The 3rd International Conference On Science
Journal of Physics: Conference Series 1341 (2019) 032006 doi:10.1088/1742-6596/1341/3/032006

Toxicological evaluation and antibacterial activity of crude protein extract from endophytic bacteria associated with Algae Eucheuma spinosum

A Sugrani1,*, A Ahmad2*, M N Djide1 and H Natsir2

1Department of Medical Laboratory Technology, Megareozy University of Makassar, Makassar, Indonesia
2Department of Chemistry, Mathematics and Natural Science Faculty, Hasanuddin University, Makassar, Indonesia
3Faculty of Pharmacy, Hasanuddin University, Makassar, Indonesia

*E-mail: ahyarahmad@gmail.com; #author with equal contribution

Abstract. This study evaluated the toxicological and antibacterial activity of crude extract of endophytic bacteria’s protein associated with the red algae Eucheuma spinosum which was produced at various incubation times. The external and internal proteins of the potential bacteria were collected on the incubation times which were 18, 24, 30, 36, 42, 48, and 54 hours. The evaluation toxicological of protein used Brine Shrimp Lethality Test (BSLT) method and the antibacterial activity of protein towards E. coli and S. aureus were measured using the diffusion method. Seven bacteria were successful to be isolated from these algae (ES01, ES11, ES21, ES22, ES23, ES24, and ES25), the Isolate ES25 was a genus of Vibrio and its protein had the potential to be produced. External protein levels were higher than internal, so it continued with the next phase. The extracted protein of (PE42) was active towards S. aureus (12.0 mm) while the extracted protein of (PE36) is active toward E. coli (9.0 mm) and also highly toxic to the larvae of Artemia salina Leach (1.596 μg/mL). The external protein of endophytic bacteria ES25 (Vibrio) associated with red alga Eucheuma spinosum was potentially produced in the time of incubation above 36 hours to get the highest toxicity and active antibacterial.

1. Introduction

An alga is one of the marine eukaryotes associated with bacteria [1]. Other algae or marine eukaryotes are associated and dependent with bacteria for growth, development, nutrient supply and as a protection against the predator attacks [2]. Bacteria associated with algae are the potential source of biotechnology products because they produce bioactive compounds with diverse characteristics [1]. Descriptive studies of algae in the last 40 years found that about 149 algae associate with bacteria. The algae came from various species, including 36 from green algae (Chlorophyta), 46 from brown algae (Phaeophyceae), 55 from red algae (Rhodophyta), and 12 unknown species [3].

Some bacteria associated with red algae (Rhodophyta) produce bioactive compounds. Bacillus amyloliquefaciens bacteria associated with Laurenciae papillosa produce polyketides as antibacterial, Pseudomonas sp. bacteria associated with Ceratodictyon spongiosis produce phenol compounds as antibiotics, the bacteria Acaryochloris sp. associated with Ahnfelttiopsis flabelliformis produce pyrole compounds as a component of photosynthesis, Pseudoalteromonas sp. associated with Digenea sp. produce peptide compounds as antibiotics, Pseudomonas sp. associated with red algae (unknown...
species) produces lactone and lipopeptides as antibiotics, *Bacillus sp.* associated with *Schizymenia dubyi* produces lactone as an antibiotic and *Alteromonas sp.* associated with *Rhodymenia sp.* produces anti-supplement peptides [3].

The use of bacteria associated with red algae, endophytes or epiphytes are familiar and well-known. But generally, what can be produced is the secondary metabolite. There were not so many research has been reported of the bioactive protein of endophytic bacteria of red algae, whereas red algae contain 10-47% of protein from dry weight [4] and this protein is active, Dali [5] reported that a protein fraction of 0-20% of red algae *Gelidium amansii* effectively inhibits *Staphylococcus aureus* with inhibition diameter > 14 mm. The bioactive protein from red algae is also potential to be produced from the endophytic bacteria which associate with because the endophytic bacteria have the potential to produce a compound which is the same as its host.

Endophytic bacteria are one of the convincing targets for future research studies. These bacteria proved that they can prevent the growth of various diseases. Of the 300,000 types of plants that exist on earth; each plant becomes a place for one or more endophytic bacteria’s life [7]. Thus, it estimates that there can be millions of different species of endophytic bacteria, but only a few of them have been explained, this means investigating bioactive compounds from endophytes can increase the chances of finding new products [8]. This study evaluates the toxicology and antibacterial activity of protein extracts of endophytic bacteria associated with *Eucheuma spinosum* algae.

