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RESEARCH ARTICLE

Screening of *Haliclona* sp. symbiont bacteria that have the potential as MDR (Multidrug-Resistant) antibacterial from Tanjung Tiram Beach

Sri Anggarini Rasyid [1], Sugireng [1], Asni Ramayana Tina [1]

[1]Medical Laboratory Technology Study Program, Faculty of Science and Technology, Mandala Waluya University, Kendari, Indonesia

Correspondence:
Sri Anggarini Rasyid
Jl. Jend. A. H. Nasution No. 37 G, Kelurahan Kambu, Kendari 93231, Indonesia

Email: anggarini.09@gmail.com

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Abstract

Sponge *Haliclona* sp. is a type of sponge living in marine waters and is found in many areas of coral reef ecosystems. This study aimed to determine the potential for sponge *Haliclona* sp. obtained from Tanjung Tiram waters, Konawe, as a source of bioactive compounds of MDR (Multidrug-Resistant) antibacterial. The isolation method of *Haliclona* sp. symbiont was carried out by pouring and purification of *Haliclona* sp. symbiont using the scratch method. The purification results obtained 12 isolates of symbiont bacteria that were successfully isolated. Then the bacterial isolate was tested for its antibacterial ability against MRSA and ESBL *Escherichia coli* bacteria qualitatively using the agar diffusion method. Bacteria that have strong inhibitory activity are identified by morphological and biochemical tests. The results of the qualitative screening test produced four bacterial isolates that have inhibitory activity against MRSA (*Methicilin Resistant Staphylococcus aureus*) pathogenic bacteria with the isolate codes H3 and H8 belonging to the strong category while H4 and H10 belonging to the medium categoy. Additionally, two bacterial isolates that have inhibitory activity against ESBL *Escherichia coli* pathogenic bacteria with the isolate codes H3 and H8 belonging to the strong category. Based on morphological observations and biochemical tests, the H3 bacterial isolate was identified as the genus *Corynebacterium* spp. and the H8 bacterial isolate was identified as the genus *Micrococcus* spp. In conclusion, *Haliclona* sp. sponge exhibits promising potential as a source of microorganism producing antibacterial compounds, particulary againts MDR (*Multidrug-Resistant*) strains.

1. INTRODUCTION

Animals belonging to the Porifera phylum, known as sponges, are components of coastal and marine ecosystems, especially coral reef ecosystems. Although not commonly used, sponges offer bioactive potential as antibacterial, anti-cancer, and antifungal agents. In the Indonesian seas, there exists between 850-1500 different species of sponges (1). It is well known that various bacteria inhabit sponges, making up about 40% of their biomass. When compared to other marine and land animals, sponges are marine invertebrates with the highest antibacterial potential. This is due to the symbiotic relationship between sponges and bacteria (2).

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Microorganisms present in water represent a promising resource for the discovery of bioactive chemicals is. It is often seen that nutrient-demanding marine bacteria form symbiosis with various other marine species, such as sponges. Sponges are one of the marine biotas that can be found both in deep and shallow coastal areas (3). Natural compounds isolated from marine sources may play an important role the discovery of new antimicrobial agents. Beyond contributing to our understanding of microbial community structure, bacteria forming symbiotic relationship with sponges have the capacity to produce bioactive chemicals, offering solutions to challenges in the delivery of bioactive substances (4). In the region of Manado, several bioactive chemicals, including novel compounds, have been identified from marine organisms, with sponges being among them (5).

Bacteria engaged in symbiosis relationship with organisms undergo biochemical interaction with their host. As a consequence of these interactions, symbiotic bacteria often produce similar bioactive compounds to those their hosts. This phenomenon raises the prospect that various in symbiosis with soft corals, for instance, may produce bioactive substances with potential applications as antibacterial agents (6).

Staphylococcus aureus and *Escherichia coli* are bacteria that often cause infections in humans, posing a significant threat to human life. Therefore, there are various ways to prevent or treat the diseases. Treatment of infections caused by bacteria can be overcome by giving antibiotics/antibacterial. Antibacterial is compound used to control the growth of harmful bacteria (7).

S. aureus is treatable with antibiotics, but several strains of *S. aureus* found to be resistant to antibiotics, such as Methicillin-Resistant *Staphylococcus aureus* (MRSA) (8). *S. aureus* and *E. coli* have developed resistance to antibiotics on the market, causing problems in medical therapy (9). Antibacterial activity screening seeks to identify bacteria capable of inhibiting the growth of these resistant strains, serving as test organisms. It is crucial that the test bacteria selected represent both Gram-positive and Gram-negative bacteria, as both *E. coli* and *S. aureus* are pathogenic to humans (10).

