Antibacterial resistance *Escherichia coli* and *Aeromonas hydrophila* on alkaloid from marine sponge *Callyspongia* sp.

M Musman¹*, V Kurnianda², A Mauliza², A Winanda²

¹Department of Chemistry Education, Faculty of Teacher Training and Education, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia.
²Department of Marine Science, Faculty of Marine and Fisheries, Universitas Syiah Kuala, Darussalam, Banda Aceh 23111, Indonesia.

*Email: musrimusman@unsyiah.ac.id

**Abstract.** Research on the bioactivity of a compound from *Callyspongia* sp. as an antibacterial agent against *Escherichia coli* and *Aeromonas hydrophila* resistent was conducted. The active metabolite was isolated based on bioassay-guided separation with several steps of chromatography. The FTIR data showed the vibration at 1637.4 cm⁻¹ as C-N imine functional group with the fingerprint region at 1449.0 cm⁻¹ indicated the presence of alkaloid as the active compound. The results of this study informed the inhibitory zone diameter of the extract against *E. coli* showed an area as wide as 8.10 mm at the concentration of 80 μg mL⁻¹ compared to the inhibitory zone diameter of the positive control Chloramphenicol as wide as 7.50 mm at the concentration of 40 μg mL⁻¹. However, the inhibitory zone of the extract against *A. hydrophila* displayed an area 8.50 mm in diameter at the concentration of 20 μg mL⁻¹ compared to the zone diameter of the positive control Chloramphenicol as wide as 7.25 mm at the concentration of 40 μg mL⁻¹. Based on the bioassay results, the alkaloid compound from *Callyspongia* sp. has potential activity against the Gram-positive bacterial resistant of *Escheria coli* and the Gram-negative bacterial resistant of *Aeromonas hydrophila*.

1. **Introduction**

Diarrhea is a common gastrointestinal disease. This disease makes sufferers become frequent bowel movement with watery stools. In brief, diarrhea results from food and beverages exposed to viruses, bacteria, or parasites [1, 2]. Diarrhea is a foremost basis of mortality and morbidity caused by *E. coli* [3] and *A. hydrophila* [4] covered worldwide. Diarrheal disease is associated with the production of extracellular enterotoxin by the bacteria *Escherichia coli* [5] or *Aeromonas hydrophila* [6, 7]. *E. coli* is a Gram-positive bacterium that often lives in the human digestive tract and harmless. However, some types of bacteria can release poisons that cause acute infections, and cause diarrhea. *Aeromonas hydrophila* is a Gram-negative bacterium that can cause several diseases, *i.e.* diarrhea [8], soft tissue, skin, and blood infections and septicemia [9, 10]. Treatment of the diarrheal disease is done by administering antibiotics. However, frequent use of the antibiotics may lead to resistance to the bacterium. Resistance is where bacterial conditions in the body do not have antibiotic effects so that when antibiotics are given they can...
cause bacteria to survive by mutating genes and will form protein synthesis in cells. Hence, natural ingredients that are safer to use in the treatment process are needed [11].

Several types of plants from natural ingredients are known to have active compounds as antibacterial, antitumor and anticancer such as mangosteen skin extract, turmeric skin extract and squeezed galangal rhizome [12]. Natural materials can also come from the sea. A large number of marine organisms have been used as raw material for medicines. One of the marine organism widely studied for the raw materials of medicines is sponges due to they have a lot of antibacterial, anticaner, antitumor properties [13].

Management of this bacterial disease is by giving commercial antibiotics. The pharmacological effects of antibiotics have a negative effect caused this disease cannot be overcome optimally due to it can be resistant. The researchers begin looking for alternative sources of medicine derived from natural marine products. Several studies have reported that medicinal sources of marine natural product origin have antibacterial, antiviral, antifungal, and antioxidant activities [14-17]. One of the marine natural sources is Callyspongia sp. Sponge [18-21]. This study focused on antibacterial exploration of resistance to E. coli and A. hydrophila against alkaloids obtained from marine sponges Callyspongia sp.

