Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 25(3): 147 – 157 (2021)
www.ejabf.journals.ekb.eg

Antibacterial Activity of Carotenoid from Bacterial Symbiont *Virgibacillus salarius* Strain 19.PP.Sc.1.6 against MDR *E. coli* and MRSA

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ARTICLE INFO
Article History:
Received: April 17, 2021
Accepted: April 30, 2021
Online: May 25, 2021

Keywords:
antibacterial,
carotenoids,
MDR *E. coli*,
MRSA,
symbionts bacteria.

ABSTRACT

Multi-Drug Resistant (MDR) *E. coli* and Methicillin- Resistant *Staphylococcus Aureus* (MRSA) are resistant bacteria and cause infection. Compounds that have the potential to be antibacterial are carotenoids produced by bacteria associated with soft corals such as *Sinularia* sp. This study aims to determine the potential of carotenoid bacteria symbionts *Virgibacillus salarius* strain 19.PP.Sc1.6 soft coral *Sinularia* sp. against the growth of MDR *E. coli* and MRSA bacteria with concentrations of 4, 6, and 8%. The carotenoid from symbiont bacteria was extracted by the maceration method using methanol as solvent. The results of the screening test showed that the average clear zone diameter for MDR *E. coli* antibacterial test at 4% concentration was 0.770 cm, 6% 0.818 cm, and 8% 0.915 cm with positive control 1.924 cm. The results of the antibacterial test against MRSA had an average diameter at a concentration of 4% of 1.218 cm, a concentration of 6% 1.318 cm, and a concentration of 8% 1.405 cm, and positive control of 2.109 cm. The results of statistical analysis showed that there were significant differences between the concentration groups and between the carotenoid pigment groups and the positive control. Based on the results of the study, it was concluded that the carotenoid pigment of the symbiont bacteria *V.salarius* strain 19 PP.Sc 1.6 has an antibacterial activity for MDR *E. coli* and MRSA growth

INTRODUCTION

Infectious disease is a disease caused by pathogenic microbes **(Janeway *et al*, 2001)**. One of the causes of infectious diseases is bacteria **(Doron and Gorbach, 2008)**. The medicine to treat bacterial infections is antibiotics. Irrational use of drugs in

antibiotics can cause resistance. The inaccuracy in choosing the type of antibiotic to the way and duration of administration is an example of the irrational use of antibiotics that causes bacterial resistance **(Ventola, 2015)**.

Bacterial resistance to various antibiotics has been widely reported. *Escherichia coli* and *Staphylococcus aureus* are examples of bacteria that are resistant. Multi Drug Resistant (MDR) *E.coli* is *E.coli* bacteria that have experienced resistance to ≥ 3 antibiotics **(Magiorakos *et al.*, 2012)**. MDR *E. coli* bacteria are resistant to ampicillin, clavulanate amoxicillin, and cefixime antibiotics. *S.aureus* bacteria that have been resistant to methicillin and β-lactam antibiotics are called MRSA (Methicillin-Resistant *S.aureus*) **(Stapleton and Taylor, 2002)**. MDR *E.coli* bacteria can cause diseases of the digestive tract such as diarrhea **(Monira *et al.*, 2017)**. MRSA bacteria can cause infectious diseases of the skin such as eczema **(Gillespie *et al.*, 2008)**. These bacteria are also the cause of nosocomial infections **(Khan *et al.*, 2017)**. Nosocomial infection or also known as Hospital Acquired Infection (HAI) is an infection that is acquired and develops while the patient is hospitalized **(Monegro, 2021)**.

One of the ingredients derived from nature that can be used as an alternative in the treatment of infection that is environmentally friendly and does not endanger environmental conservation is symbiont bacteria **(Hunt dan Vincent, 2006)**. Marine biota symbiont bacteria, namely microorganisms that live together with other living things, can actually produce secondary metabolites that are similar to their host **(Proksch *et al.*, 2002).** There are a variety of bioactive compounds produced by secondary metabolites produced by marine organisms such as alkaloids, terpenoids, polysaccharide sulfates, peptides, pigments, and newly discovered chemical compounds. **(Venugopal, 2009; Pangestuti and Se-Kwon Kim, 2011)**.

