriott Beach had a significant number of halophilic bacterial isolates compared to #63 Beach. The halophilic bacterial isolates belong to the Family Halomonadaceae. The 3 halophilic bacterial isolates A, B, and G, from Marriott Beach were Gram-negative, cocci, and isolate M from #63 Beach was Gram-positive, cocci. The four isolates were circular, had a shiny surface, smooth texture, and had entire margins. Marriott beach: Isolate A was yellow-pigmented and flat elevation; Isolate B was pink pigmented and raised in elevation, while Isolate G was light orange pigmented and flat in elevation. #63 beach isolate M was light pink pigmented and raised in elevation. Isolate G from Marriott Beach and Isolate M from # 63 Beach showed maximum growth at 1.0M and 1.5M NaCl. The optimum salinity for all the halophilic bacterial isolates was 0.5M NaCl. The optimum temperature for the halophilic bacterial isolates' growth was 37°C. Isolate G, from Marriott Beach, was the only halophilic bacteria that could tolerate a temperature high of 45°C. The isolates showed no antimicrobial action against *S. aureus* and *Bacillus* sp. There were significant differences between the pH and temperature between the two locations. There was not a significant difference between the salinity of both locations.

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Detection of *Salmonella typhimurium* ATCC 14028 in supplement health product liquid preparation using Real-Time PCR (qPCR)

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Abstract. *Sophian A, Purwaningsih R, Lukita BL, Ningsih EC. 2020. Detection of* Salmonella typhimurium *ATCC 14028 in supplement health product liquid preparation using Real-Time PCR (qPCR). Biofarmasi J Nat Prod Biochem 18: 65-69.* Detection of *Salmonella typhimurium* ATCC 14028 using Real-Time PCR (qPCR) on health supplement products was carried out in the microbiology and molecular biology testing laboratory of the Food and Drug Supervisory Office in Gorontalo. The purpose of this study was to provide an alternative testing method reference in the testing of liquid supplement health supplement products in the market. The sample consisted of 35 samples of liquid supplement health supplements spike with positive control of *Salmonella typhimurium* ATCC 14028 phase 2. The method used in the study was qPCR analysis using the SYBR Green method, whereas DNA isolation using the direct PCR method. Data analysis was performed based on 2 main criteria: (i) Ct (Cycle threshold) analysis, which looks at the value of the sample Ct and compares it with controls, and (ii) analysis of melting temperature (Tm), which is the melting point at the temperature at which melting occurs and comparing the melting point to the positive control. The results showed that Salmonella typhimurium ATCC 14028 was detected in the sample at an average Ct value of 14.43 and an average Tm value of 86.05; for the specificity, LOD and positive control tests were all amplified. For negative controls, Ct and Tm values were not detected. Based on these data, it can be concluded that real-time PCR (qPCR) can be used to detect *Salmonella typhimurium* ATCC 14028 in liquid supplement health supplement products.

Keywords: Health supplement, phase, qPCR, *Salmonella typhimurium*, SYBR green

Abbreviations: HEA: Hekton Enteric Agar, LOD: Limit of detection, TSA: Tryptic Soy Agar, TSB: Tryptic Soy Broth, XLD: Xylose Lysine Desoxycholate Agar

INTRODUCTION

Health supplements are products intended to fulfill nutritional needs, maintain, enhance or improve health functions, have nutritional value or physiological effects, and contain one or more ingredients in the form of vitamins, minerals, amino acids, and other non-plant ingredients which can be combined with plants. Health supplements can be made in the form of tablets, pills, capsules, oral liquids, powders, and granules or gummies. Health supplements must be made by using safe, useful, and quality raw materials by the provisions of the Indonesian Pharmacopoeia, Indonesian Herbal Pharmacopoeia, other countries' pharmacopeia, or recognized scientific references (NADFC 2019). To support the safety of the quality of health supplement products available on the market, it is necessary to have the latest test methods developed according to the advancement of science and technology. One such latest method is molecular analysis to detect pathogenic bacterial contamination in health supplements.

Molecular analysis to identify pathogenic bacteria using real-time PCR has advantages over conventional methods. The average time required if the identification of pathogenic bacteria is made conventionally is about 3-5 days, whereas it only takes 50-52 hours if the PCR method is used as an isolation technique (24 hours for enrichment samples, 24 hours for selective enrichment, and 1.5 hours for real-time PCR analysis).

One of the quality check parameters of health supplement products is that they should be free from pathogenic *Salmonella* bacteria: therefore, the mechanism of *Salmonella* identification is becoming increasingly necessary. The polymerase chain reaction (PCR) is among the several molecular techniques used in detecting *Salmonella* most commonly used is the polymerase chain reaction (PCR). Real-time PCR analysis (qPCR) shows high sensitivity and reproducibility in amplifying certain DNA fragments and can measure the presence of *Salmonella* DNA (Oliveira et al., 2018).

In recent years, several studies on the detection of *Salmonella* in various samples such as meat and poultry (Catarame et al. 2006); raw pork sausages (Wang et al. 2004); pasteurized milk, ground beef, and sprouts (Mercanoglu et al. 2005); water and milk (Jothikumar et al. 2003); samples of prepared foods, raw meat, and poultry (Cheung et al. 2004); minced beef, fish and raw milk (Perelle et al. 2004); raw and ready-to-eat beef products (Ellingson et al. 2004); and chicken intestines, cloacal

swabs, chicken carcasses (Eyigor et al. 2003; Eyigor et al. 2002) have been carried out.

According to Brooks et al. (2007), *Salmonella* can cause enteric fever caused by *Salmonella typhi* called Typhoid fever, Septicemia, a fever caused by *Salmonella choleraesuis*, and Gastroenteritis, a digestive tract disease caused by food poisoning that contains *Salmonella typhimurium*.

NADFC Regulation No. 17 of 2019 concerning Quality Requirements for Health supplements regulates the contamination limits of pathogenic bacteria in health supplements, where herbal-based health supplements may not contain pathogenic bacteria like *Salmonella* spp. Health supplement quality requirements must be applied before supplements are in circulation during health. Therefore, monitoring the product is very important to ensure that the circulating products are free from pathogenic bacterial contaminants. The quality requirements for health supplements must comply with the Indonesian Pharmacopoeia and Indonesian Herbal Pharmacopoeia provisions. Based on this, the research was conducted to develop alternative methods for detecting *Salmonella* in liquid supplement health supplement products.

MATERIALS AND METHODS

Materials

The sample materials used in this study were liquid health supplements, distilled water, TSB enrichment media, TSA, XLD, and HEA selective media; the PCR kit uses QuantiNova SYBR Green (Qiagen).

Sample setup

A total of 35 samples of liquid supplement health supplements spike with positive control of *Salmonella typhimurium* ATCC 14028 phase 2 were used during the present study.

Isolation in selective media

For isolation, 10 grams sample for solid samples were weighed out while it was pipette out 10 ml for a liquid sample. To this, 90 mL TSB was added and then incubated at 35-37C for 18-24 hrs. After incubation, one sample was scratched on the HEA and XLD selective media and then incubated at 35-37C for 18-24 hrs. Observations of colonies growing on selective media were recorded at regular intervals.

DNA isolation

DNA isolation was not carried out because the direct PCR method was used. The results of isolation from selective media were then enriched on the media to be skewed. The enrichment results stabilized in physiological NaCl and equalized to standard 1 MacFarland. The results of this equalization were then used as DNA templates.

qPCR analysis

Cycling and melt curve analysis was carried out using qPCR (QIAGEN 5 Plex) with the 2-step cycling method: Denaturation 95^{o} C for 45 sec and Annealing/Extention 60^{o} C for 45 sec. The primer used to detect *Salmonella typhimurium* using InvA Forward primers (5'-ATC AGT ACC AGT CTT ATC TTG AT-3 '), reverse (5'-TCT GTT TAC CGG GCA TAC CAT-3 ').

Reaction setup

The total volume of the master mix was 10 µL consisting of 5 µL Sybr green master mix, 1 µL forward primer, 1 µL reverse primer, 1 µL water-free RNase, and 2 µL DNA template.

Positive control

The positive control used was *Salmonella typhimurium* ATCC 14028 phase 2, which was enriched and etched on the media to make it tilt. *Salmonella typhimurium* ATCC 14028 colony was then dredged in physiological NaCl and equalized to standard 1 MacFarland.

Negative control

NTC (No Template Control) was negative control, a master mix combined with primers and free water from nucleic acids. The total negative control volume was 10 µL consisting of; master mix Sybr green, 1 µL forward primer, 1 µL reverse primer, and 3 µL RNase free water.

Specificity

Specificity testing was done by mixing the *Salmonella typhimurium* ATCC 14028 colony with the colony of *Escherichia coli* ATCC 25922 and *Listeria monocytogenes* ATCC 7644 in a ratio of 1: 1: 1 using the reference standard 1 MacFarland.

LOD

For LOD analysis, it was done by adding a 10: 1 sample DNA template from a positive DNA control template. In the positive control, the DNA template used was 2 µL, then the DNA template for LOD was 0.2 µL.

Data analysis

Data analysis was carried out based on 2 main criteria, which included: (i) Ct (Cycle threshold) analysis which looked at the value of sample Ct and compared it with controls. (ii) Analysis of melting temperature (Tm), the melting point at the temperature at which melting occurs, compares the melting point to the positive control.

RESULTS AND DISCUSSION

Isolation in selective media

The results of observations on HEA and XLD selective media can be seen in Figure 1. Based on the isolation results on HEA selective media, the colony was grey to black in the middle, while on XLD media, it was translucent to black in the center.

Figure 1. *Salmonella* profile on selective media HEA (blue) and XLD (red)

The choice of HEA and XLD selective media is because these two selective media are specific media for the isolation of *Salmonella* pathogenic bacteria. Andrews et al. (2007) explained that the profile of *Salmonella* on HEA and XLD media has a similar shape, which is the presence of black spots or not in the middle of the colony. A different matter was stated by Murray et al. (1999), which states that the HEA and XLD media are often used together to detect *Salmonella* because both of these media can also isolate *Shigella*. Both HEA and XLD have H2S indicators that can detect *Salmonella* from lactose-positive indicators.