2. Materials and methods
   2.1. Materials
   The tools used in the research were analytical balance, incubator, autoclave, UV-Vis spectrophotometer, pH meter, blender, cold centrifuge, magnetic stirrer, petri dish, knife, laminar flow, shaker incubator, vortex, eppendorf tube, micropipette, aerator, incandescent lamps, water baths, magnifying glass, stopwatch, ose wire, light microscope, mortar, stamper, test tube, dropper pipette, blank disk, erlenmeyer.

   2.2. Preparation of algae samples
   The surfaces of Algae were sterilized using the method of Kaaria [9], Etminani and Harighi [10] which has been modified. The red alga was washed by sterile seawater, dried with tissue paper, immersed in sodium hypochlorite 5% and Tween20 0.25% for 5 minutes, 4 times cleaned by sterile water and passed on bunsen lamps.

   2.3. Isolation of Endophytic Bacteria
   The planting of bacteria associated with red algae was processed by using a distribution method [10]. The sample of sterile red algae was put into the blender and it was added with sterile seawater, then it took 10 mL of the juice and it was put into a diluted bottle which contained 90 mL of sterile seawater (10⁻¹ dilution) the multilevel dilution was made up to 10⁻⁵.

   After that, the series of dilution (10⁻¹ to 10⁻⁵) was taken 100 μL and spread into Nutrient Agar (NA) medium and incubated in an upside down position at 37°C for 48 h. While on the incubation process, the colonies growth was observed during an incubation period. The colonies with clear zones were considered as the active isolate because they can compete with another isolated bacterium. After the incubation process over, the isolate was purified by using the scratch method. The endophytic bacteria colonies were observed in shape, elevation, edges, color, size, and surface of the colonies.

   2.4. Test of Bacterial Morphology
   The Test used the gram staining method, bacteria were placed on a glass slide, then it added one drop crystal violet for one minute, and then it was cleaned and poured by water. Further, it added one drop of Mordant iodine (Merck) Gram for one minute, then it was cleaned again by water. After that, Ethyl alcohol 95% was added until the crystal violet cannot be dissolved and these were cleaned with water. Finally, safranin was added to the bacteria for 45 seconds and cleaned with water and dried.
After all the process was completed, the bacteria were observed by microscope. Gram-positive bacteria are positive if the cells appear dark blue or purple, while gram-negative bacterium appears pink cells. Besides, the coloring test also determines the morphology of the bacteria (coci, basil, vibrio and or spirillum) [11].

2.5. **Simple Biochemical Test**
The biochemical test was done by applying Bergey's Manual of Determinative Bacteriology method [12] the method examined Triple Sugar Iron Agar Test (TSIA), Indole Motility (SIM) Sulfide Test, Methyl Red-Voges Proskauer Test (MR-VP), Simon Citrate Agar Test (SCA), and carbohydrate fermentation test.

2.6. **Determination of Optimum Bacterial Production Time**
One ose bacterium was inoculated in 200 mL of Nutrient Broth (Merck®) seawater and then it was cultivated by Incubator Shaker Series with temperature (37°C, 150 rpm, and 72 hours). Every 6 hours of incubation time, Optical Density (OD) of fermentation media was measured using UV-VIS spectrophotometer (at 600 nm wavelength). The data of medium growth was described by the growth curve [13].

2.7. **Protein Isolation**
A pure isolate of endophytic bacteria was inoculated on the production medium and incubated at 37°C on a shaker. Bacterial growth was characterized by small or large granules emergence, discoloration, and thickness of the media. Bacterial culture was collected for 72 hours every six-hour interval. Furthermore, the culture was centrifuged (5000 rpm, 4°C, for 30 minutes) to separate the filtrate and the cells. The filtrate is a crude extract of extracellular proteins. The obtained cell was crushed and added 100 mL of Tris-HCl buffers. The cell fragments were freeze-thawed for 2-3 times and they were sonicated for 3 x 10 minutes on ice to help the fragmentation of the cells. The cell fragments were centrifuged (5,000 rpm, 4°C, 30 minutes) to obtain crude extracts of intracellular proteins. The crude extracts of protein (extracellular and intracellular) were kept in the refrigerator before doing the next test and purification process.