2. Material and Methods

2.1. Materials

This research utilized Kern analytical scales, infrared resonance spectroscopy, Eyela N-1000 rotary evaporator, Memmert INB 500 incubator, Tommy SX-300/500/700 autoclave, SafeFast Elite 212 SD laminar air flow, UV UVGL-25 lamps, Akebono hotplate, Jouan oven, LG refrigerator, thin layer chromatography NP-SiO2, open column chromatography NP-SiO2, Pyrex Petri dish, Pyrex glass beaker, Pyrex reaction tube, Pyrex split funnel, micropipette Pipetteman P20 F123563 (vol. 2-20 μL), and Eppendorf micro pipets (volume 100-1000 μL).

2.2. Biomaterial

– Specimen of sponge. Sponge took at Sabang, Indonesia in March 2017. Samples were extracted using methanol 96% and phytochemical test at the Marine Chemistry Laboratory.

– The specimen of resistance bacteria. Resistant bacteria Escherichia coli O157: H7 were taken from Regional Public Hospital (RSUD) dr. Zainoel Abidin, Banda Aceh, and resistant bacteria Aeromonas hydrophila were got from Department of Brackish Water Hatchery (Badan Pembenihan Air Payau), Aceh Besar. Both the resistant bacteria were cultured in Microbiology Laboratory at Faculty of Medicine, Syiah Kuala University.

– Preparation of resistance bacteria. Resistant bacteria of E. coli and A. hydrophila were cultivated on Nutrient Agar (NA) medium. Furthermore, NA medium was sterilized using an autoclave for 2 hours at 121 °C. After sterilization, added 30 μg mL⁻¹ chloramphenicol antibiotics into the petri dish and incubated for 24 hours at 30 °C [13].

– Antibacterial cultivation and growth condition. E. coli and A. hydrophila were maintained on Nutrient Agar at 37 °C. Mid-log-phase bacilli obtained based on Mc. Farland in the range of 0.08-0.1 was equivalent to 1x10⁸ CFU mL⁻¹ for E. coli and 1.5x10⁸ CFU mL⁻¹ for A. hydrophila were incubated for 24 hours at 37 °C (aerobic conditions) based on OD₆₂₅= 0.1.

2.3 Methods

– Extraction and isolation. The sponge Callyspongia sp. (200 g dry weight) cut into small pieces. Then, the sponge was macerated using a methanol solvent for 72 hours and evaporated. The result of the extracts obtained methanol fraction (16.89 gram). Methanol fraction (16.89 g) was partitioned using chloroform: methanol: water (1:1:1 v/v). The results showed that semi-polar fraction (0.8 g) and polar fraction (16.09
Based on bioassay guidelines, the polar fraction showed inhibitory zone [100 μg mL\(^{-1}\) (9.25 mm)]. Furthermore, the polar fraction (16.09 g) was purified using open column chromatography NP-SiO\(_2\) (dichloromethane: methanol gradient) obtained 3 fractions. The bioassay guidelines informed that the third fraction (0.3 g) exhibited inhibitory zone [100 μg mL\(^{-1}\) (10.25 mm)].

– Test of bioassay. 2.8 grams of Nutrient Agar (NA) medium was dissolved into 100 mL of aquadest then inserted into autoclave along with petri dish and Erlenmeyer for 2 hours at 121 °C for sterilization. After the autoclave process was completed, the NA media together the resistant bacteria were added into Erlenmeyer. The NA medium was separated and then inserted into a petri dish and allowed to form a gel. Blank disks were added to the agar medium and then added the extract with concentrations of 20, 40, 60, 80 and 100 μg mL\(^{-1}\) on the disk respectively. The negative control was DMSO 2% and positive control used chloramphenicol with the concentration of 40 μg mL\(^{-1}\). The petri dish was put into the incubator for 12 hours in temperature 37 °C, then was measured zone of bacterial inhibition [13, 22].

3. Results and Discussions

The bioactivity test of the third fraction extract was done against resistant bacteria *Escherichia coli* and *Aeromonas hydrophila*. This bioactivity test was performed at different concentrations in order to saw the differences in the effect of different concentrations on resistant *E. coli* and *A. hydrophila* bacteria. The extract signaled the *Callyspongia* sp. has potential activity against resistant bacteria *E. coli* and *A. hydrophila* based on inhibition zone fashioned (see Table 1). In the bioassay, the DMSO 2% was applied as a negative control due to at concentrations <3% DMSO is not toxic to cell survival, and chloramphenicol 40 μg mL\(^{-1}\) was added as a positive control [23].