Real-time PCR analysis

PCR real-time analysis was performed using the qualitative SYBR green method, and the results are presented in Table 1.

Based on the analysis of 35 samples, the obtained results showed that all samples detected were positive for *Salmonella typhimurium* ATCC 14028, as seen from samples compared with positive controls. The detection limit used was 1:10 from the sample template. This comparison was set because there is no definite standard of LOD in detecting *Salmonella typhimurium* ATCC 14028. It is determined by itself with a dilution system 10 times smaller than the DNA template. The specificity test was made from 2 groups of gram-negative and gram-positive bacteria to see if there was an influence on the ability of the method to detect specific DNA. The *Escherichia coli* ATCC 25922 were selected because these bacteria belong to the same gram-negative group as *Salmonella typhimurium* ATCC 14028. Listeria monocytogenes ATCC 7644 represents a group of gram-positive bacteria.

The results of cycling analysis by looking at the Ct value showed that the qPCR analysis showed *Salmonella typhimurium* ATCC 14028 in samples detected at Ct 14.43, LOD with a spike of positive control 10 times smaller than the sample turned out to be able to be detected at Ct 14.8, specificity was detected at Ct 19.47 difference. This is quite far from the value of Ct samples, and LOD against Ct specificity indicates that the addition of templates from Escherichia coli ATCC 25922 and Listeria monocytogenes ATCC 7644 influence the ability to detect Ct values. Several factors can also cause positive control at 15.7 different Ct values. The PCR direct concentration method and DNA template purity were not carried out in the measurement process. The template concentration between the sample and control was not the same. The Ct values could be different. Negative controls give unamplified results. This gives information that the master mix or in the test process does not occur contamination of the sample and positive control, as shown in Figure 2.

The results of the melt curve analysis by looking at the Tm value showed that the qPCR analysis showed *Salmonella typhimurium* in samples detected at Tm 86.05, LOD at Tm 86.0, specificity at Tm 83.8, and positive control at Tm 85.2. While the negative control did not show any Tm value due to not being amputated in the qPCR amplification process, as shown in (Figure 3). As with the Ct analysis, the difference in the value of Tm was also directly proportional to the difference in the value of Ct. This was because the DNA template used was not analyzed for its purity and concentration. DNA isolation PCR direct technique was used, and the concentration of samples, positive control, LOD, and sensitivity are unknown.

Table 1. qPCR data analysis

qPCR analysis	*Treatment*				
	Sample	**Negative control (NTC)**	**LOD**	**Specificity**	**Positive control**
Ct values	14.43	-	14.8	19.47	15.7
Tm values	86.05	-	86.0	83.8	85.2

Note: Ct and Tm values are the average value of 35 data replications

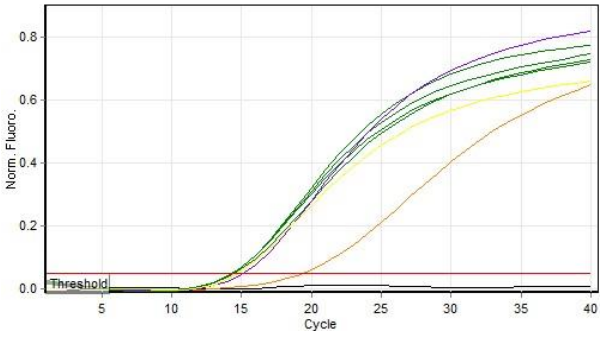

Figure 2. Ct analysis

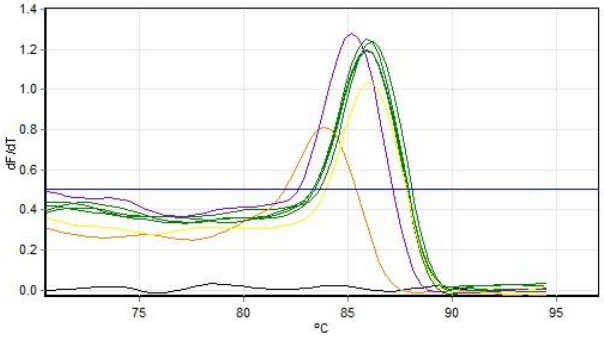

Figure 3. Melt curve analysis (Tm)

Discussion

Detection of *Salmonella* in liquid supplement health supplement products using real-time PCR was carried out by a qualitative method using the Quantinova SYBR Green (Qiagen) kit. This study was conducted using a sample of 35 liquid supplement packs on the spike with *Salmonella typhimurium* ATCC 14028, while for specificity used, *Escherichia coli* ATCC 25922 and *Listeria monocytogenes* ATCC 7644.

XLD media gave positive results of *Salmonella* by positive control, namely translucent spherical colonies with black spots in the middle. These results align with research conducted by Maddocks et al. (2002) and Nye (2002). According to Braid et al. (1995), the color change is based on observations of the HEA selective media (Figure 1) shows that on selective media, the colonies are greenish-blue with or without black at the center of the colony. Many colonies can be glossy black or appear to be almost entirely black in large concentrations or cultures (BAM 2007). HEA is a selective media that does not have the ability to selectivity like XLD. Gaillot et al. (1999) tested 508 *Salmonella* samples using HEA, with 16 results being detected as false positive; in other words, 96% showed positive results, and 4% showed false-positive results.

According to Oliveira et al. (2018), molecular analysis for the detection of *Salmonella* using real-time PCR showed considerable sensitivity and reproducibility in the amplification of certain fragments. It could measure the presence of *Salmonella* DNA. Molecular research in species detection has been developed in various forms of modification. A study of the identification of *Salmonella* using real-time PCR, conducted by Piknova et al. (2002) on 75 types of *Salmonella* strains using the SYBR green method, gave 100% detectable results. The real-time PCR analysis was performed using the 2-step cycling method: Denaturation 95ºC for 45 sec and annealing/extension 60º C for 45 sec.

The genes used in this study were invA genes with primary sequences forward (5'-ATC AGT ACC AGT CTT ATC TTG AT-3'), reverse (5'-TCT GTT TAC CGG GCA TAC CAT-3'). The invA gene as the primer was selected because the invA gene is a specific gene to identify *Salmonella typhimurium* ATCC 14028. The use of invA genes for *Salmonella* detection using real-time PCR has been developed and validated by Malorny et al. (2003). According to Patel et al. (2006), validation is very important in making standard methods to provide accurate results.

The Tm value in the qPCR analysis is influenced by the composition and size of the nucleotides. Fluorescent signals give information when DNA bands begin to separate after the annealing process. The melt curve produces a specific single peak of each band detected. However, in each melt curve analysis, the presence of double peaks can sometimes also occur, but even so, the double peaks that occur do not say that this method is not specific (Dwight 2011).

To evaluate real-time PCR tests to detect pathogens in various matrices, both the sensitivity and specificity of the test need to be determined. The analytical sensitivity of a test has been defined as the smallest amount detected from the analyte in question. In contrast, analytical specificity is the extent to which the test does not cross-react with other analytes (Bohaychuk et al., 2006).

Health Supplements are products intended to supplement nutrition. The conclusion that can be drawn from this study is the use of real-time PCR with the SYBR Green method to identify *Salmonella typhimurium* ATCC 14028 in 35 samples giving 100% detected results.

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Evaluation of the antiproliferative activity of red propolis hydroalcoholic extract and its fractions obtained by partition

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Abstract. *Squarisi IS, De Freitas KS, Lemes DC, Ccana-Ccapatinta GV, Mejia JAA, Bastos JK, Veneziani RCS, Ambrosio SR, Tavares DC. 2020. Evaluation of the antiproliferative activity of red propolis hydroalcoholic extract and its fractions obtained by partition. Biofarmasi J Nat Prod Biochem 18: 70-73.* The present study aimed to evaluate the cytotoxicity of red propolis hydroalcoholic extract (RPHE) and its fractions obtained by partition, hexanes (HF), dichloromethane (DF), ethyl acetate (AF), and *n*-butanol (BF) on tumor and non-tumor cell lines. For this purpose, the XTT colorimetric assay was performed on human lung fibroblasts (GM07492A, non-tumor cell), breast adenocarcinoma (MCF-7), glioblastoma (U343), and cervix adenocarcinoma (HeLa) cells. The results showed that RPHE, HF, and DF presented not only cytotoxic potential to all tumor cell lines but also to normal cell lines, indicating selectivity absence. HF presented the lowest IC_{50} (half minimal inhibitory concentration; 33.8-133.3 µg/mL), with a significant difference from those observed for RPHE (137.0-262.7 µg/mL). BF and AF revealed an IC_{50} higher than 1250 µg/mL in all cell lines. The results showed that red propolis has substances with antiproliferative activity, indicating that its hexane fraction may have substances with antitumor potential.

Keywords: cytotoxicity, red propolis, XTT colorimetric assay

INTRODUCTION

Propolis is a product of honeybee hives, containing mainly beeswax and a resin obtained from various plant sources such as apical buds, young leaves, and exudates (Salatino & Salatino 2018). Red propolis, with *Dalbergia ecastaphyllum* (Daugsch et al., 2008; Silva et al., 2008) and *Symphonia globulifera* (Ccana-Ccapatinta et al., 2020) as its main botanical sources, has recently stood out as a natural medicinal product due to its various biological properties such as anti-inflammatory (Batista et al. 2018), antitumoral (Salatino & Salatino 2018) antioxidant, cytotoxic (de Oliveira Reis et al., 2019), antimicrobial (Miranda et al. 2019) activities and healing capacity (Picolotto et al. 2019). The biological activity of red propolis is mainly related to isoflavones, which act in synergy with the other compounds. Other compounds, such as vestitol, neovestitol, biochanin A, and liquiritigenin, are identified in the fractions. The extracts of Brazilian red propolis are also considered important markers associated with different biological factors' effects (Rufatto et al., 2018; Nani et al., 2018).