Marine organisms that have antibacterial activity against pathogenic bacteria are symbiont bacteria associated with *Sinularia* sp. **(Sulistiyani, 2010)**. *Sinularia* sp. produce secondary metabolites that can be used as antibacterials **(Tanod *et al,* 2018)**. One type of compound produced by *Sinularia* sp. is a carotenoid pigment **(Wagner, 1999)**. According to research conducted by **Wiguna *et al.*, (2015)**, carotenoids produced from soft coral symbionts can be used as antibacterial.

The purpose of this study is to determine the antibacterial activity of the carotenoid bacteria symbiont *Sinularia* sp. Especially against bacteria that have experienced resistance, namely MRSA and MDR *E.coli*.

MATERIALS AND METHODS

Sampling

The sampling method used in this research was purposive method; samples of *Silunaria* sp. were taken from Panjang Island. Sample *Sinularia* sp. taken from a depth of about 2 m. Samples were taken at coordinate points 6°34’37.35”S 110°37’52.01”E and

6°34'41.38"S 110°37'54.12"E in Panjang Island (**Fig. 1**). Sampling was conducted on January 19, 2019. Colony of *Silunaria* sp. taken with a size of 3-5 cm put in a plastic container filled with sterile sea water and then temporarily stored in a coolbox **(Kusmita *et al.* 2017; 2021**).

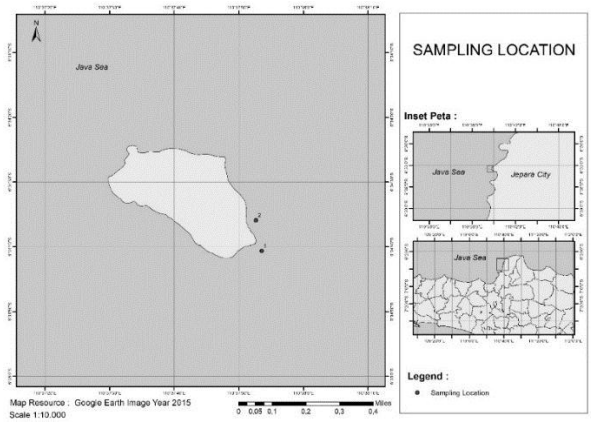


Fig 1. Sampling location of *Sinularia* sp. from Panjang Island

Isolation of bacterial symbiont of *Sinularia* sp.

The isolation of microorganisms associated with soft corals was carried out by the distribution method. Sample *Sinularia* sp. as the results of the sampling were washed with sterile sea water. After that, the sample was cut and crushed. The soft corals that had been crushed were put into petri dishes which partly contain sterile sea water. The sample that has been diluted with sterile water is then carried out a series of dilutions. Dilution was carried out by taking 10 ml of each sample with a sterile pipette, then putting it in an erlemeyer flask containing 90 ml of sterile sea water and a sample dilution of 10^{-1} would be obtained. Samples that had been carried out with 10^{-1} dilution were taken 1 ml of the sample with a sterile pipette and put into a test tube containing 9 ml of sterile sea water and a 10^{-2} dilution would be obtained. This method was also used to obtain a sample dilution of 10^{-3}; 10^{-4}; and 10^{-5}. Each dilution series that had been obtained was 1 ml sample and put into a sterile petri dish poured with Zobell 2216E agar media. The petri dishes were then incubated at 30^{o}C for 1-2 days.

Bacterial Culture

Bacterial culture aims to multiply bacteria which would be used for the next process. Pure bacteria were taken with a round loop and then planted in 5 ml of sterile liquid Zobell media in a test tube then shaken for 24 hours. After 24 hours, the bacterial

culture was mixed into 45 ml of sterile liquid Zobell media, then shaken again for 2 x 24 hours. Then the second culture was mixed again into 450 ml of new sterile liquid Zobell media and shaken for 3 x 24 hours. The culture was made as many as 8 replications so that the final result was 4 liters. After that the bacteria were separated from the liquid media using a centrifuge. Then the bacterial pellets (sediment) were taken and put into a vial for the extraction process.