Referring to the inhibition zone classification, the diameter zone of 0-13, 13-20, and 20-28 mm is categorized as weak, moderate, and strong, respectively [24]. The positive control used was chloramphenicol with a concentration of 40 μg mL\(^{-1}\) giving an inhibition zone of 7.50 mm in diameter as well as the negative control used was DMSO 2% giving a result of 0.00 mm in diameter. Both of control showed the inhibition zone belong to the weak category. However, the bioassay results (see Table 1) showed that the higher concentration of extract used to give effect the greater inhibition zone formed both in *E. coli* and *A. hydrophila*. resistant bacteria compared to the control. The performance of extract on the bacteria could be predicted in several mechanisms including disrupting the function of membranes, inhibiting the synthesis of bacterial cell proteins, and interfering the synthesis of DNA/RNA [25, 26].

| No. | Sample Name               | Concentration | Inhibition zone (mm) E. coli | A. hydrophila |
|-----|---------------------------|---------------|-----------------------------|--------------|
| 1   | Neg. control (DMSO)       | 2%            | 0.00                        | 0.00         |
| 2   | Pos. control (Chloramphenicol) | 40 μg mL\(^{-1}\) | 7.50                        | 7.25         |
| 3   | Extract                   | 20 μg mL\(^{-1}\) | 7.00                        | 8.50         |
| 4   | Extract                   | 40 μg mL\(^{-1}\) | 7.15                        | 9.85         |
| 5   | Extract                   | 60 μg mL\(^{-1}\) | 7.25                        | 9.86         |
| 6   | Extract                   | 80 μg mL\(^{-1}\) | 8.10                        | 10.15        |
| 7   | Extract                   | 100 μg mL\(^{-1}\) | 8.25                        | 10.25        |
The FTIR spectrum (see Figure 1) of the extract designated several functional groups characterized by various vibration. General absorption at a wavenumber of 3271.4 cm\(^{-1}\) has a type of alcohol compound with O-H functional groups confirmed in fingerprints with wavenumbers of 1113.5 cm\(^{-1}\) and 1015 cm\(^{-1}\) which were types of alcohol compound with C-O functional group [27]. General absorptions at wavenumbers 2949.0 cm\(^{-1}\) and 2835.5 cm\(^{-1}\) have alkane compound with CH moieties, then at a wavenumber of 1637.4 cm\(^{-1}\) informed the type of imine moiety with CN functional group confirmed by fingerprints. A fingerprint at wavenumbers of 1449.0 cm\(^{-1}\) and 1407.2 cm\(^{-1}\) designated a type of imine compound with a CN functional group [28]. The results of the FTIR data interpretation identified a vibration absorption in the wavenumber area 1637.4 cm\(^{-1}\) which showed the vibration of the CN imine functional group. It was confirmed by the fingerprint area at wavenumbers 1449.0 cm\(^{-1}\) suggesting the active compound was an alkaloid secondary metabolite [29].

4. Conclusion
The alkaloid substance extracted from the sponge Callyspongia sp. showed the potent properties for antibacterial resistance to 1) Gram-positive Escherichia coli at the minimum concentration of 80 \(\mu g\) mL\(^{-1}\) (8.10 mm in diameter of inhibition zone) and the optimum concentration of 100 \(\mu g\) mL\(^{-1}\) (8.25 mm in diameter of inhibition zone), and 2) Gram-negative Aeromonas hydrophila at the minimum concentration of 20 \(\mu g\) mL\(^{-1}\) (8.50 mm in diameter of inhibition zone) and the optimum concentration of 100 \(\mu g\) mL\(^{-1}\) (10.25 mm in diameter of inhibition zone).

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References
[1] Igbinosa I H, Igumbor E U, Aghdasi F, Tom M and Okoh A I 2012 Scientific World Journal 2012 625023
[2] Pal M 2018 J Food Microbiol 2 9–10
[3] Khalil I A, Troeger C, Blacker B F, Rao P C, Brown A, Atherly D E, Brewer T G, Engmann C M, Houpt E R, Kang G, Kotloff K L, Levine M M, Luby S P, MacLennan C A, Pan W K, Pavlinac P B, Platts-Mills J A, Quadri F, Riddle M S, Ryan E T, Shoultz D A, Steele A D, Watson J L, Sanders J W, Mokdad A H, Murray C J L, Hay S I and Reiner Jr R C 2018 *Lancet Infect Dis* **18** 1229–40