Bio-guided fractionations are efficient methods to improve the development of new drugs, highlighting the activity of each group of compounds, individually or in combination with others (Dos Santos et al., 2019). Thus, the present study aimed to evaluate the cytotoxicity of the red hydroalcoholic extract (RPHE) and its fractions, namely, hexanic (HF), dichloromethane (DF), ethyl acetate (AF), and *n*-butanol (BF), on tumor and non-tumor cell lines.

MATERIALS AND METHODS

Obtention and profiling of red propolis hydroalcoholic extract (RPHE) and its fractions

Red propolis (2 kg) was purchased from Cooperativa de apicultores de Canavieiras (COAPER) in the city of Canavieiras (Bahia state, Brazil,) in April of 2018. RPHE was prepared by exhaustive maceration of 500 g of red propolis in 1.5 L of ethanol: H_2O (7:3 v/v) for seven days. The extract was then filtered, concentrated, and lyophilized to result in 75g of RPHE. After resuspending RPHE in 500 mL of methanol: H_2O (8:2 v/v), the fractioning of this extract was executed through a four-times partition with 500 mL of hexanes, dichloromethane, ethyl acetate, and *n*-butanol respectively to result in HF (22.8g), DF (43.6g), AF (0.90g), and BF (4.34g) after evaporation.

The chemical profiles of RPHE and its fractions were obtained by HPLC-DAD analysis using a Synergi Polar-RP (150 x 4.60 mm, 4 µm) column as a stationary phase and a mobile gradient phase of H_2O (A) and acetonitrile (B) starting from 23% up to 100% of (A) in 32 min at 1.2 mL/min. The detection wavelength was 220 nm, and the identification of the compounds was performed by co-

injection of standard concentrations available in our laboratory (Figure 1): liquiritigenin (1), formononetin (2), liquiritigenin (3), vestitol (4), neovestitol (5), medicarpin (6), 7-O-methylvestitol (7), a mixture of guttiferone E and xanthochymol (8), oblongifolin B (9).

Evaluation of antiproliferative activity

In this study, three different tumor cell lines were used: human breast adenocarcinoma (MCF-7), human cervical adenocarcinoma (HeLa), and human glioblastoma (U343). A normal human cell line (lung fibroblasts, GM07492A) was included to evaluate the possible selective activity of the natural medicinal product under investigation. Different cell lines were maintained as monolayers in plastic culture medium (HAM-F10 + DMEM, 1:1, Sigma-Aldrich) supplemented with 10% fetal bovine serum (Nutricell), antibiotics (0.01 mg/mL streptomycin and 0.005 mg/mL penicillin; Sigma-Aldrich) and 2.38 mg/mL HEPES (Sigma-Aldrich). Cells were incubated at 36.5°C in humidified 5% CO_2 atmosphere.

According to the manufacturer's guidelines, the samples' antiproliferative activity was evaluated using the in vitro toxicology colorimetric assay-XTT Kit (Roche Diagnostics). For the experiments, 1 x 10^4 cells were seeded in 96-well microplates; each well received a maximum of 100 µL culture medium (HAM F10 + DMEM, 1:1) supplemented with 10% fetal bovine serum-containing different sample concentrations, which ranged 9.77 to 1250 µg/mL. Negative (untreated), solvent (1% DMSO), and positive (25% DMSO) control wells were also included. After incubation with the substances at 37 °C for 24 h, the culture medium was removed, and the cells were washed with 100 µL PBS to remove treatments and exposed to 100 µL HAM-F10 culture medium without phenol red. Then, 25 µL of XTT were added to each well, and the microplates were incubated at 37 °C for 17 h. The absorbance of the samples was determined using a multi-plate reader (Asys-UVM 340/Microwin 2000 ELISA) at a wavelength of 450 nm and a reference length of 620 nm. The number of soluble products formed (formazan) was proportional to the number of viable cells. The negative control group was designated as 100%, and the results were expressed as a percentage of the negative control. The experiments were performed in triplicate.

Statistical analysis

Cytotoxicity was assessed using the IC_{50} value (50% cell growth inhibition) as a response parameter, calculated with the GraphPad Prism 5.0 program (GraphPad Software, San Diego, CA, USA), plotting cell survival against the respective concentrations of the natural products tested. One-way ANOVA was used to compare means ($p < 0.05$).

RESULTS AND DISCUSSION

Results

Table 1 presents the results expressed by the inhibitory concentration of 50% cell growth (IC_{50}). The results showed that, for each cell line-GM07492A (human lung fibroblasts, non-tumor cells), MCF-7 (breast adenocarcinoma), U343 (glioblastoma), and HeLa (cervix adenocarcinoma)-RPHE revealed an IC_{50} of 137.0, 262.7, 138.2 and 137.4 µg/mL, HF an IC_{50} of 33.8, 133.3, 33.8 and 34.5 µg/mL, DF an IC_{50} of 78.9, 237.6, 71.6 and 99.6 µg/mL, and AF and BF an IC_{50} of higher than 1250 µg/mL in all cell lines. RPHE, HF, and DF, therefore, showed cytotoxic activity in all tumor cell lines and the normal cell line, indicating selectivity absence. HF presented the highest cytotoxic potential with the lowest IC_{50}, being significantly different from those observed for RPHE. The cytotoxicity of HF might be attributed to its chemical composition, consisting predominantly of polyprenylated benzophenones **8** and **9** (guttiferone E, xanthochymol, and oblongifolin B), described in the literature as pro-apoptotic compounds.

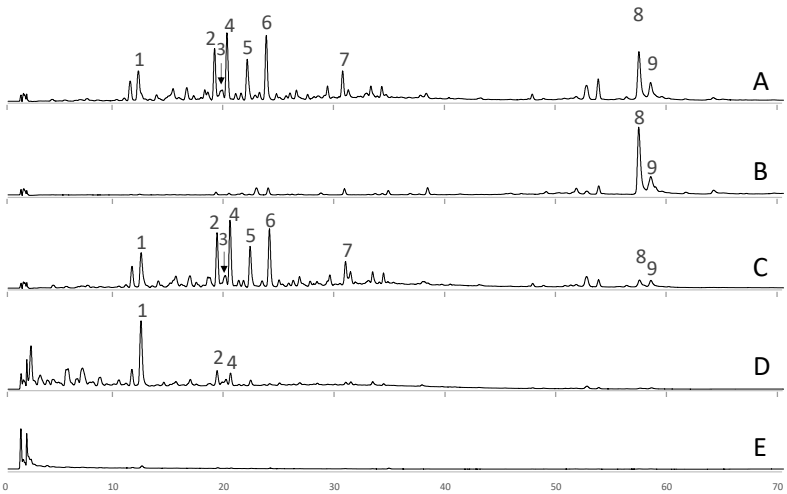

Figure 1. HPLC-DAD profile of red propolis hydroalcoholic extract (RPHE; A) and its fractions obtained in *n*-hexanes (HF; B), dichloromethane (DF; C), ethyl acetate (AF; D), and *n*-butanol (BF; E). Identity of main constituents: liquiritigenin (1), formononetin (2), liquiritigenin (3), vestitol (4), neovestitol (5), medicarpin (6), 7-O-methylvestitol (7), a mixture of guttiferone E and xanthochymol (8), oblongifolin B (9)

Table 1. Inhibitory concentrations (IC_{50}, µg/mL) obtained from RPHE-treated non-tumoral (GM07492A) and tumoral (MCF-7, U343 and HeLa) cell cultures and its fractions.

Treatment	Cell line			
	GM07492A	MCF-7	U343	HeLa
RPHE	137.0 ± 4.2	262.7 ± 5.9	138.2 ± 2.3	137.4 ± 0.3
HF	33.8 ± 0.6[a]	133.3 ± 1.3[a]	33.8 ± 0.3[a]	34.5 ± 0.1[a]
DF	78.9 ± 1.7[a]	237.6 ± 26.6	71.6 ± 0.6[a]	99.6 ± 27.3
AF	> 1250	> 1250	> 1250	> 1250
BF	> 1250	> 1250	> 1250	> 1250

Note: RPHE: red propolis hydroalcoholic extract; HF: hexanes fraction; DF: dichloromethane fraction; AF: ethyl acetate fraction; BF: *n*-butanol fraction. GM07492A: non-tumor human lung fibroblast; MCF-7: human breast adenocarcinoma; U343: human glioblastoma; HeLa: human cervical adenocarcinoma. Values are mean ± SD, n: 3. [a]Significantly different from the RPHE treatment ($p < 0.05$)

Discussion

In this sense, Lin et al. (2019) reported cytotoxicity of a mixture of guttiferone E and xanthochymol in five types of human cancer cell lines: leukemic (HEL, IC_{50} = 11.27 µg/mL; K562, IC_{50} = 10.92 µg/mL), cervical (HeLa, IC_{50} = 5.46 µg/mL), breast (MCF-7, IC_{50} = 4.68 µg/mL) and lung (A549, IC_{50} = 6.10 µg/mL). These results demonstrate that these two substances present in the hexanes fraction of red propolis corroborate their cytotoxic potential.

Novak et al. (2017) observed that a fraction of the ethanolic extract of Brazilian red propolis, containing xanthochymol and formononetin, showed an antiproliferative effect in acute promyelocytic leukemia cell lines (HL-60, IC_{50} = 20.5 µg/mL), human chronic myeloid leukemia (K562). IC_{50} = 30.3 µg/mL), multiple myeloma (RPMI8226, IC_{50} = 32.6 µg/mL) and murine melanoma (B16F10, IC_{50} = 25.7 µg/mL). The fraction showed a more promising antiproliferative effect than that observed by the ethanolic extract.