Extraction of symbiont bacteria pigments *V. salarius* strain 19.PP.Sc1.6

Extraction was carried out using cold methanol **(Kusmita *et al.*, 2021)**. Pellets were extracted until the bacteria become colorless or pale, which indicates the carotenoid has been completely removed. The extract obtained was then separated from the pellets with a centrifuge at 6500 rpm for 10 minutes, then the extraction was filtered then exposed to nitrogen gas to dry to remove the solvent.

Moleculer identification

Based on previous research, bacteria were moleculer identification using PCR, sequencing, and phylogenetic trees. The results showed that the type of bacteria obtained was *V. salarius* strain 19.PP.Sc1.6 **(Kusmita *et al.*, 2001).**

Bacterial Resistance Test of MDR *E.coli* and MRSA

The resistance test was carried out to prove that the bacteria used for the test were bacteria that had experienced resistance. *E.coli* bacteria resistance test uses the antibiotic amoxicillin clavulanate, cefixime, and ampicillin. And the antibiotics used for MRSA resistance tests were chloramphenicol, tetracycline, and cefixime.

Antibacterial Activity Test

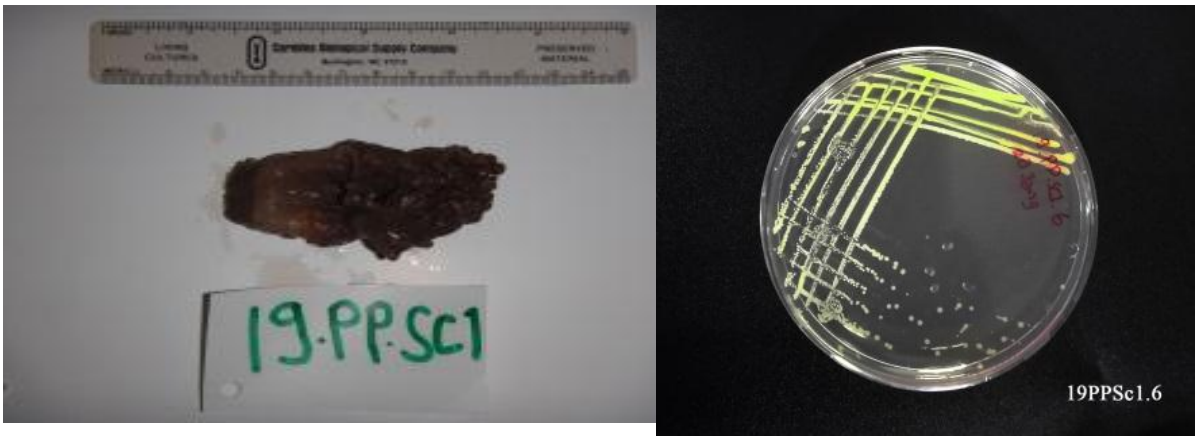
Media of Manitol Salt Agar (MSA) or sterile liquid Eosyn Methylene Blue Agar (EMB) was put into a sterile 30 ml petri dish consisting of 10 ml of MSA or EMB as the first layer, allowed to solidify at room temperature. Inserted 5 cylinder cups were placed on the mark that had been made. A total of 20 ml of MSA or EMB mixed with 50 µl of bacterial suspension was poured into the erlenmeyer, shaken then put into a petri dish as a second layer. The media was allowed to solidify then the cylinder cup was taken out.

The carotenoid pigment extract with various concentrations was dissolved with DMSO solvent. Each pigment extract was inserted into the well as much as 50 µl. The positive control used was ciprofloxacin and the negative control used was DMSO. The

petri dishes containing the media were incubated at 37^{o}C for 24 hours. The presence of clear areas on the media surrounding each extract indicated antibacterial activity against the growth of MDR *E. coli* and MRSA bacteria. The diameter of the resulting resistance area was measured using a caliper.