2.8. **Determination of Protein Levels**
Protein level was measured by the Lowry method [14], protein samples were added with 0,9 mL of Lowry A reagent, and then it was shaken and left for 15 minutes. 3 mL of Lowry B (Folin ciocalteu) reagent was added in protein; it was shaken and left for 30 minutes. After that, the absorbance was determined with spectrophotometer at λ 500 nm.

2.9. **Antibacterial Activity**
Antibacterial activity test used strains of gram-positive bacteria (S. aureus) and strains of gram-negative bacteria (E. coli), this test followed the method of disk diffusion [15]. Bacterial strains were obtained from the Hasanuddin University Medical Research Center (HUM-RC), Indonesia.

The antibacterial activity test in this research consisted of two types; they were the qualitative and quantitative antibacterial test. The antibacterial qualitative test aims only to determine the presence or absence of bacterial isolate activity, while the antibacterial quantitative test determined the inhibition zone of isolates and their proteins.

The medium was inoculated with tested bacteria using the swap method. Sterile paper disks (6.2 mm) were dipped into the test sample and these were placed on the agar media. Petri dishes were wrapped by plastic wrap and kept in an incubator at 37°C for 1-2 days.

2.10. **Toxicology Test**
The toxicology test used Brine Shrimp Lethality Test (BSLT) method, the *Artemia salina* leach was hatched using sea-water, and the hatching container was covered by aluminum foil and lit by
incandescent lamps. The hatching process took the time for 48 hours. Test compounds were made in the concentrations of 1, 10, 100 ppm in seawater. Ten *Artemia salina* leach larvae were put in a vial, the vial contained test compound and seawater. After 24 hours, the live larvae were counted. The data from observations were tabulated and calculated using probit analysis to obtain Lethal Concentration 50 (LC$_{50}$) values [16].

### 3. Results and Discussion

The function of sterilization a surface of an algae sample, was to clean other particles, epiphytic microorganisms on the sample so it was expected that the bacteria growth is endophytic bacteria. Then, the sample was crushed by grinding until smooth, which aims to enlarge the surface area of the algae tissue, and to optimize planted endophytic bacteria [6].

The isolation of bacteria from *Eucheuma spinosum* obtained seven bacterial isolates (ES01, ES11, ES21, ES22, ES23, ES24, and ES25) (Figure 1). The qualitative antibacterial test showed the potential of activity from seven bacterial isolates, these tests against pathogenic bacteria *E. coli* and *S. aureus* (Table 1). The clear zone was the characteristic of the isolates bacteria can compete with other bacteria around them.

![Figure 1. Endophytic bacteria isolated from *Eucheuma spinosum*](image)

| No | Isolates Code | Bacterial Test | E. coli | S. aureus |
|----|---------------|----------------|---------|-----------|
| 1  | ES01          |                | +       | -         |
| 2  | ES11          |                | +       | -         |
| 3  | ES21          |                | +       | -         |
| 4  | ES22          |                | +       | -         |
| 5  | ES23          |                | +       | +         |
| 6  | ES24          |                | +       | -         |
| 7  | ES25          |                | +       | +         |

(+) No activity, (+): Exist Activity

Colonies with stable clear zones were taken and scratched several times to obtain pure bacterial isolates. Pure bacterial colonies contained one morphological characteristic in common only [6]. From
the purification process, each one bacterial isolate was made in duplo as working culture and stock culture [17].

In general, endophytic bacteria abundant population and they are specific microbes because they directly interact with bioactive compounds from algae. This interaction occurs when the algae cell wall damaged, so that the bacteria enter and interact with bioactive algae compounds [18]. The interaction between bacteria and algae is a form of mutualism symbiosis. Algae provide the place and nutrients which are needed by bacteria, while bacteria encourage the growth and protect the surface of algae from pathogenic organisms [19].