[4] Mansour A M, Elkhalek R A, Shaheen H I, Mohammady H E, Refaey S, Hassa K, Riddle M, Sanders J W, Sebeny P J, Young S Y N and Frenck R 2012 *J Infect Dev Ctries* **6** 842–846

[5] Kartsev N N, Fursova N K, Pachkunov D M, Bannov V A, Eruslanov B V, Svetoch E A and Dyatlov I A 2015 *PLoS One* **10** e0123357

[6] Sha J, Lu M and Chopra A K 2001 *Infect Immun* **69** 6370–81

[7] Qamar F N, Nisar M I, Quadri F, Shakoor S, Sow S O, Nasrin D, Blackwelder W C, Wu Y, Farag T, Panchalingham S, Sur D, Qureshi S, Faruque A S G, Saha D, Alonso P L, Breiman R F, Bassat Q, Tamboura B, Ramamurthy T, Kanungo S, Ahmed S, Hussain A, Das S K, Antonio M, Hussain M J, Mandomando I, Tennant S M, Kotloff K L, Levine M M and Zaidi A K M 2016 *Am J Trop Med Hyg* **95** 774–780

[8] Bunya I A and Obais I K 2018 *Journal of Global Pharma Technology* **10** 272–280

[9] Chao C M, Lai C C, Tang H J, Ko W C and Hsueh P R 2013 *Eur J Clin Microbiol Infect Dis* **32** 543–7

[10] Coutinho A S, de Morais O O, Gomes C M and de Oliveira C da Motta 2013 *J Infection* **41** 595–6

[11] Allan B J and Stevenson R M W 1981 *Canadian Journal of Microbiology* **27** 1114–1122

[12] Grube A, Assmann M, Lichte E, Sasse F, Pawlik J R and Köck M 2007 *J Nat Prod* **70** 504–509

[13] Mbah J A, Ngemenya M N, Abawah A L, Babiaka S B, Nubed L N, Nyongbela K D, Lemuh N D and Efange S M N 2012 *Annals of Clinical Microbiology and Antimicrobials* **11** 1–10

[14] Van Soest R W M, Boury-Ésnault N, Vacelet J, Dohrmann M, Erpenbeck D, De Voogd N J, Santodomingo N, Vanhoorne B, Kelly M and Hooper J N A 2012 *PLoS ONE* **7** e35105

[15] Abdelmohsen U R, Yang C, Horn H, Hajjar D, Ravasi T and Hentschel U 2014 *Mar Drugs* **12** 2771–2789

[16] Brinkmann C M, Marker A and Kurthböke D I 2017 *Diversity* **9** 40

[17] Chaudhary A, Naughton L M, Montáncher I, Dobson A D W and Rai D K 2017 *Mar Drugs* **15** 272

[18] Perdicaris S, Vlachogianni N and Valavanis A 2013 *Nat Prod Res* **1** 114

[19] Shaala L A, Youssef D T A, Ibrahim S R M G and Mohamed G A 2016 *Natural Product Research* **30** 2783–90

[20] Ibrahim H A H, El-Naggar H A, El-Damhougy K A, Bashar M A E and Senna F M A 2017 *The Journal of Basic and Applied Zoology* **78** 7

[21] Mioso R, Marante F J T, de Souza B R, Borges F V P, de Oliveira S B V and de Laguna I H B 2017 *Molecules* **22** 208

[22] Kurnianda V and Setiawan A 2015 *International Journal of Pharmaceutical Biological and Chemical Sciences* **4** 30–33

[23] Widayanti S M, Permana A S and Kusumaningrum H D 2009 *Journal Postharvest Pertanian* **6** 61–88 [in Indonesia]

[24] Greenwood D 1995 *Antimicrobial Chemotherapy* (Oxford, New York, Tokyo: Oxford University Press) pp13–61

[25] Cowan M M 1999 *Clinical Microbiology Reviews* **12** 564–82

[26] Radulovic N S, Blagojevic P D, Stojanovic-Radic Z Z and Stojanovic N M 2013 *Current Medicinal Chemistry* **20** 932–52
[27] Stuart B H 2004 *Infrared Spectroscopy: Fundamentals and Applications* (Chichester, West Sussex, England: John Wiley & Sons, Ltd.) p 76
[28] Layer R W 1963 *Chem Rev* 63 489–510
[29] Wang Y and Poirier R A 1997 *J Phys Chem A* 101 907–12