RESULTS

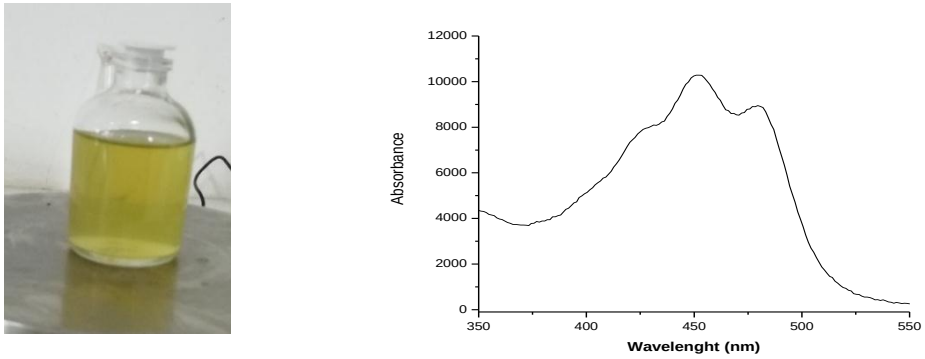
Soft coral *Sinularia* sp. taken from Panjang island, Jepara Regency, Central Java, Indonesia. The samples were isolated from the symbiont bacteria *V.salarius* 19.PP.Sc1.6 (**Fig. 2**.)



(a) (b)

Fig. 2. *Sinularia* sp (a) and isolate of the symbiont bacteria *V.salarius* strain 19.PP.Sc.1.6

Carotenoid producing bacteria *V.salarius* strain 19.PP.Sc1.6. The carotenoid extract of the symbiont bacterium *V.salarius* strain 19.PP.Sc1.6 is shown in **Fig. 3a.** and the spectrum pattern of the extract is shown in **Fig. 3b.**



(a) (b)

Fig. 3. Carotenoid extract (a) and spectrum pattern of carotenoid extract (b) symbiont bacteria *V.salarius* strain 19.PP.Sc1.6

The bacterial resistance test to be tested was carried out on MDR *E. coli* and MRSA bacteria shown in **Fig 4.**

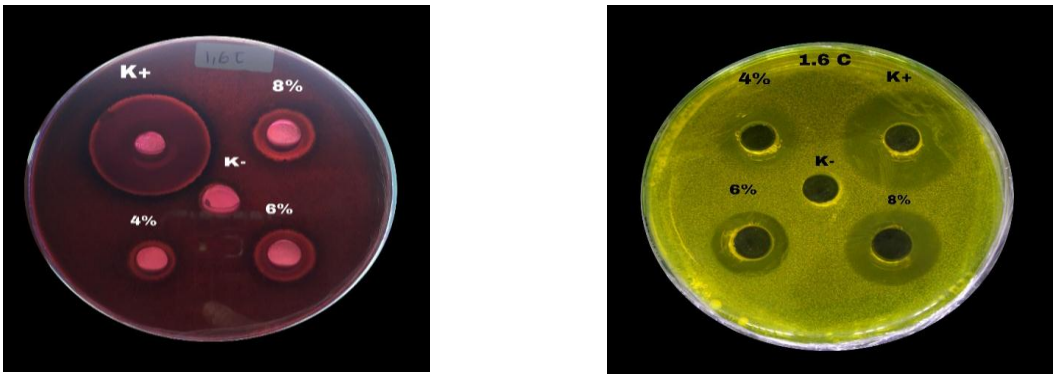
(a) (b)

Fig. 4. The results of the MDR *E. coli* (a) and MRSA (b) bacterial resistance test

The results of the antibacterial activity test of the carotenoid extract of the symbiont bacteria *V.salarius* strain 19.PP.Sc1.6 against MDR *E. coli* and MRSA are shown in **Table 1** and **Fig. 5.**

Table 1. Antibacterial activity of extract carotenoid *V. salarius* strain 19 PP.Sc1.6 against of MDR *E. coli* and MRSA

No	Concentration (%)	Inhibition Zone (cm)	
		MDR *E. coli*	**MRSA**
1	4	0.770±0.016	1.218±0.005
2	6	0.818±0.004	1.318±0.006
3	8	0.915±0.011	1.405±0.008



(a) **(b)**

Fig. 5. Inhibition Test Results for Carotenoid Pigments in Symbionts 19.PP.Sc 1.6 against MDR *E. coli* (a) and MRSA (b)