Through the MTT test, the ethyl acetate fractions of the red propolis extract revealed cytotoxic activity in HT-29 (human colorectal adenocarcinoma) and HCT-116 (human colorectal carcinoma), and non-tumor cell line Vero (monkey kidney epithelium). IC_{50} values ranged from 40.32 to 105.23 µg/mL, with the lowest IC_{50} values corresponding to the tumoral lines tested, indicating selectivity. Chemical analysis of the fractions indicated the presence of formononetin, vestitol, biochanin A, liquiritigenin, and the guttiferone E xanthochymol mixture (Santos et al., 2019).

The antiproliferative activity of the hydroethanolic extract of red propolis and two of its fractions (J and L) was evaluated in human laryngeal epidermoid carcinoma cells (Hep-2) by Da Silva Frozza et al. (2017). The chemical profile of fraction J revealed the presence of formononetin, liquiritigenin, medicarpin, vestitol, isovestitol, and (3S)-ferreirin, and fraction L, only fraction L liquiritigenin. The results showed an IC_{50} of 145.40 µg/mL for the extract, 60.96 µg/mL for the J fraction, and 74.60 µg/mL for the L fraction. Thus, the fractions showed a greater cytotoxic effect than the extract. Fractionation of the extract leads to a decrease in the number of chemicals present in the fraction. It may increase the concentration of active compounds compared to the exposure of cells to the crude extract (da Silva Frozza et al., 2017).

The results showed that red propolis presents substances with antiproliferative activity, indicating that the hexane fraction may have substances with antitumor potential. Further studies should be conducted with guttiferone E, xanthochymol, and oblongifolin B, substances predominantly identified in the hexane fraction.

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Review: The endless nutritional and pharmaceutical benefits of the Himalayan gold, *Cordyceps;* Current knowledge and prospective potentials

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Abstract. *Elkhateeb WA, Daba GM. 2020. Review: The endless nutritional and pharmaceutical benefits of the Himalayan gold, Cordyceps; Current knowledge and prospective potentials. Biofarmasi J Nat Prod Biochem 18: 74-81.* As a traditional medicine, *Cordyceps* has long been used in Asian nations for maintaining vivacity and boosting immunity. Numerous publications on various bioactivities of *Cordyceps* have been investigated in both in-vitro as well as *in vivo* studies. Nevertheless, the role of *Cordyceps* is still arguable whether it acts as a food supplement for health benefits or a real healing drug that can be prescribed in medicine. The *Cordyceps* industry has developed greatly and offers thousands of products commonly available in a global marketplace. This review will focus on introducing the ecology of *Cordyceps* and their classification. Moreover, elucidation of the richness of extracts originating from this mushroom in nutritional components was presented, with a description of the chemical compounds of *Cordyceps* and its well-known compounds such as cordycepin and cordycepic acid. Furthermore, highlights on natural growth and artificial cultivation of famous *Cordyceps* species were presented. The health benefits and reported bioactivities of *Cordyceps* species as promising antimicrobial, anticancer, hypocholesterolemic, antioxidant, antiviral, anti-inflammatory, organ protective agent, and enhancer for organ function were presented.

Keywords: *Cordyceps*; cultivation; secondary metabolites; traditional medicine

INTRODUCTION

Commonly seen nowadays on shelves of pharmacies and drug stores and recommended for many benefits such as boosting immunity, the genus *Cordyceps* is an ascomycetous traditional medicinal mushroom that is famous for having numerous bioactive compounds. *Cordyceps* have different common names: insect mushroom, caterpillar fungus, Himalayan gold, etc. Still, the name *Cordyceps* originates from the Latin words (cord), which means 'club,' and (ceps), referring to 'head.' The fruiting bodies of these fungi appear from the head of different life stages of various orders of insect species (Zhou et al. 2009; Smiderle et al. 2014; Chen et al. 2000; Peterson 2008; Wang and Yao 2011; Dworecka-Kaszak 2014). The genus *Cordyceps* is classified under the order Hypocreales, family Ophiocordycipitaceae, and phylum Ascomycota. Some genera belong to families Cordycipitaceae and partial Clavicipitaceae, as shown in Table 1 (Pu and Li 1996; Buenz et al. 2005; Sung et al. 2007; Kepler et al. 2012).

Species belonging to the genus *Cordyceps* have a golden history due to their safe use in traditional oriental medicines (Paterson 2008). Also, they were utilized 2000 years ago in China for curing different infectious diseases (Singh et al. 2008; Zhou et al. 2009). Although *Cordyceps* has a wide-reaching distribution, most species have been described from Asia (Boesi Alessandro and Francesca Cardi 2009).

The most famous and widely used species of *Cordyceps* is *C. sinensis* (Berk.) Sacc (syn. *Ophiocordyceps sinensis* (Berk.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora). Currently, the preferred scientific name is *Cordyceps sinensis* (Berk.) Sacc. (Devkota et al. 2006). This species has a wide host range, including different species of Lepidopteran larvae (Wang and Yao 2011), numerous Thitarodes caterpillars, and its most common host, the Himalayan bat moth *Hepialus armoricanus* (Chen et al. 2000). *Cordyceps militaris* (also known as orange caterpillar mushroom) is rich in bioactive compounds and hence has medical-biological activities in a similar way to *Cordyceps sinensis* (Shrestha and Sung 2005; Gong et al. 2006; Ma et al. 2007; Huang et al. 2009; Das et al. 2010; Dong et al. 2012). This review discussed ecology, classification, nutritional components, chemical composition, natural growth and artificial cultivation, health benefits, and reported bioactivities of famous Cordyceps species.

CORDYCEPS ECOLOGY

Many *Cordyceps* species grow by feeding on insect larvae and sometimes on mature insects. *Cordyceps* grows on insects, crickets, cockroaches, bees, centipedes, black beetles, and ants. From the genus *Cordyceps*, there are several species known to have medical value, only a few are cultivated, and the most common and well-known are

Cordyceps sinensis (Figure 1) and *Cordyceps militaris* (Figure 2) (Halpern Georges 2007). *Cordyceps* may also grow on other arthropods and the fungus *Elaphomyces* Nees.

Table 1. The current classification system of *Cordyceps* (*Cordyceps* sensu lato)

Genus	No. of species
Partial Clavicipitaceae	
Drechmeria	2
Hypocrella	50
Metacordyceps	4
Metarhizium	35
Nomuraea	3
Pochonia	3
Podocrella	4
Regiocrella	2
Sphaerocordyceps	2
Tyrannicordyceps	5
Total: 10	110
Ophiocordycipitaceae	
Blistum	1
Didymobotryopsis	3
Elaphocordyceps	1
Haptocillium	8
Hirsutella	78
Hymenostilbe	22
Ophiocordyceps	155
Paraisaria	2
Perennicordyceps	4
Polycephalomyces	12
Purpureocillium	3
Syngliocladium	5
Synnematium	1
Tolypocladium	39
Trichosterigma	1
Total: 15	335
Cordycipitaceae	
Akanthomyces	13
Ascopolyporus	7
Beauveria	31
Beejasamuha	1
Cordyceps	175
Coremiopsis	2
Engyodontium	5
Gibellula	21
Hyperdermium	2
Insecticola	2
Isaria	83
Lecanicillium	21
Microhilum	1
Phytocordyceps	1
Pseudogibellula	1
Rotiferophthora	27
Simplicillium	8
Torrubiella	66
Total: 18	467
Total: 3 families, 43 genera, and 912 species	

Note: The data is counted from Catalogue of Life: http://www.catalogueoflife.org/, accessed in June 2017)

Figure 1. *Cordyceps sinensis* (Collector: Soraj25: Locality: Nepal, Mid-Western, Jumla, Nepal, hosted by http://mycoportal.org)

Figure 2. *Cordyceps militaris* (Collector: Marjan Kustera: Locality: Gabrovac, Serbia, hosted by http://mycoportal.org).

Classification of the genus *Cordyceps* was previously within the family Clavicipitaceae, order Hypocreales (the old genus *Cordyceps* Fr.). Currently, many genera have segregated from the genus *Cordyceps*, such as *Metacordyceps*, *Tyrannycordyceps* (placed in family Clavicipitaceae); *Elphcordyceps*, *Ophiocordyceps* (in family Ophiocordycepitaceae); *Sphaerocordyceps*, and remaining *Cordyceps* species (placed in *Incertae sedis* within Hypocreales). Due to their special nutritional and healing values, *Cordyceps* is widely spreading in China, with a huge existing domestic market. In the Chinese market, *Cordyceps* is known as 'Dongchong Xiacao (worm in winter, an herb in summer; *O. sinensis* (Berk.) Sung et al. 2007), which is the most expensive type and only produced from the Tibetan Plateau; other *Cordyceps* species in the markets are termed 'fake Dongchong Xiacao.'

THE NUTRITIONAL COMPONENTS IN *CORDYCEPS*

Cordyceps is rich in various compounds considered nutritional such as vitamins (K, B1, B2, B12, and E); essential amino acids. Additionally, many mono-, di-, and oligosaccharides and many complex polysaccharides were found in *Cordyceps* besides trace elements (Na, K, Zn, Ca, Mg, Al, Fe, Cr, Cu, Mn, Zr, Pi, Se, Si, Sr, Ti, Ga, V, and Ni), proteins, nucleosides, and sterols. *Cordyceps* contains considerable quantities of polysaccharides, representing 3-8% of the mushroom's total weight, and these polysaccharides usually originate from fruiting bodies. *Cordyceps* polysaccharides are considered the major biologically active compounds besides nucleotides (Zhou et al., 2009; Mishra and Yogesh, 2011; Elkhateeb et al., 2019).

CHEMICAL COMPOSITION OF THE MOST COMMON *CORDYCEPS* SPECIES

Cordyceps sinensis (Berk.) Sacc.) is considered the most expensive and well-studied *Cordyceps* species. According to different chemical analyses, *C. sinensis* contains proteins, polysaccharides, fats, fiber, and carbohydrates, the famous bioactive compounds cordycepin (30-deoxyadenosine) and cordycepic acid (D-mannitol), and different vitamins (Ohta et al. 2007; Zhou et al. 2009).