Sponge *Haliclona* sp. is a particular type of sponge that thrives in marine waters and is found in many areas of coral reef ecosystems. It is well known that the content of secondary metabolites from the sponge has the power to ward off and inhibit harmful germs. Sponges have the potential to be developed in the realm of pharmacology, namely as antibiotics, making them one of the interesting marine biotas to study (11). Bacteria, widely distributed in nature, serve as producers of antibiotics with extensive commercial use. Nature is the main source of discovery of new antibacterials. The exploration of bioactive compounds from these bacteria has become a significant area of interest for researchers. Therefore, this study aims to isolate bacterial strains from *Haliclona* sp., sourced from the Tanjung Tiram Beach in South Konawe Regency.

2. MATERIALS AND METHODS

This study is exploratory in nature, aiming to identify *Haliclona* sp. symbiont bacteria with the potential as antibacterial agents against Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Extended-Spectrum Beta-Lactamase (ESBL).

2.1. Sampling

The bacterial isolates employed in this research were sourced from *Haliclona* sp. collected at Tanjung Tiram beach, North Moramo Sub-district, South Konawe Regency, Southeast Sulawesi. Subsequently, sea cucumbers were carefully placed into sterile plastic containers and stored in a cool box for transportation to the laboratory.

2.2. Isolation and Antibacterial assay

First, the sample is collected from the host organism. Then, the sample is subjected to various laboratory techniques to separate and purify the bacteria. Theses techniques encompass culturing the bacteria on specific growth media, utilizing differential staining methods for bacteria identification, and conducting biochemical tests for further characterization. The isolated bacteria are then studied to understand their role in the symbiotic relationship with *Haliclona* sp. and to explore their potential applications in various fields. The symbiotic bacteria of *Haliclona* sp. were obtained by employing the pour plate method on NA (Nutrient Agar) media. Following the incubation period, the observation of colony morphology was carried out to identify the isolates that were successfully purified using the streak plate technique.

These purified isolates were subsequently preserved on agar slants as stocks of pure isolates, in preparation for subsequent examinations. The production of bacterial secondary metabolites is a complex process that involves various stages and mechanisms. These metabolites, which are distinct from primary metabolites, play important roles in the survival and adaptation of bacteria in their environments.

The production of bacterial secondary metabolites is intricately influenced by multiple factors, including both genetic and environmental factors. Bacterial secondary metabolites have diverse functions, such as antimicrobial activity, signaling, and competition for resources. Understanding the production of these metabolites is crucial for various applications, including drug discovery, agriculture, and biotechnology. Researchers employ various techniques and approaches to study and manipulate the production of bacterial secondary metabolites, with the goal of harnessing their potential benefits for human health and other fields (12).

Secondary metabolite compounds are synthesized by bacteria during the stationary phase of growth. To initiate this process, a bacterial culture constituting 10% (10^7 cells/mL) of the total volume is inoculated into a 50 mL production media Nutrient Agar (Merck, Darmstadt, German). The culture is subsequently incubated using an incubator shaker for the optimal duration. Following incubation, the production media is centrifuged at a speed of 10.000 rpm for a duration of 10 minutes to separate the supernatant (13).

The evaluation of secondary metabolite compounds against multidrug-resistant bacteria, specifically MRSA and ESBL *E. coli,* was conducted. An experiment was designed to assess the efficacy of bacterial metabolite compounds as antagonists. The bacterial secondary metabolites, in the form of supernatant (free cells), were tested. In the experiment, paper discs measuring 5 mm in diameter were inoculated with bacterial supernatant containing a cell density of 10^7 cells/mL (14). These discs were then placed on the surface of agar media inoculated with MRSA and ESBL *E. coli* bacteria, also at a density of 10^7 cells/mL. The plates were incubated at 37°C for 24 hours. The clear zones formed around the discs were measured using digital calipers. During incubation, antimicrobial compounds diffuse into the agar medium. The results were observed by measuring the drag diameter (mm) and comparing it with Kirby Bauer's CLSI standard (15). It was observed that the symbiotic bacteria of *Haliclona* sp. exhibited activity, as evidenced by the formation of clear zones around the colonies (16). The positive control utilized the antibiotic erythromycin (Kimia Farma, Jakarta, Indonesia) with a concentration of 0.01%, while the negative control employed sterile aquadest.

2.3. Identification of the Symbiont Bacteria from *Haliclona* sp.

Characterization and identification were carried out for bacterial isolates that had a strong zone of inhibition based on the Bergey's Manual of Determinative instructions by observing cell and colony morphology (Microscopic) (Table 1), then and biochemical tests.