Description:
4%: Concentration of carotenoid pigments 4%
6%: Concentration of carotenoid pigments 6%
8%: Concentration of carotenoid pigments 8%
K +: Ciprofloxacin
K-: DMSO

DISCUSSION

V. salarius strain 19.PP.Sc1.6 is an sample of bacterial symbiont associated with a softcoral *Sinularia* sp. **(Kusmita, *et al.*, 2021)**. *Sinularia* sp is a soft coral class Cnidaria, phylum Alcyonaria from the family Alcyoniidae. Taxonomic identification is based largely on examination of the spicules, and sufficiently reliable taxonomic keys are available to guide species identification **(Manuputty, 2016).**

These bacteria produce carotenoid pigments which are indicated by their yellow color. **Gross (1991)** states that carotenoid pigments have color absorption from yellow, red to orange. To ensure that the extract obtained is a carotenoid, the spectral pattern is measured using UV-Vis spectrophotometry. Based on the measurement results showed that the bacterial extract of the symbiont *V.salarius* strain 19.PP.Sc.1.6 has a pattern and absorption of carotenoids. Carotenoids have an absorption pattern of 3 (three) peaks **(Rodriguez Amaya, 2001)** and their absorption at a wavelength of 300-600 nm **(Gross, 1991).**

In previous research, the biological activity of the carotenoid bacteria symbiont *V.salarius* strain 19.PP.Sc1.6 was as an antioxidant and sunscreen. Carotenoid extract from these bacteria has the highest activity compared to carotenoid extracts from other bacteria **(Kusmita, *et al.*, 2021)**. In this research, activity testing will be carried out against bacteria that have experienced resistance

The resistance test is a test carried out to determine the sensitivity of bacteria to antibiotics **(Ventola, 2015)**. The results of the resistance test showed that the bacteria used for the test were bacteria that were already resistant, as evidenced by the absence of an inhibition zone. Thus, the two bacteria that will be used for testing are bacteria that have experienced resistance to several antibiotics.

Based on the results of antibacterial tests against MDR *E. coli* bacteria for a concentration of 4% had an average clear zone of 0.770 cm, at a concentration of 6% of 0.818 cm, and at a concentration of 8% of 0.915 cm. In the antibacterial test against MRSA a concentration of 4% produced a clear zone of 1.218 cm, for a 6% concentration of 1.318 cm, and at a concentration of 8% of 1.405 cm. The concentration of carotenoid pigment samples from the symbiont bacteria *V.salarius* strain 19.PP.Sc1.6 was higher, the resulting inhibition zone also increased. The higher the sample concentration, the larger the compounds that diffuse into the planted bacteria will further inhibit the growth of MDR *E. coli* and MRSA bacteria.

The ability of the carotenoid sample of the symbiont bacteria *V.salarius* strain 19.PP.Sc1.6 to inhibit the growth of MDR *E. coli* and MRSA bacteria is due to carotenoids which are a group of terpenoids **(Stephen *et al.*, 2016)**. According to **Radjasa *et al.*(2009)**, carotenoids are compounds that can be used as antibacterials. The carotenoid mechanism in inhibiting bacterial growth reacts with porin (trans membrane

protein) on the outer membrane of the bacterial cell wall and then forms a strong polymer bond that results in the destruction of porin. Where porin is the way out of nutrients for bacteria. So that if porin is damaged, the supply of nutrients for bacterial growth will not exist and will cause the bacteria not to grow and then die **(Cowan, 1999).**

