

# 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. 2018. 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: 78-83. Hardcoral-associated bacteria are potential sources of natural product compounds with wide range of activities, i.e. antibacterial activity, antiviral, anticancer, antifungal, etc. *Virgibacillus salarius* PHC-44-04 is a gram-positive bacterium that was 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 crude extract antibacterial test from *V. salarius* PHC-44-04 after being cultivated in 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 liquid-liquid extraction method. The liquid medium containing bacterial cell was separated using centrifuge with 8000 rpm 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 pellet was 0.0320. The pellet crude extract has higher mass but not having antibacterial activity against MDR-EA. The result showed only supernatant crude extract of *V. salarius* showed antibacterial activity against 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 at 500 µg/mL with zone of inhibition (ZOI) diameter of  $11.77 \pm 0.8730$  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 are diseases, which could be transferred from one person to another directly, or indirectly by vectors. The causes of infectious disease mostly are pathogen which belongs to bacteria, viruses, fungi, and parasite. Those bacteria because infectious diseases have been evolved become multidrug-resistant bacteria. This resistance is the response of natural selection, mutation, gene transfer, irrational use of antibiotics, etc. (National Institute of Allergy and Infectious Diseases (NIAID) 2011). According to Suwantar and Carroll (2016), gram-negative bacteria are easier to evolve becoming Multidrug-Resistant Gram-negative bacteria (MDRGN) with 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 already resistant to some kind of 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 the new antibiotic candidate is urgently needed.

The exploration of natural products for drug or antibiotic discovery has been begun long ago by 1908 when the first antibiotic was discovered (Silver 2011). Since then, a lot of sources have been explored to find some interesting compounds which have antibacterial activity, not only from land but also from marine environment which considered more unique and has a lot of potential compounds. The source of natural product from marine organisms are coming mostly from the

invertebrate i.e. sponges, corals (hard corals and soft corals), tunicates, nudibranch 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 an antibacterial, antiviral, anti-inflammatory, cytotoxic, etc. But the direct exploration from the coral organisms as well as invertebrate needs tons of individuals to yield low amount of active compounds, which later give negative impact on the environment. Thus, many researchers prospect the potential from the symbiont or associated microorganisms.

Bacterial-symbiont in the marine invertebrate especially corals has become the main source for the exploration of antibacterial compounds for drug discovery. This culture-dependent method began with isolation of bacterial symbionts, screening, liquid cultivation and extraction and purification of antibacterial compounds. Liquid cultivation and extraction play an important role for the next processes, because most bacteria cannot retain the compound after being cultivated from solid to liquid medium. According to the 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 cultivated in liquid medium, to know which part of the bacterial culture has antibacterial activity and to 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 environment. Hard corals were identified using Veron (2000) instructions by observing the shape and structure of corallite using stereomicroscope. Then, the structure of the corallite was being matched with the guide book. After that, the specimen was being stored into 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). The pathogen of MDR *E. aerogenes* (MDR-EA) was obtained from Dr. Karyadi Hospital, Semarang. MDR-EA was cultured a day before antibacterial assay in solid medium (Muller Hilton Agar). Then, pick 3-4 colonies of MDR and placed in to the sterile physiologic salt solution and measure until reach 0.5 McFarland or density of  $1 \times 10^8$  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 ( $29 \pm 2^\circ\text{C}$ ). The harvest was done by centrifugation to separate the cell from the medium at 6000 rpm as long as 10 minutes. The medium was mixed with ethyl acetate (EtOAc) and the cell with methanol (MeOH) in 1:1 (v/v) ratio. Both suspensions were homogenized by shaking for 15 minutes, then separated the organic layer form water using separatory funnel (Pyrex). The organic layer was evaporated using rotary evaporator (Buchi R-124) at  $40^\circ\text{C}$ . Further, the mixture of pellets with MeOH was separated using filter paper. The extraction result was collected in to the clean vial bottle and being concentrated using nitrogen gas.

### Antibacterial test of crude extract

The antibacterial test against MDR-EA was conducted using 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 on to *Muller Hinton Agar* (MHA) medium. As many as 30 µl extracts of each concentration were dropped on to each paper disc and those being placed on the MHA medium containing swapped MDR-EA bacterium. This experiment was using Chloramphenicol 30 µg/disc as positive control and EtOAc or MeOH as the negative control depending on which solvent used for extraction. The plates were incubated at  $37^\circ\text{C}$  for overnight and observed the clear zone. All experiment measurement data were performed with Vernier Caliper from Tricle Brand in three replicates and expressed as mean  $\pm$  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 septocostals are interconnected. Under watercolor was light brown or grayish.