The characteristic of endophytic bacteria was carried by macroscopic and microscopic observations. Macroscopic observations are needed to determine the differences in the properties of colonies in solid media included the elevation, edges, color, size, and the surface of colonies (Table 2). Microscopic observations were carried out by the Gram staining method, the result observations showed from seven isolate bacteria, one isolate was gram-positive bacteria (ES01) and another were gram-negative bacteria (ES11, ES21, ES22, ES23, ES24, and ES25). On the observation of cell form, one isolate had the shape of basil spores (ES01), one isolate had the shape of basil's without spores (ES11), and five isolates had the shape of cocobacil (ES21, ES22, ES23, ES24, and ES25), the results of a gram stained and cell shape can be seen in Figure 2.

![Figure 2](image_url)

**Figure 2.** The picture of the gram staining of *Eucheuma spinosum* endophyte bacteria; bacteria ES01 (1); bacteria ES11 (2); bacteria ES21 (3); and bacteria ES22, ES23, ES24, ES25 (4).

Pelczar and Chan [20] explained that the bacteria can be divided into two types; they are positive and negative gram bacteria. The lipid substance of negative gram bacteria dissolved during the cleaning process by alcohol, the pores and the permeability on the cell wall enlarged so that the absorbed colour substance was easily waived and the bacteria became colorless. Whereas at gram-positive bacteria denatured the protein on the cell wall by alcohol. The protein became hard and stiff, the pores shrink, less of permeability, so the purple complex of iodine crystals were maintained and the cell remain purple.

Based on the macroscopic and microscopic test, it presumed that from the seven isolate bacteria, there were four isolates found as the same isolate bacteria, they were ES22, ES23, ES24, and ES25
because they had the same characteristics which the same macroscopic, microscopic, and biochemical test. So that from seven bacterial isolates were chosen into four selected isolates they are ES01, ES11, ES21 and ES25 (one of the four isolates considered as same).

Table 2. The characteristic result of endophyte bacteria of red algae Eucheuma spinosum.

| Sample | Elevation | Ledges | Color     | size  | Surface       |
|--------|-----------|--------|-----------|-------|---------------|
| ES01   | Convex    | Average| Milky white| Small | Smooth        |
| ES11   | Convex    | Average| Milky white| Small | Shiny Smooth  |
| ES21   | Convex    | Average| Milky white| Point | Shiny Smooth  |
| ES22   | Flat      | Average| Yellowish white| Small | Shiny Smooth  |
| ES23   | Flat      | Average| Yellowish white| Small | Shiny Smooth  |
| ES24   | Flat      | Average| Yellowish white| Small | Shiny Smooth  |
| ES25   | Flat      | Average| Yellowish white| Small | Shiny Smooth  |

The four selected isolates were tested further to select the potential isolates to be the source of bioactive protein. The test was applying quantitative antibacterial test using agar diffusion method, seawater as negative control and chloramphenicol as a positive control, the results of this antibacterial test showed that all selected bacterial isolates had activity, but none of these isolates exceeded the value activity of positive control (Table 3).

Table 3. The quantitative of antibacterial test results.

| No | Isolates Code | Inhibition zone (mm)* |
|----|---------------|-----------------------|
|    |               | S. aureus | E. coli |
| 1  | ES01          | 8.4       | 8.1     |
| 2  | ES11          | 8.4       | 8.0     |
| 3  | ES21          | 8.7       | 8.2     |
| 4  | ES25          | 9.2       | 8.5     |
| 5  | (-)           | 6.2       | 6.2     |
| 6  | (+)           | 23.3      | 21.6    |

*) = Mean from twice of measurements

The test results of the selected bacteria with quantitative antibacterial tests showed that ES25 bacteria had the highest activity compared to another isolate bacteria (ES01, ES11, and ES21) with a 9.2 mm barrier zone against S. aureus and 8.5 mm against E. coli, so the ES25 bacteria was the target to be optimized for bioactive protein production.

Furthermore, to identify the types of ES25 bacteria associated with Eucheuma spinosum red algae, besides doing the macroscopic and microscopic tests, biochemistry test is needed (Table 4). Based on these tests, ES25 bacteria have similarities with the type of Vibrio bacteria. Ihsan and Retnaningrum [21] explains that the members of Vibrio bacteria have biochemical properties such as oxidation, catalase, and positive nitrate reduction, aerogenic fermentation in glucose, and some species can degrade gelatin, chitin, lipidamilum, and aesculin. Other than that, the members of the genus Vibrio have characteristics such as short stem, gram-negative, non-spore, yellow, orange and green colonies.