The therapeutic potentials of *Cordyceps* depend mainly on the key actions of increased oxygen utilization, ATP production, and sugar metabolism stabilization. Many bioactive compounds originating from Cordyceps are responsible for those effects, such as cordycepin, cordycepic acid, polysaccharides, vitamins, and trace elements. The full bioactive compounds existing in *C. sinensis* are not yet identified. However, at least two compounds, cordycepic acid and cordycepin, have been identified and recommended as important bioactive compounds. Of all *Cordyceps* species, *C. militaris* is the only species that has been successfully cultivated and most intensively studied. The majority of *Cordyceps* products available in the markets are developed from the fruiting bodies of cultivated *C. militaris*. According to reported chemical investigations, *C. militaris* contains cordycepin, adenosine, polysaccharide, mannitol, trehalose, polyunsaturated fatty acids, δ-tocopherol, p-Hydroxybenzoic acid, and β-(1→3)-D-glucan (Reis et al. 2013; Liu et al. 2014; Smiderle et al. 2014; Wen et al. 2017; Elkhateeb et al. 2019).

CORDYCEPS NATURAL GROWTH AND ARTIFICIAL CULTIVATION

The natural growing fruiting bodies of *Cordyceps* are rare, and their collection is an expensive process. Moreover, natural populations of key *Cordyceps* species are decreasing rapidly due to over-collection (Stone 2008; Zhang et al. 2012), presenting the need for increased *in-vitro* cultivation using the artificial medium. While over 400 species under the genus *Cordyceps* have been identified, only 36 species have been successfully cultivated in artificial media (Sung et al. 1999; Yin and Qin 2009). The first large scale fruiting techniques used for growing *Cordyceps* reduced the natural growing cycle from 5 to 2 years; this technique included breeding the host larvae, Thitarodes (Hepialus), then placing about 100 larvae into shoe carton sized plastic containers covered with lids, which are filled with grassland soil containing roots and tubers of their natural foods collected from the wild, besides other roots from cultivation. Spores of *C. sinensis* were inoculated after two years, and about 10% of the larvae were actually taken over by *Cordyceps* and grew stromata (Yue et al., 2013). On the other hand, Arora et al. (2013) succeeded in using submerged conditions for culturing *Cordyceps sinensis* at pH 6 and a temperature of 15ºC.

In previous studies, the medium used for the growth of *C. sinensis* was sabouraud's dextrose supplemented with yeast extract broth medium, and different additives, carbon, and nitrogen sources were also investigated (Arora et al. 2009). The highest number of conidia was obtained under the physical stress of freeze-shock (Ren and Yao 2013). Sucrose has been reported as the best-tested carbon source for the growth of *C. Sinensis*. Similarly, beef extract and yeast extract were the best nitrogen sources (Seema et al., 2012). Furthermore, using folic acid meaningfully increased the yield, and adding calcium chloride and zinc chloride as micro and macronutrient increased the yield significantly. One of the optimum artificial techniques for culturing *C. sinensis* was utilizing sterile rice media at 9-13°C for 40-60 days. Then to induce stroma production, the temperature was lowered to 4°C and 13°C for 40 days to develop fruiting bodies (Cao et al. 2015). It should be highlighted that the growth of *Cordyceps* mycelium is mainly affected by some environmental factors and many factors such as temperature, growth media, and pH (Calam 1971). Still, after testing various media, potato dextrose agar was confirmed to be the optimum medium at pH ranging between 8.5-9.5 at 20- 25°C (Ruhul et al. 2008).

Artificial cultivation is achieved by inoculating reared larvae with cultured strains, and the infected larvae are monitored and fed indoors for one or two years. After that, *C. sinensis* could be collected. (Lo et al. 2013). *C. militaris* cultivation is much easier than *C. sinensis* in both solid and liquid media using different carbon and nitrogen sources since *C. militaris* can complete its life cycle when cultured *in-vitro* (Shrestha et al. 2004, Xiong et al. 2010). The artificial cultivation of *C. militaris* mycelium on synthetic media has recently been advanced, especially for cordycepin production, using different methods such as surface culture (Masuda et al. 2007) and submerged culture (Mao et al. 2005). Usually, *C. militaris* stromata production requires 35-70 days (Zhang and Liu 1997; Du et al. 2010). Culture duration depends on many conditions, such as the amount of medium, shape, and volume of the culture container. The development of *C. militaris* stroma cultivation *in-vitro* started with using insects to grow

stromata by Leatherdale (1970), followed by laboratory trials using various organic substrates by Yue et al. (1982). For commercial production of *C. militaris* stromata, cereals, including rice, have been widely used (Wen et al., 2008; Chen et al., 2011). Also, using substrates such as wheat grains, cottonseed coats, corn cobs, corn grain, bean powder, millet, and sorghum has shown promising results (Chen and Wu 1990; Zhang and Liu 1997; Gao and Wang 2008, Wei and Huang 2009). The optimum organic substrate currently used is a mixture of rice and silkworm pupae (Shrestha et al. 2005, Sung et al. 2006; Jin et al. 2009). Furthermore, brown rice, malt, and soybean were important sources of nutrition for C. militaris compared to chemical media (Xie et al. 2009). *C. militaris* cultivation needs a relatively low level of nitrogen (Gao et al. 2000) which may explain lower yields when using insects compared to higher yields reached when cereals were used in the culture. Plant hormones such as colchicines, 2, 4-D, and citric acid triamine can promote *C. militaris* stroma production. Also, potassium, calcium, and magnesium salts can increase the yield of fruiting bodies (Xiao et al., 2010).

Mycelia production for the large-scale production of bioactive compounds is also achievable and has been performed in submerged culture (Huang et al., 2006; Xie et al., 2009; Das et al., 2010). *C. militaris* cultivation has been improved, successfully producing a high yield of stromata and elevated cordycepin content (Sun et al., 2009; Du et al., 2010). The production of fruiting bodies has been studied for three successive generations (Hong et al. 2010; Xiao et al. 2010; Shrestha et al. 2012; Xiaoli et al. 2014).

CORDYCEPS AND HEALTH BENEFITS

The genus *Cordyceps* species are extensively studied due to the uncountable number of medical-biological activities used by their extracted compounds, with various medical and nutritional values. In traditional Chinese medicine, the main use of *Cordyceps* has been to treat asthma and other bronchial conditions and give energy and sexual power. Recent research now confirms the competence of *Cordyceps* in many other fields. One of the advances of modern research has been the discovery of cordycepin, which has a potent antibacterial action against the majority of bacterial species that have currently developed resistance to other commonly used antibiotics. *Cordyceps* showed remarkable activity during treating tuberculosis and human leukemia, as shown in many clinical trials in Asia and elsewhere (Halpern Georges 2007). *Cordyceps* was shown to improve the maximum amount of oxygen and improve respiratory function. Other components produced by *Cordyceps sinensis* include the deoxynucleoside 2', 3' deoxyadenosine. Also, Quinic acid derived from cordycepin obtained from *Cordyceps* exerts antimicrobial and antiviral activities. Many studies have reported the potency of *Cordyceps sinensis* in healing heart rhythm disturbances such as chronic heart failure and cardiac arrhythmia (Mishra and Yogesh, 2011; Wang et al., 2012).

Anticancer activities of *Cordyceps*

Various *Cordyceps-originated* bioactive compounds have a promising anticancer activity that was previously reported (El-Hagrassi et al., 2020). For example, cordycepin showed antitumor activity against B16 melanoma cells (Yoshikawa 2004 and 2007). In addition, cordycepin inhibited the mammalian target of rapamycin complex 1 in gallbladder cancer cells in-vitro, resulting in loss of cancer cell viability and apoptosis (Wong et al. 2010; Ferreira et al. 2010; Wu et al. 2014). *C. militaris* was found to inhibit U937 cell growth in a dose-dependent manner and limit human leukemia (Park 2005). *Cordyceps* has shown promising results in slowing and inhibiting the growth of cancer cells (Santhosh Kumar et al. 2014) and, in some cases, could reduce tumor size (Nakamura et al. 2003). Clinical trials on cancer patients have been conducted in many Asian countries, showing talented results in reducing tumor size (Wang et al. 2001), improving tolerance for chemotherapy and/or radiation (Zhou et al. 1998), and stimulating the immune system which, hence, enhances the efficiency of chemotherapy (Shin et al. 2003). Crude extract of *C. militaris* showed potent anticancer activity in a xenograft mouse model with RMA cell-derived tumors (Park et al., 2017). Additionally, some *Cordyceps* species have anti-leukemia activities and better suppressive effects of chemotherapy on bone marrow function as a model for cancer treatment (Liu et al. 2008; Wong et al. 2010; Ferreira et al. 2010).

Hypoglycemic and hypocholesterolemic effects

Cordyceps are found to regulate and lower blood sugar levels by improving glucose metabolism and conserving hepatic glycogen (Zhao et al., 2002; El-Hagrassi et al., 2020). *Cordyceps* can increase the secretion of glucokinase and hexokinase, which are glucose-regulating enzymes secreted by the liver (Kim et al., 2017). Polysaccharides are the key player in showing the hypoglycaemic activity of *Cordyceps*. For example, CS-F30, a polysaccharide extracted from *C. sinensis* culture mycelium, has been reported for its promising hypoglycaemic effect (Kiho 1996). Additionally, the plasma glucose level was reduced quickly after intravenous administration of CS-F30 in normal and streptozocin-induced diabetic mice (Kiho 1996). Another polysaccharide (CS-F10) was purified from a hot-water extract of *C. sinensis* cultured mycelia and consists of galactose, glucose, and mannose in a molar ratio of 43:33:24. CS-F10 lowers the plasma glucose level in normal, adrenaline-induced hyperglycaemic, and diabetic mice. Hypercholesterolemia is an indicator of a high risk of cardiovascular attack. El-Hagrassi et al. (2020) reported the role of *C. militaris* in lowering the total cholesterol level and the level of triglycerides. A hot-water extract of *C. sinensis* mycelia has been found to reduce total cholesterol concentration in the serum of mice by reducing LDL (low-density lipoprotein) hypocholesterolemic activities (El-Hagrassi et al., 2020). A hot-water extract of *C. sinensis* mycelia has been found to reduce total cholesterol concentration in the serum of mice by reducing LDL) and very-low-density lipoprotein and increasing good cholesterol concentration (HDL cholesterol) (Koh 2003).