According to the coral identification book *The Indo Pacific Coral Finder* (Kelley 2009) and 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 plate. The zooxanthellae microsymbliont was responsible for the color. The growth of coral colonies was massive and encrusting. Corallite does not have clear wall, present on both surfaces, irregular, small, and without wall. Septocostae within the close corallite is united with each other and developed well become dominant appearance.

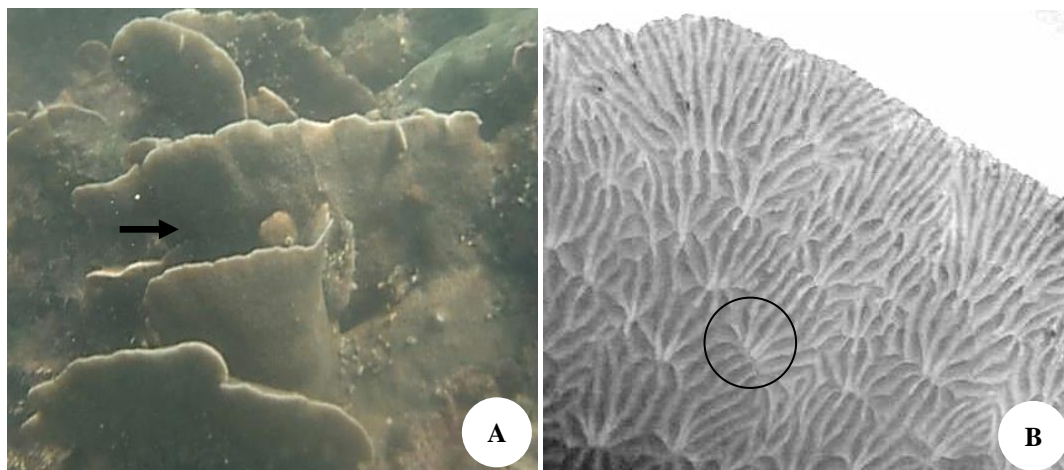
This genus of coral is found in Panjang Island waters, in the dept of 0.5 up to 7 meters. The other coral families grow in these waters according to Munasik et al. (2012) including 25 genera, which belong to 11 different families. Those families consist of Acroporidae, Agariciidae, Dendrophyllidae, Faviidae, Fungiidae, Merulinidae, Mussidae, Oculinidae, Ectinidae, Pociloporidae, and Poritidae. The genus *Pavona* that was identified in this research belongs to family Agariciidae. 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.

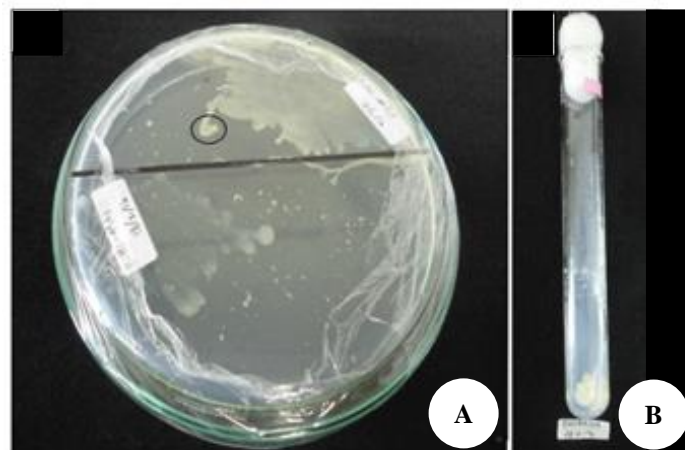
#### Characterization of *Virgibacillus salarius* PHC-44-04

Hard corals harbor various microbiome as a consortium for providing any chemicals for growth, development, and also protection of the host (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 composition of microbial communities in the mucus, tissue, and skeletal parts.