Before the protein produced, it must be known well the optimum cultivation time growth of ES25 bacterial, which aims to determine the right time to obtain the maximum bacterial culture [13]. The method used on this activity was an indirect method based on Optical Density (OD). The observations showed that isolate ES25 bacteria had a lag phase (adaptation) on the 0-12 hour of growth. At this phase, bacteria adapted to their environment, on the log (exponential) phase at the 12-54 hour growth, the bacteria grew rapidly, and the stationary phase at the time of the 54-72 hour growth, the number of
cell populations remained still because the number of dividing cells was the same as the number of dying cells, while the death phase cannot be detected yet in this study because of the less volume of bacteria’s culture on this death phase there should be reduction of rate caused by less growing material of bacteria such as vitamins and mineral element.

| Tests | Results |
|-------|---------|
| TSIA  | Slant Acid |
|       | Butt Acid |
|       | Gas - |
|       | H₂S - |
|       | Indol - |
| SIM   | Motility + |
|       | H₂S - |
| MRVP  | MR + |
|       | VP - |
|       | Citrat - |
|       | Urea - |
|       | Glucose + |
|       | Lactose - |
|       | Sucrose + |
|       | Manitol + |

The chosen time to produce protein was log phase (exponential), the growth time between 12 hours to 54 hours (Figure 3), the log (exponential) phase was chosen because in this phase the cells produced many metabolites substances which were needed in order to growth [22]. Proteins secreted by bacteria, both extracellular and intracellular proteins were produced in the log (exponential) phase, the growth time among 18, 24, 30, 36, 42, 48, and 54 hours, the results external and internal protein levels from ES25 isolates bacteria can be seen on (Table 5) and (Figure 3).

| Protein Production | Protein content (mg/mL) |
|--------------------|-------------------------|
| Time (hours)       | External | Internal |
| 18                 | 1.340    | 0.696    |
| 24                 | 1.346    | 0.755    |
| 30                 | 1.495    | 0.840    |
| 36                 | 1.534    | 0.850    |
| 42                 | 1.546    | 0.949    |
| 48                 | 1.560    | 1.073    |
| 54                 | 1.600    | 1.242    |

Seven extracellular proteins were collected based on their incubation time (PE18, PE24, PE30, PE36, PE42, PE48, and PE54). PE18 for external bacterial protein was incubated 18 hours, PE24 external bacterial protein was incubated 24 hours, PE30 bacterial external protein was incubated 30 hours, PE36 bacterial external protein was incubated 36 hours, PE42 bacterial external protein was incubated 42 hours, PE48 bacterial external protein was incubated 48 hours and PE54 protein external bacteria incubated 54 hours.
Figure 3. Optical Density Curves, Extracellular and Intracellular Protein Levels for ES25 endophytic bacteria

These extracellular proteins were tested further towards antibacterial activity against pathogenic bacteria *E. coli* and *S. aureus* (Table 6). The highest inhibitory activity against *S. aureus* from protein PE42 (12.0 mm), while the highest inhibitory activity against *E. coli* in protein PE36 (9.0 mm) at a 24-hour incubation time. The antibacterial activity of these external proteins was not effective because according to Dali [5], an antibiotic was considered effective in inhibited bacterial growth if the inhibition diameter was ≥ 14 mm. The ineffectiveness of extracellular protein activity because of the tested protein was still in a crude extract protein form.

Table 6. Inhibition zone of External Proteins ES25 endophytic bacteria.

| External Protein | *Staphylococcus aureus* | *Eschericia coli* |
|------------------|-------------------------|------------------|
|                  | 24 hour     | 48 hour     | 24 hour     | 48 hour     |
| PE18             | 6.2         | 6.2         | 6.2         | 6.2         |
| PE24             | 6.2         | 6.2         | 6.2         | 6.2         |
| PE30             | 11.0        | 6.2         | 8.0         | 6.2         |
| PE36             | 10.0        | 6.2         | 9.0         | 7.0         |
| PE42             | 12.0        | 6.2         | 7.0         | 6.2         |
| PE48             | 11.0        | 6.2         | 6.2         | 6.2         |
| PE54             | 10.0        | 6.2         | 7.0         | 7.0         |
| (-)              | 6.2         | 6.2         | 6.2         | 6.2         |
| (+)              | 17.0        | 16.0        | 16.0        | 8.0         |

*) = Mean from twice of measurements
Inhibition zone of protein fraction and positive control against \textit{S. aureus} (gram-positive bacteria) are wider than inhibition zones \textit{E. coli} (gram-negative bacteria). It because the cell wall structure of gram-positive bacteria is simpler, making it easier for antibacterial compounds to enter the cell and find a target to work, but not for the gram-negative bacterial, it cell walls is more complex and three-layer, they are the outer layer of lipoprotein, middle layer in the form of peptidoglycan and inner layer lipopolysaccharide [20].