Improving kidney functions and liver disorders

Some clinical trials revealed that the administration of *C. sinensis* could significantly improve kidney function and boost the overall immunity of patients diagnosed with chronic renal failure (Guan et al. 1992). Additionally, treating patients with gentamicin-induced kidney damage helped recover 89% of normal kidney function in a relatively short time (Zhou et al. 1990). The mode of kidney enhancing the action of *Cordyceps* is explained by its ability to protect sodium pump activity of tubular cells, increase 17- ketosteroid and 17-hydroxycorticosteroid levels in the human body, reduce the content of calcium in certain tissues, and accelerate the regeneration of tubular cells (Zhou et al. 1990; Xu et al. 1995; Wang et al. 1998). *Cordyceps* is commonly used for the treatment of chronic hepatitis B and C. Using the antiviral drug, lamivudine, plus mixed extracts of *Cordyceps* with other medicinal mushrooms showed promising results for treating hepatitis B (Wang and Shiao 2000; Ng and Wang, 2005; Zhou et al. 2009). On the other hand, daily consumption of *Cordyceps* improved liver functions in patients who have post-hepatic cirrhosis (Zhu and Liu 1992; Zhou et al. 2009). *Cordyceps* extracts are used to help in the healing of both chronic hepatitis B and C (Wang and Shiao 2000).

***Cordyceps* as antioxidant and antiaging activities**

One of the most potent bioactivities reported for *Cordyceps* extracts is their ability to protect cells from being damaged by free radicals. This activity corresponds to polysaccharide fraction (Yu et al. 2007; Wang et al. 2012). *Cordyceps sinensis* has potent antioxidant and anti-aging properties (Yamaguchi et al., 2000; Ji et al., 2009). Also, many studies elucidated the antioxidant effect of extracts obtained from *C. militaris* (El-Hagrassi et al., 2020). The fruiting bodies extract of *C. militaris* showed strong DPPH radical scavenging activity, which indicated high antioxidant activity. In contrast, the fermented mycelia extract had stronger total antioxidant activity and reduced capability (Dong et al. 2014). *Cordyceps* has been used for centuries as a remedy for weakness and fatigue by residents living in the high mountains of Tibet to give them energy which is achieved by increasing cellular ATP (Holliday et al. 2008). Nowadays, athletes utilize Cordyceps to overcome weakness and fatigue, increase endurance and boost energy levels (Liu et al. 1997). Moreover, clinical trials connecting chronic fatigue with aging patients revealed that treatment with *C. sinensis* improved fatigue and dizziness, increasing cold intolerance and amnesia (Mizuno et al. 1999; Chen et al. 2013; Wu et al. 2014).

***Cordyceps* for organs and glands protection**

Cordyceps sinensis also has clear effects on other organ systems (Chen 1995; Zhang and Yuan 1997; Guo and Guo 2000; Xu 2006). For example, C. sinensis has sedative, anticonvulsant, and cooling effects on the central nervous system. In the respiratory system, *C. sinensis* has a potent relaxant action on bronchi, noticeably increases secretion of adrenaline from the adrenal glands, and participates in tracheal contraction caused by histamine; it also has an expectorant antitussive and anti-asthmatic effects, and it also inhibits pulmonary emphysema. In the endocrine system, *C. sinensis* has effects as a male hormone. Polysaccharides extracted from *Cordyceps* can increase corticosterone levels in plasma. *Cordyceps* has been used in traditional medicine for decades to improve men's fertility. A study has proven the positive effect of using *C. militaris* mycelium on sperm motility, morphology, productivity, and enhancement of sexual activity (Lin et al. 2007). *Cordyceps* extracts contain adenosine, deoxyadenosine, corresponding adenosine type nucleotides, and nucleosides, which help stabilize heartbeat and correct heart arrhythmias (Pelleg and Porter 1990).

***Cordyceps* as anti-inflammatory**

Commonly, the famous cordycepin is the compound causing the anti-inflammatory activity of numerous *Cordyceps* species (Won and Park 2005; Kim et al. 2006; Yang et al. 2011). *C. militaris* ethanolic extracts of cultured fruiting bodies and mycelia exerted an anti-inflammatory activity on the chick embryo chorioallantoic membrane angiogenesis and the croton oil-induced ear edema in mice (Won and Park 2005). On the other hand, an alkaline extract of *C. militaris* showed a strong anti-inflammatory effect against formalin-induced nociception and LPS-induced peritonitis in mice due to containing a potent anti-inflammatory compound (linear b- (1R3)-D-glucan) (Smiderle et al. 2014; Park et al. 2015). Adenosine is also extracted from *Cordyceps* species with numerous activities related to avoiding tissue damage as anti-inflammatory properties (Nakav et al. 2008; Tsai et al. 2010; Liu et al. 2015).

***Cordyceps* as antiviral agents**

Many studies reported that cordycepin extracted from *C. militaris* could inhibit infection with the human immunodeficiency virus (Mueller et al. 1991). Cordycepin has shown antiviral activities against different viruses such as plant viruses and human viruses (influenza virus, Epstein-Barr virus, murine leukemia virus) (Ryu et al. 2014). The mode of killing or inhibiting actions of cordycepin is not fully understood, but several studies have elucidated the ability of cordycepin to inhibit numerous protein kinases (Glazer and Kuo 1977; Jin et al. 2011; Elkhateeb et al. 2019). On the other hand, crude extract of fruiting bodies of *C. militaris* showed promising in-vitro antirotavirus SA-11 agent activities (El-Hagrassi et al., 2020).

***Cordyceps* side effects and safety**

Cordyceps is generally safe in the recommended dosage, and no major side effects were reported. (Das et al. 2010).

CONCLUDING REMARK

Cordyceps and its bioactive components and metabolites are golden mines with therapeutic potential against various fatal diseases. Developing new techniques

capable of cultivating species other than *Cordyceps militaris* can contribute to producing enough quantities of bioactive compounds such as cordycepin and cordycepic acid, which also may lower the cost of this expensive medicinal mushroom. On the other hand, further *in vivo* studies should be conducted in order to evaluate the clinical activities of Cordyceps metabolites which can be a step toward certifying its use as a medical drug.

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Crude extract from a hardcoral-associated bacterium *Virgibacillus salarius* PHC-44-04 inhibiting growth of Multidrug-Resistant *Enterobacter aerogenes* human pathogen

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Abstract. *Ayuningrum D, Muchlissin SI, Trianto A, Radjasa OK, Sabdono A. 2020. Crude extract from a hardcoral-associated bacterium Virgibacillus salarius PHC-44-04 inhibiting growth of Multidrug-Resistant Enterobacter aerogenes human pathogen. Biofarmasi J Nat Prod Biochem 18: 82-87.* Hardcoral-associated bacteria are potential sources of natural product compounds with a wide range of activities, i.e., antibacterial activity, antiviral, anticancer, antifungal, etc. *Virgibacillus salarius* PHC-44-04 is a gram-positive bacterium isolated in prior research from hard coral *Pavona* sp. collected from Panjang Island, Jepara, Indonesia. This bacterium at the screening phase was showing high antibacterial activity against Multidrug-Resistant *Enterobacter aerogenes* (MDR-EA). Therefore, this paper has aim to deliver the result of the crude extract antibacterial test from *V. salarius* PHC-44-04 after being cultivated in a liquid medium, to know the efficiency of using supernatant and pellet extract, and to determine in which concentration was the minimum for antibacterial activity. The cultivation of bacterial isolates was using liquid medium Nutrient Broth, and the production of crude extract was using the liquid-liquid extraction method. The liquid medium containing bacterial cells were separated using a centrifuge at 8,000 rpm and became supernatant and pellet. The supernatant was extracted using ethyl acetate, while the pellet was extracted using methanol. The antibacterial test from both crude extract from supernatant and pellet was using disk diffusion method with several concentration as follows: 15 µg/mL, 30 µg/mL, 60 µg/mL, 90 µg/mL, 180 µg/mL, 250 µg/mL, 350 µg/mL, and 500 µg/mL. Each concentration was repeated in three replicates. The crude extract produced from 700 mL supernatant was 0.0667 g and from 50mL pellets was 0.0320. The pellet crude extract has a higher mass but does not have antibacterial activity against MDR-EA. The result showed an only supernatant crude extract of *V. salarius* showed antibacterial activity against the MDR-EA bacterium. The minimal crude extract concentration to inhibit the growth of MDR-EA was 60 µg/mL. Meanwhile, the best concentration for exhibiting antibacterial activity was 500 µg/mL with a zone of inhibition (ZOI) diameter of 11.77 ± 0.8730 mm. Thus, supernatant should be the main source of crude extract production rather than the pellet to get high antibacterial activity.

Keywords: Antibacterial activity, crude extract, MDR-*Enterobacter aerogenes*, *Pavona*, *Virgibacillus salarius*

INTRODUCTION

Infectious diseases could be transferred from one person to another directly or indirectly by vectors. The causes of infectious disease mostly are pathogens which belong to bacteria, viruses, fungi, and parasites. Those bacteria, because infectious diseases have evolved, have become multidrug-resistant bacteria. This resistance responds to natural selection, mutation, gene transfer, irrational use of antibiotics, etc. (National Institute of Allergy and Infectious Diseases (NIAID) 2011). According to Suwantarat and Carroll (2016), gram-negative bacteria are easier to evolve, becoming Multidrug-Resistant Gram-negative bacteria (MDRGN) with the highest contribution of various cases associated with admission to medical wards, respiratory tract origin, and hospital-onset of infection in Southeast Asia. Multidrug-Resistant *Enterobacter aerogenes* (MDR-EA) is the main source of nosocomial infection, as many as 53% (Cornejo-Juarez et al. 2015), with some diseases such as bacteremia, urinary tract infection, and wound infection (Khan et al. 2015). This kind of Multidrug-Resistant (MDR) bacteria is already resistant to some antibiotics, e.g., imipenem (89.7%), cefoxitin (50.3-74.2%), ciprofloxacin (51.4%) and levofloxacin (54.4%) (Biendo et al. 2008; and Lu et al. 2012). Thus, the search for a new antibiotic candidate is urgently needed.