The antibacterial activity of MRSA bacteria was greater than that of MDR *E. coli*. These results are because the MDR *E. coli* bacteria have a cell wall structure that is more complex than the MRSA bacteria. MDR *E. coli* bacteria are gram-negative bacteria that are resistant to several antibacterials this is due to the three layers of cell walls in these bacteria, so that some compounds are unable to damage the tissue from the cell walls of *E. coli* bacteria. **(Jawetz *et al*., 2007)**. The cell wall of gram-negative bacteria contains three polymers, namely the outer lipoprotein layer, the lipopolysaccharide middle layer, the peptidoglycan inner layer, and the outer membrane in the form of a bilayer (having better resistance to compounds that enter or leave the cell and cause toxic effects). According to **Alberts (2002),** the cell wall that denatures the most easily is the cell wall composed of polysaccharides compared to the cell wall composed of phospholipids. MRSA bacteria, which are gram-positive cell wall bacteria, contain peptidoglycan, teichoic acid, and teikuronic acid. According to **Silhavy *et al*. (2010)**, teichoic acid as a constituent of gram-positive bacterial cell walls is a water-soluble polymer that functions as a transport for positive ions to enter and exit. This water-solubility indicates that the cell wall of gram-positive bacteria is more polar, so that bioactive compounds that are polar can easily enter the cell wall and damage the polar petidoglycan layer rather than the nonpolar lipid layer.

CONCLUSION

Carotenoid extract from symbiont bacteria *V.salarius* strain 19.PP.Sc1.6 associated with *Sinularia* sp. has antibacterial activity against MDR *E. coli* and MRSA bacteria.

ACKNOWLEDGEMENT

This research was partly funded by the Ministry of Research, Technology, and Higher Education, Indonesia under the Program Beasiswa Pendidikan Pascasarjana Dalam Negeri (BPPDN) decree number 2903.9/D3/PG/2017 and Hibah Disertasi Doctor number 225-16/UN7/6.1/PP/2020.

REFERENCES

Alberts, B.; Johnson, A. and Lewis, J. (2002). Molecular Biology of the Cell. 4th edition. New York: Garland Science; The Plant Cell Wall. Available from: https://www.ncbi.nlm.nih.gov/books/NBK26928/

Cowan, M. (1999). Plant Product as Antimicrobial Agent. Clinical Microbiology Review., 12(4): 564 – 582

Doron, S. and Gorbach, S. L. (2008). Bacterial Infections: Overview. International Encyclopedia of Public Health., 273–282. https://doi.org/10.1016/B978-012373960-5.00596-7

Gillespie, S. H. and Bamford, K. B. (2000). Medical Microbiology and Infection at a Glance Janett P Gillespie M.B. MRCGP, General Practitioner London.

Gross, J. (1991). Pigmen in Vegetables. Van Nastrand Reinhold, New York, 351 pp.

Hunt, B. and Vincent, A.C.J. (2006). Scale and sustainability of marine bioprospecting for pharmaceuticals. Ambio., 35 (2): 57 – 64.

Janeway C.A.; Jr Travers P. and Walport M. (2001). Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science; Infectious agents and how they cause disease. Available from: https://www.ncbi. nlm.nih.gov/books/NBK27114/

Jawetz,; Melnick, and Adelberg. (2007). Medical Microbiology. 23th Edition. Jakarta : EGC

Kaur, D. C. and Sadhana, S. C. (2015). Study of Antibiotic Resistance Pattern in Methicillin Resistant Staphylococcus Aureus with Special Reference to Newer Antibiotic. Journal of global infectious diseases., 7(2): 78-84. doi:10.4103/0974-777X.157245

Khan, H. A.; Baig, F. K. and Mehboob, R. (2017). Nosocomial infections: Epidemiology, prevention, control and surveillance, Asian Pacific Journal of Tropical Biomedicine., 7(5): 478-482. https://doi.org/10.1016/j.apjtb.2017.01.019.

Kusmita, L.; Mutiara, E.V.; Nuryadi, H.; Pratama, P.A.; Wiguna, A.S. and Radjasa, O.K. (2017). Characterizatio of carotenoid pigments from bacterial symbionts of soft-coral Sarcophyton sp. from North Java Sea. Int Aquat Res., 9:61–69. doi: 10.1007/s40071-017-0157-2.

Kusmita, L.; Nuryadi, H.; Widyananto, P.A.; Muchlissin, S.; Sabdono, A. and Radjasa, O.K. (2021). Bioactivity of carotenoid produced by soft coral symbiotic microorganisms from Panjang and Karimunjawa Island, central Java, Indonesia. BIODIVERSITAS., 22(2): 732 – 740. DOI: 10.13057/biodiv/d220233.