Antibacterial activity tests continued for up to 48 hours and the overall extracellular protein inhibition activity decreased both against \textit{E. coli} or \textit{S. aureus}, this indicated that extracellular proteins from ES25 endophytic bacteria were bacteriostatic. According to Nemeth [23], an antibacterial can be considered as bacteriostatic when the antibacterial is only effective to inhibit the growth of bacteria’s test but does not kill bacteria.

In addition to the antibacterial activity test, the external proteins of ES25 endophytic bacteria associated with \textit{Eucheuma spinosum} also evaluated the toxicology; this test used the BSLT method. The test result of external protein fraction were categorized based on the classification of LC$_{50}$ toxicity according to Ibrahim and Nur [24], if LC$_{50}$<20 µg/mL (very toxic), if the LC$_{50}$ is at 20-100 µg/mL (toxic), if the LC$_{50}$ value is at 100-500 µg/mL (medium), if the LC$_{50}$ value is at 500-1000 µg/mL (weak) and if the LC$_{50}$> 1000 µg/mL (non-toxic), this results found that from the seven external protein fractions, four of them were in the very toxic and three in the toxic category, but the smallest toxicity value among the very toxic was PE36 external protein with LC$_{50}$=1.596 µg/mL (Table 7), the smaller a toxicity value, the more toxic a test compound.

| External Protein | Value LC$_{50}$ (µg/mL) | Category |
|------------------|------------------------|----------|
| PE18             | 23.662                 | Toxic    |
| PE24             | 8.197                  | Very Toxic |
| PE30             | 11.060                 | Very Toxic |
| PE36             | 1.596                  | Very Toxic |
| PE42             | 11.300                 | Very Toxic |
| PE48             | 92.612                 | Toxic    |
| PE54             | 51.885                 | Toxic    |

Table 7. External Protein LC$_{50}$ value of ES25 endophytic bacteria

Conclusion
There were seven isolates of endophyte bacteria successfully isolated from these algae (ES01, ES11, ES21, ES22, ES23, ES24, and ES25). Isolate ES25 was a genus of \textit{Vibrio} and it potentially produced protein. The external protein level was higher than internal, and it continued with the next phase. PE42 external protein was active against \textit{S. aureus} with a diameter of an inhibition zone 12.0 mm; while the PE36 external protein was active against \textit{E. coli} and highly toxic to shrimp larva \textit{Artemia salina} Leach with the diameter of the inhibitory zone 9.0 mm and a toxicity value (LC$_{50}$) 1.596 µg/mL.

Acknowledgment
The author thanks Andi Batari Toja, S.Pd, the corrector for this research article and also thanks to the Endowment Fund for Education (LPDP) Ministry of Finance was supported this research (Contract number: PRJ-5155/LPDP.3/2016) through the scheme flagship scholarship for Indonesian domestic lecturers (BUDI-DN).

References
[1] Chakraborty K, Thilakan B, Raola VK, and Joy M 2017 Antibacterial polyketides from \textit{Bacillus amyloliquefaciens} associated with edible red seaweed (\textit{Laurenciae papillosa}) \textit{Food. chem.} 218 427-434.
[2] Egan S, Harder T, Burke C, Steinberg P, Kjelleberg S, and Thomas T 2013 The seaweed
Identification of bacteria

[3] Goecke F, Labes A, Wiese J, and Imhoff J F 2010 Chemical interactions between marine macroalgae and bacteria Marine Ecology Progress Series, 409 267-299.