The exploration of natural products for drug or antibiotic discovery was begun long ago, not starting in 1908 when the first antibiotic was discovered (Silver 2011). Since then, many sources have been explored to find some interesting compounds that have antibacterial activity, not only from land but also from the marine environment, which is considered more unique and has a lot of potential compounds. The source of natural products from marine organisms comes mostly from the invertebrates, i.e., sponges, corals (hard corals and soft

corals), tunicates, nudibranchs, and many more (Ayuningrum et al., 2019; Kristiana et al., 2019). Corals are a source of potential compounds that have a lot of activities as antibacterial, antiviral, anti-inflammatory, cytotoxic, etc. But the direct exploration of the coral organisms and invertebrate needs tons of individuals to yield low amounts of active compounds, which later negatively impact the environment. Thus, many researchers prospect the potential from the symbiont or associated microorganisms.

Bacterial-symbiont in marine invertebrates, especially corals, has become the main source for exploring antibacterial compounds for drug discovery. This culture-dependent method began with isolating bacterial symbionts, screening, liquid cultivation, and extraction and purification of antibacterial compounds. Liquid cultivation and extraction play an important role in the next processes because most bacteria cannot retain the compound after being cultivated from solid to liquid medium. According to prior research, we have successfully isolated a bacterium from a hard coral Pavona sp. According to morphological and molecular characterization, the bacterium was identified as *Virgibacillus salarius* PHC-44-04. The screening process showed that this bacterium has strong activity against MDR-EA. This research aims to know the crude extract antibacterial activity of *V. salarius* PHC-44-4 after cultivation in a liquid medium, know which part of the bacterial culture has antibacterial activity, and estimate the best concentration to inhibit the growth of MDR-EA. That information is important to prepare the scaling-up process of the bacterial culture in order to purify the antibacterial compounds without losing the antibacterial activity from solid to liquid medium.

MATERIALS AND METHODS

Chemical and reagents

Nutrient Broth (Hi-media), Nutrient Agar (Hi-media), Muller Hilton Broth (Hi-media), Muller Hilton Agar (Hi-media), Aquabides (Onemed), Ethyl Acetate (Merck), Methanol (Merck), Dimethyl Sulfoxide (Merck), Paperdisc blank (Advantec, Japan), Paperdisc with Antibiotic for Positive control used chloramphenicol 30 µg/disc (Oxoid), Nitrogen Gas (PT. Samator, Semarang, Indonesia).

Hard coral specimen identification

Hard Coral sampling was conducted in January 2016 at Panjang Island, Jepara, Indonesia. Hard corals were collected using SCUBA gears (SCUBAPRO) at a depth of 3-6 m. Hard corals were placed separately into plastic zip-lock to avoid contact with the environment. Hard corals were identified using Veron's (2000) instructions by observing the shape and structure of corallite using a stereomicroscope. Then, the corallite structure was matched with the guidebook. After that, the specimen was stored in alcohol 96% for long-term preservation.

Bacterial strains and pathogen preparation

Virgibacillus salarius PHC-44-04 was isolated from hard coral *Pavona* sp. from previous research by Ayuningrum et al. (2017). MDR E. aerogenes (MDR-EA) pathogen was obtained from Dr. Karyadi Hospital, Semarang. MDR-EA was cultured a day before antibacterial assay in a solid medium (Muller Hilton Agar). Then, pick 3-4 colonies of MDR and placed in into the sterile physiologic salt solution and measure until they reach 0.5 Mc Farland or density of 1 x 10^8 CFU/mL (Bacteria solution were compared using standard Mc-Farland from Hi-media.

Production of crude extract

The production of crude extract was started by cultivating in liquid culture of 500 mL marine NB. The cultures were shaken at 110 rpm for 3 days at room temperature (29±2°C). The harvest was done by centrifugation to separate the cell from the medium at 6,000 rpm for 10 minutes. The medium was mixed with ethyl acetate (EtOAc) and the cell with methanol (MeOH) in a 1:1 (v/v) ratio. Both suspensions were homogenized by shaking for 15 minutes. Then, the organic layer from the water was separated using a separatory funnel (Pyrex). The organic layer was evaporated using a rotary evaporator (Buchi R-124) at 40°C. Further, the mixture of pellets with MeOH was separated using filter paper. The extraction was collected into the clean vial bottle and concentrated using nitrogen gas.

Antibacterial test of crude extract

The antibacterial test against MDR-EA was conducted using the disk diffusion method (Yoghiapiscessa et al., 2016). Some concentrations used in this test were consisting of 15 µg/mL, 30 µg/mL, 60 µg/mL, 90 µg/mL, 180 µg/mL, 250 µg/mL, 350 µg/mL, dan 500 µg/mL with three replications of each concentration. The MDR-EA bacterium was refreshed a day before, then measured to be 0,5 McFarland, and after that, being swapped onto *Muller Hinton Agar* (MHA) medium. As many as 30 µl extracts of each concentration were dropped onto each paper disc and placed on the MHA medium containing swapped MDR-EA bacterium. This experiment used Chloramphenicol 30 µg/disc as the positive control and EtOAc or MeOH as the negative control, depending on which solvent was extracted. The plates were incubated at 37°C overnight and observed in the clear zone. All experiment measurement data were performed with Vernier Caliper from Tricle Brand in three replicates and expressed as mean ± SD (n=3).

RESULTS AND DISCUSSION

Identification of hard coral

Hard coral specimens from Panjang Island, Jepara, have been identified as *Pavona* sp. (Figure 1). Colonies in the form of short sheets, thick and upright. Corallites were present on both surfaces, irregular, small without walls, and interconnected septocostals. Under watercolor was light brown or grayish.

According to the coral identification book *The Indo-Pacific Coral Finder* (Kelley 2009) and the website *Corals*

of the Worlds (coral.aims.gov.au), the coral specimen was identified as *Pavona* sp. The characterization of *Pavona* sp. was already described in Suharsono (2008) showed that the body of coral has greenish-brown color and shape of the plate. The zooxanthellae microsymbiont was responsible for the color. The growth of coral colonies was massive and encrusting. Corallite does not have a clear wall; present on both surfaces, irregular, small, and without a wall. Septocostae within the close corallite is united and developed well to become a dominant appearance.

This genus of coral is found in Panjang Island waters, in the dept of 0.5 up to 7 meters. The other coral families grow in these waters, according to Munasik et al. (2012), including 25 genera, which belong to 11 different families. Those families consist of Acroporidae, Agariciidae, Dendrophyllidae, Faviidae, Fungiidae, Merulinidae, Muscidae, Oculinidae, Ectinidae, Pociloporidae, and Poritidae. The genus *Pavona* identified in this research belongs to the family Agaricidae. The classification of *Pavona* sp. (Hoeksema 2015) is as follows, Kingdom: Animalia, Phylum: Cnidaria, Class: Anthozoa, Subclass: Hexacorallia, Order: Scleractinia, Family: Agariciidae, Genus: *Pavona,* and Species: *Pavona* sp.

Characterization of *Virgibacillus salarius* PHC-44-04

Hard corals harbor various microbiomes as a consortium for providing any chemicals for the host's growth, development, and protection (Rosenberg and Gobna, 2011; Kuang et al., 2015;). Initiation of hard coral-associated bacteria depends on its reproductive strategies; the associated bacteria can move vertically (during reproduction) or horizontally through the water column at each stage of coral life, passively moving through the water column, carried away by the current, and suspended sediments from benthos (Sweet et al. 2010; Rosenberg and Gobna 2011). That makes the different compositions of microbial communities in the mucus, tissue, and skeletal parts.

We previously described a *Pavona* sp.-associated bacterium that has a similarity with *Virgibacillus salarius* (Ayuningrum et al. 2017). The bacterium colony was white in color and shape round with medium size (Figure 2), which was expected to have a strong antibacterial compound.