We previously described a *Pavona* sp.-associated bacterium 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 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 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 mass, 0,0667 g, and pellet crude extract had mass 0.0320 g (Table 1). The results showed that pellet contains more polar compounds, which different from supernatant that contains less 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 purpose to determine the effectiveness of the solvent to extract the compounds inside the supernatant and pellet. Ethyl acetate solvent (polarity index 4.4) use 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 able to dissolve compounds that have antibacterial activity such as flavonoids and polyhydroxy phenolic (Wardhani and Sulistyani 2012). Meanwhile, the use of methanol to extract the compounds in the pellets is because methanol (polarity index 5.1) able to bind all the chemical components which 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 supernatant (Yoghiapiscessa et al. 2016). The temperature was remaining low in evaporation process has purpose to keep the active compounds stay 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 with pellet crude extracts showed no activity (Table 2, Figure 3). The lowest activity is at concentration of 15 µg/mL with a mean inhibition zone 9.57 mm and the largest inhibition zone was 11.77 mm in the highest concentration of 500 µg/mL, which means the higher crude extract concentration, the bigger inhibition zone. The inhibition zones of crude extracts against MDR-AB were shown in Figure 3.

Antibacterial activity test in this research using 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 low concentration of sulfonamide, 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 concern 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 concentration of 500 µg/mL. 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 secreted out of the cell. This confirmed by Kristiana et al. (2020), the antibacterial activity of supernatant must be caused by compounds that are secreted by bacteria. So, when centrifuged to be separated between the active compounds remaining in the pellet and primary metabolites such as protein in the cells (pellets).

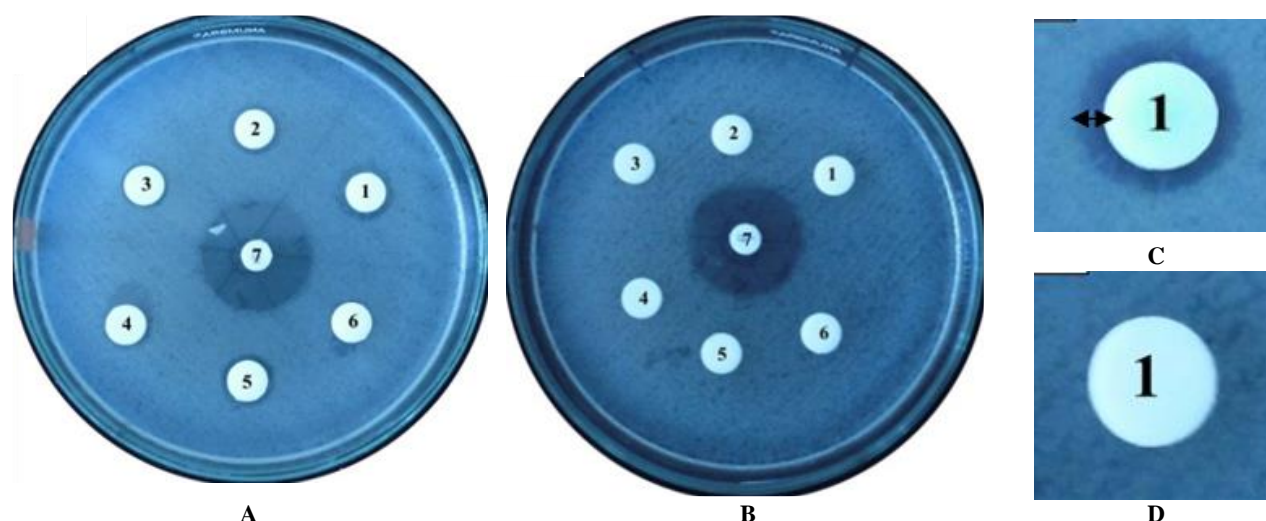
**Table 1.** The production of crude extract *Virgibacillus salarius* PHC-44-04

	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 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 gives influences to the diameter of inhibition zone. This is related to the enzymes or other substances produced by bacteria which then give 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 damage the active substance (Ramadhan et al. 2015). MDR *E. aerogenes* is bacterium that has 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 formed. The other factors that affect the diameter of inhibition zone are disc ability to absorb extract, pH of the medium, reaction between active ingredients with medium, incubation temperature, and so on.

In conclusion, to retain the antibacterial activity from solid medium to liquid, bacterial isolate should be cultivated in seed culture before moved to big scale culture. 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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