[4] Fan X, Bai L, Zhu L, Yang L, and Zhang X 2014 Marine algae-derived bioactive peptides for human nutrition and health J. of agr. and food chem. 62 9211-9222

[5] Dalì S, Natsir H, and Ahmad A 2011 Antibacterial Bioactivity Protein Fraction of Red Algae Gelidium amansii from Takalar Cikoang Aquatic, South Sulawesi Pharmacy and Pharmacology Magazine 15 47-52

[6] Rau C H, Yudistira A, Simbala H E I 2018 Isolation, molecular identification using Gen 16S RNA, And test the antibacterial activity of endophyte Simbion bacteria that is isolated from Halimanea algae PHARMACON 7 53-61

[7] Ryan R P, Germaine K, Franks A, Ryan D J, and Dowling D N 2008 Bacterial endophytes: recent developments and applications FEMS microbiology letters 278 1-9.

[8] Yu H, Zhang L, Li L, Zheng C, Guo L, Li W, and Qin L 2010 Recent developments and future prospects of antimicrobial metabolites produced by endophytes Microbiological research. 165 437-449

[9] Kaaria P, Matiru V, Wakibia J, Bii C, and Ndung'u M 2015 Antimicrobial Activity of Marine Algae Associated Endophytes and Epiphytes from the Kenya Coast Int. J. Curr. Microbiol. App. Sci. 4 17-22

[10] Etminani F and Harighi B 2018 Isolation and Identification of Endophytic Bacteria with Plant Growth Promoting Activity and Biocontrol Potential from Wild Pistachio Trees Plant Pathol. J. 34 208-217

[11] Ayitto A S and Onyango D M 2016 Isolation And Identification by Morphological and Biochemical Methods Of Antibiotic Producing Microorganisms from the gut of Macrote rms Michael seni in Maseno, Kenya J. Appl. Biol. Biotech. 4 027-033

[12] Sawale A A, Kadam T A and Mitkare S S 2013 Isolation and Characterization of Secondary Metabolites from Halophilic Bacillus Species from Marin drive in Mumbai J. App. Pharm. Sci. 3 182-188.

[13] Campbell J 2011 High-throughput assessment of bacterial growth inhibition by optical density measurements Curr. Protoc. Chem. Biol. 3 1-20

[14] Sugrani A, Natsir H, Djide M N, Ahmad A 2019b Biofunctional protein fraction from red algae (Rhodophyta) Eucheuma spinosum as an antibacterial and anticancer drug agent Int. Res. J. Pharm. 10 64-69

[15] Mostafa A A, Al-Askar A A, Almaary K S, Dawoud T M, Sholkamy E N, Bakri M M 2018 Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases Saudi J. of Bio. Sci. 25 361–366

[16] Sugrani A, Natsir H, Djide M N, Ahmad A 2019a Antibacterial and anticancer activity of protein sponges collected from the waters of Kapoposang island of south Sulawesi, Indonesia Int. Res. J. Pharm. 10 82- 87

[17] Noverita F D, Sinaga E 2009 Isolation and test of the antibacterial activity of endophytic mushrooms of the leaves and rhizomes of Zingiber officinale Val Indonesian Phar. J. 4 171-176

[18] Sammour R, 2012 Biotechnology - Molecular Studies and Novel Applications for Improved Quality of Human Life (Croatia: InTech) p 28-30

[19] Hollants J, Leliaert F, De-Clerck O, and Willems A 2012 What We can Learn from Sushi: A Review on Seaweed-Bacterial Associations FEMS Microbiology Ecology 1-16.

[20] Silhavy T J, Kahne D, and Walker S 2010 The bacterial cell envelope Cold Spring Harbor perspectives in biology 2 1-16.

[21] Ihsan B and Retnaningrum E 2017 Isolation and Identification of bacteria Vibrio sp. on the shells (Meretrix meretrix) in the Trenggalek district Journal Harpodon Borneo 10 23-27.
[22] Setyati W A, Martani E, and Zainuddin M 2015 Growth kinetics and activity Protease with Isolate 36k from Mangrove ecosystem, Karimunjawa, Jepara Indonesian J. of Mar. Scie. 20 163-169

[23] Nemeth J, Oesch G, and Kuster S P 2015 Bacteriostatic versus bactericidal antibiotics for patients with serious bacterial infections: systematic review and meta-analysis J Antimicrob Chemother. 70 382–395

[24] Ibrahim and Nur E 2015 Cytotoxicity study on Maerua pseudopetalosa (Glig and Bened.) De Wolf tuber fractions African Journal of Plant Science. 9 490-497