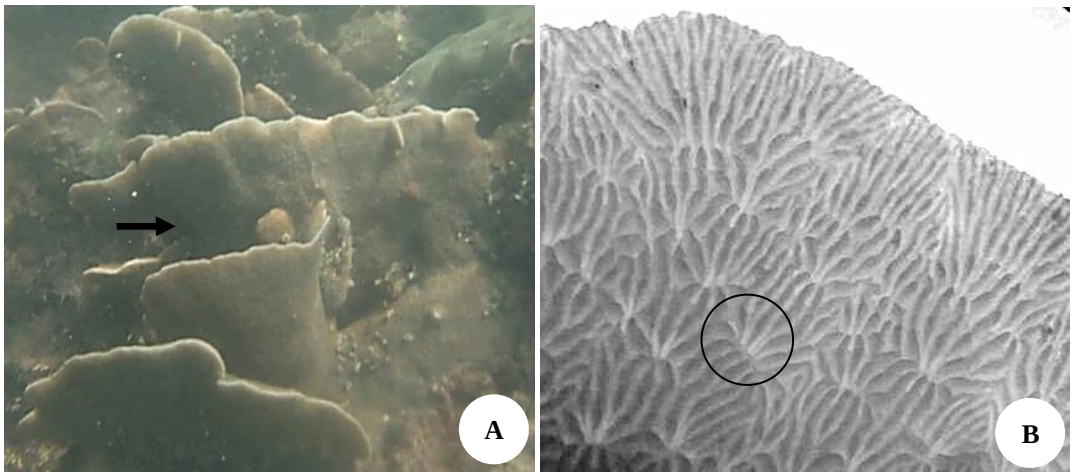

Figure 1. A. The underwater photography or hard coral *Pavona* sp., the colony of hard coral (black arrow), B. Corralite (black circle) observation using a stereo microscope

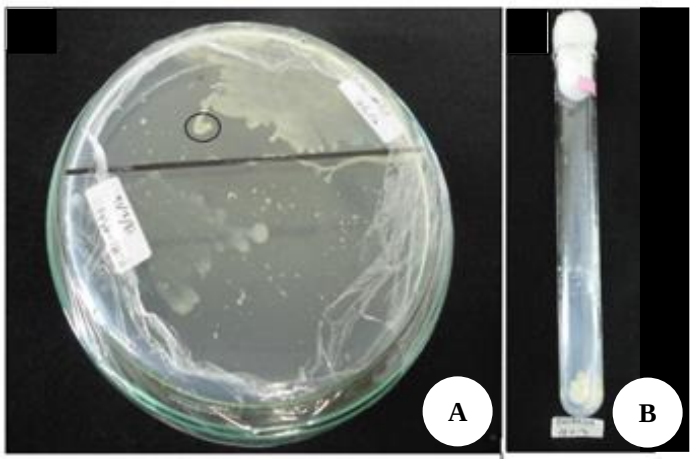

Figure 2. A. The morphology of *Virgibacillus salarius* PHC-44-04 colony (black circle), B. The preservation of the strain in agar slant

Production of crude extract

The supernatant crude extract had a mass of 0,0667 g, and the pellet crude extract had a mass of 0.0320 g (Table 1). The results showed that the pellet contains more polar compounds, which is different from the supernatant, which contains fewer polar compounds. Nevertheless, both extracts had the same paste forms and greenish-yellow colors.

The extraction method influences the compounds inside the crude extract. The use of different solvents has the purpose of determining the solvent's effectiveness in extracting the compounds inside the supernatant and pellet. Ethyl acetate solvent (polarity index 4.4) is used to extract semi-polar and volatile compounds because this solvent can dissolve semi-polar compounds, i.e., flavonoid aglycone, non-toxic, and hygroscopic (Harborne, 1987). Moreover, ethyl acetate is able to dissolve compounds that have antibacterial activity, such as flavonoids and polyhydroxy phenolics (Wardhani and Sulistyani 2012). Meanwhile, the use of methanol to extract the compounds in the pellets is because methanol (polarity index 5.1) can bind all the chemical components that are more polar than ethyl acetate. The volume of supernatant to solvent has a ratio of 1: 1; this reason was to minimize the saturation level of solvent in binding to the bioactive compounds inside the supernatant (Yoghiapiscessa et al. 2016). The temperature remained low during the evaporation process to keep the active compounds in the crude extract.

Antibacterial assay

After being extracted, a total of 8 concentrations of crude extracts, both supernatant, and pellet, were tested against MDR-EA. All concentrations of supernatant crude extracts demonstrated inhibitory activities against MDR-AB; in contrast, the pellet crude extracts showed no activity (Table 2, Figure 3). The lowest activity is at a concentration of 15 µg/mL with a mean inhibition zone of 9.57 mm. The largest inhibition zone was 11.77 mm in the highest 500 µg/mL concentration, which means the higher the crude extract concentration, the bigger the inhibition zone. The inhibition zones of crude extracts against MDR-AB are shown in Figure 3.

The antibacterial activity test in this research uses Muller Hinton Agar (MHA) as the medium because it is recommended by the Food and Drug Association (FDA) and the World Health Organization (WHO) to test the antibacterial activity mainly for aerobic bacteria and facultative anaerobic bacteria. This medium contains a low sulfonamide concentration, trimethoprim, and tetracycline inhibitors and provides optimal pathogen growth. Furthermore, giving the negative control (solvent) in the antibacterial test function to determine if the negative control shows a positive result, the extract is toxic to the bacteria. To choose the positive control should be concerned about the sensitivity of the antibiotic against MDR-AB which in this research, we use chloramphenicol 30 µg/disc. The largest inhibition zone was 11.77 mm in the highest 500 µg/mL concentration. This different result found in pellets extract showed no activity at all. It showed that only the supernatant extract has activity against MDR-AB. This means that the active compounds in bacterial secondary metabolites are secreted from the cell. This is confirmed by Kristiana et al. (2020); compounds secreted by bacteria must cause the antibacterial activity of supernatant. So, when centrifuged, it is separated between the active compounds remaining in the pellet and primary metabolites such as protein in the cells (pellets).

Table 1. The production of crude extract *Virgibacillus salarius* PHC-44-04

	Culture volume (mL)	Solvent volume (mL)	Mass of crude extract (g)	Form	Colour
Supernatant	700	700	0.0667	Paste	Greenish-yellow
Pellet	50	50	0.0320	Paste	Greenish-yellow

Table 2. The inhibition zone of crude extract *Virgibacillus salarius* strain PHC-44-04 against MDR-EA

Concentration (µg/mL)		Diameter of Inhibition zone (mm)	
		Supernatant	Pellet
	15	9.57 ± 0.4497	8 ± 0.0000
	30	9.60 ± 0.2944	8 ± 0.0000
	60	10.12 ± 0.6575	8 ± 0.0000
	90	10.53 ± 0.8576	8 ± 0.0000
	150	10.63 ± 0.4497	8 ± 0.0000
	250	10.70 ± 0.2160	8 ± 0.0000
	350	10.84 ± 0.6556	8 ± 0.0000
	500	11.77 ± 0.8730	8 ± 0.0000
Control (+)	Chloramphenicol	21.63 ± 0.6650	21.85±0.8879
Control (-)	Ethyl acetate	8.00 ± 0.0000	-
	Methanol	-	8.00 ± 0.0000

Note: the disc diameter was 8mm

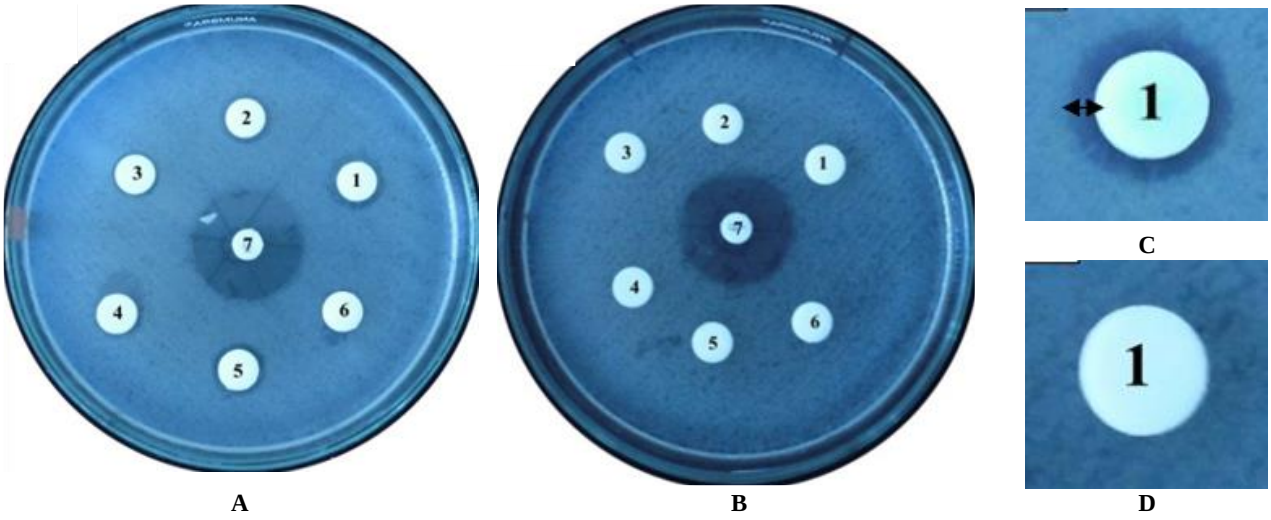

Figure 3. The antibacterial activity of supernatant (A) and pelet (B) showing inhibition zones against MDR-EA. (C) Close-up of disc containing supernatant extracts with concentration of 15 µg/mL inhibition zone of 9,57 mm. (D) Close-up of disc containing no inhibition zone. Number 1: 15µg/mL, 2: 30µg/mL, 3: 60µg/mL, 4: 90µg/mL, 5: 150µg/mL, 6: 250µg/mL. 7: Positive control (antibiotics)

The test results indicate that the concentration of each extract gives different sizes in the zone of inhibition. The higher concentration of crude extract, the greater zone of inhibition formed. This finding also supports the previous reports by Gonemali et al. (2018) and Bhalodia and Shukla (2011). Besides, the type of tested bacteria also influences the diameter of the inhibition zone. This is related to bacteria's enzymes or other substances, giving different effects to the active substance contained in isolate PHC-44/04. This enzyme can be toxic to antibacterial agents, thereby inactivating the active substance by destroying or damaging the active substance (Ramadhan et al., 2015). MDR *E. aerogenes* is a bacterium with complex resistance mechanisms such as the enzyme beta-lactamase, which can damage the active side of antibiotics, and have an efflux pump (Dzidic et al. 2008 and Nikaido 2009. This is what causes the small zone of inhibition to form. The other factors that affect the diameter of the inhibition zone are the disc's ability to absorb extract, pH of the medium, the reaction between active ingredients with medium, incubation temperature, etc.

In conclusion, to retain the antibacterial activity from solid medium to liquid, bacterial isolate should be cultivated in seed culture before moving to big-scale culture. The only supernatant crude extract shows antibacterial activity therefore to get antibacterial compounds, liquid-liquid extraction should be conducted from the cultivation medium, not the bacterial cell. Supernatant crude extract of *V. salarius* has maximum activity against MDR-EA activity with a minimal concentration of 60 µg/mL.

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