Magiorakos, A.P.; Srinivasan, A.; Carey, R.B.; Carmeli, Y.; Falagas, M.E. and Giske, C.G.; (2012). Multidrug-resistant, extensively drug-resistant and drug

resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical Microbiology and Infection., 18 : 268 – 281.

Manuputty, A.E.W. (2016). Karang Lunak (Octocorallia : Alcyonacea) di Perairan Biak Timur The Common Soft Corals (Octocorallia : Alcyonacea) in East Biak Waters. Oseanologi dan Limnol di Indonesia., 1(12):47–59

Monegro, A.F.; Muppidi, V. and Regunath, H. (2021). Hospital Acquired Infections. [Updated 2020 Sep 3]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan-. Available from: https://www.ncbi.nlm.nih.gov/ books/ NBK441857/

Monira, S.; Shabnam, S. A.; Ali, S. I.; Sadique, A.; Johura, F. T.; Rahman, K. Z.; Alam, N. H.; Watanabe, H. and Alam, M. (2017). Multi-drug resistant pathogenic bacteria in the gut of young children in Bangladesh. Gut pathogens., 9: 19. https://doi.org/10.1186/s13099-017-0170-4

Pangestuti, R. and Se-Kwon Kim. (2011). Biological activities and health benefit effects of natural pigments derived from marine algae. Journal of Functional Foods., 3(4): 255-266. https://doi.org/10.1016/j.jff.2011.07.001.

Proksch, P.; Edrada, R.A. and Ebel, R. (2002). Drugs from the seas – Current Status and Microbiological Implications. Appl. Microbiol. Biotechnol., 59 : 125-124.

Radjasa, O.K.; Limantara, L. and A. Sabdono. (2009). Antibacterial activity of a pigment producing-bacterium associated with *Halimeda* sp. From land-locked marine lake Kakaban, Indonesia. J. Coast. Dev., 12 : 100 – 104.

Rodriguez-Amaya D.B. and Kimura M. (2004) HarvestPlus Handbook for Carotenoid Analysis. Washington, DC and Cali: International Food Policy Reasearch Institute (IFPRI) and International Center for Tropical Agliculture (CIAT)

Silhavy, T. J.; Kahne, D. and Walker, S. (2010). The bacterial cell envelope. Cold Spring Harbor perspectives in biology., 2(5):a000414. https://doi.org/10.1101/ cshperspect.a000414

Stapleton, P. D. and Taylor, P. W. (2002). Methicillin resistance in Staphylococcus aureus: mechanisms and modulation. Science progress., 85 (Pt1): 57–72. https://doi.org/10.3184/003685002783238870

Stephen, I.B.; Chien, J.T. and Chen, B.H. (2016). Improved high performance liquid chromatographic method for determination of carotenoids in the microalga Chlorella pyrenoidosa. Journal of Chromatography A., 11029 (1-2) : 193 – 199.

Sulistiyani, Nugraheni, S.A.; Khoeri, M.M.; Sabdono, A. and Radjasa, O.K. (2010). Antibacterial Activity of Bacterial Symbionts of Softcoral *Sinularia* sp. against Pathogenic Resistant Bacteria. J. Coast. Dev.,13 (2) : 113 – 118.

Tanod, WA.; Aristawati, A.T.; Putra, M.Y. and Muliadin. (2018). Soft coral (*Sinularia* sp.) Extracts with Antibacterial Activity. Omni-Akuatika., 14 (1):108-117

Ventola C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. P & T : a peer-reviewed journal for formulary management., 40(4): 277–283.

Venugopal, V. (2009). Marine products for healthcare: Functional and bioactive nutraceutical compounds from the ocean. New York : CRC Press.

Wagner, W.L.; Herbst, D.R. and Sohmer, S.H. (1999). Manual of The Flowering Plants of Hawaii. Hawai.

Wiguna, A.S.; Kusmita, L. and Radjasa, O.K. (2016). Uji Aktivitas Antibakteri Pigmen Karotenoid Dari Bakteri Simbion Karang Lunak *Sarcophyton* sp. Terhadap Pertumbuhan Bakteri Patogen *Staphylococcus aureus* ATCC 25923. IJPST., 3 (3) : 92 – 98.