3. RESULTS AND DISCUSSION

3.1. *Haliclona* sp. Sponge Symbiont Bacteria Isolates

The sample utilized in this study was obtained from *Haliclona* sp. collected at Tanjung Tiram beach, South Konawe. The collection process involved snorkeling at a depth of 1-3 m above the water surface. Sponge specimens of *Haliclona* sp. were specifically selected for the isolation and subsequent testing of their antibacterial potential.

Figure 1. *Haliclona* sp. sea sponge (used in this study)

Haliclona sp. has the characteristics of a porous body like foam or sponge and a hard surface like a stone. Features of *Haliclona* sp. includes its two multicellular (dipoblastic) body layers, radial symmetry or asymmetry, rudimentary tissue formation, and gelatin content (mesenchyme) (17).

The morphological characteristics of bacteria isolates from *Haliclona* sp. were observed and documented Table 1, depicting macroscopic colony features of 12 bacterial isolates after 24 h incubation on NA medium. The recorded characteristics include colony colour, shape, border and height. Notably, the obtained of 12 isolates exhibited nearly identical colony morphology, with milky white or yellowish-white color, both round and irregular shape. Insulated edges are solid and contoured, with flat, raised and raised bumps. In general, the morphological characteristics of colonies may not serve as the basis for identification at the genus level, but can only be used as

the basis for the differences between isolates. Variations in colony characteristics indicate differences between bacterial isolates (17).

According to the separation results of nutrient agar medium and the observation of colony morphology, 12 strains of symbiotic bacteria were obtained, namely H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11 and H12 (Table 1).

Table 1. Morphological characteristics of different bacterial isolates

Isolates code	Shape	Form	Colour	Margin	Elevation	Surface
H1	Circular	Circular	White	Entire	Flat	Smooth
H2	Circular	Circular	White	Undulate	Raised	Smooth
H3	Circular	Circular	Cream	Undelate	Convex	Smooth
H4	Iregular	Circular	White	Undelate	Flat	Smooth
H5	Iregular	Circular	White	Lobate	Raised	Smooth
H6	Circular	Circular	White	Entire	Raised	Smooth
H7	Iregular	Circular	White	Undelate	Raised	Smooth
H8	Iregular	Circular	White	Undelate	Convex	Smooth
H9	Iregular	Circular	White	Undelate	Raised	Smooth
H10	Circular	Circular	White	Entire	Raised	Smooth
H11	Iregular	Circular	White	Lobate	Raised	Smooth
H12	Circular	Circular	White	Entire	Raised	Smooth

3.2. Potential Inhibitory Power of Symbiont Bacteria Isolates Against MRSA and ESBL *Escherichia coli*-Producing Bacteria

The results of the study on *Haliclona* sp. revealed the impact of commensal bacteria on MRSA bacteria, indicating the inhibitory effect of commensal bacteria on MRSA growth. The measured diameter of the zone of inhibition for the MRSA test bacteria, which served as the positive control (erythromycin), was larger than the zone formed around the paper disk extract from sponge *Haliclona* sp. Specifically, the inhibition zones for separates isolates H3 (12.30mm), H4 (6.76mm), H8 (10.25mm), H10 (8.20mm) and positive control (19.25mm). Detailed information is presented in Table 2.

Table 2. Inhibition of *Haliclona sp.* commensal bacteria against MRSA bacteria and ESBL *Escherichia coli*-producing bacteria

Isolate code	MRSA bacteria		ESBL *Escherichia coli*-producing bacteria	
	Diameter of Zone of Inhibition (mm)	Category*	Diameter of Zone of Inhibition (mm)	Category*
H1	-	-	-	-
H2	-	-	-	-
H3	12.30	Sensitive	13.70	Sensitive
H4	6.76	Intermediate	-	-
H5	-	-	-	-
H6	-	-	-	-
H7	-	-	-	-
H8	10.25	Sensitive	10.80	Sensitive
H9	-	-	-	-
H10	8.20	Intermediate	-	-
H11	-	-	-	-
H12	-	-	-	-
Control + (Erythromycin)	19.25	Intermediate	13.25	Intermediate
Control - (Sterile aquadest)	-	-	-	-

*Criteria for resistance according to diameter of Zone inhibition interpretive standards of infrequently isolated or fastidious bacteria defined by the CLSI were employed as breakpoints for ciprofloxacin, erythromycin, and tetracycline.

Comparison of the inhibition zone diameters of negative control (aquadest), positive control (erythromycin) and *Haliclona* sp. symbiont bacteria against MRSA bacteria is presented in the following Figure 2. In a standard antibacterial susceptibility test, such as a disc diffusion assay, the inhibition zone diameter is a measure of the effectiveness of an antimicrobial agent against a specific bacterial strain. The larger the inhibition zone, the more effective the antimicrobial agent is against the tested bacteria.

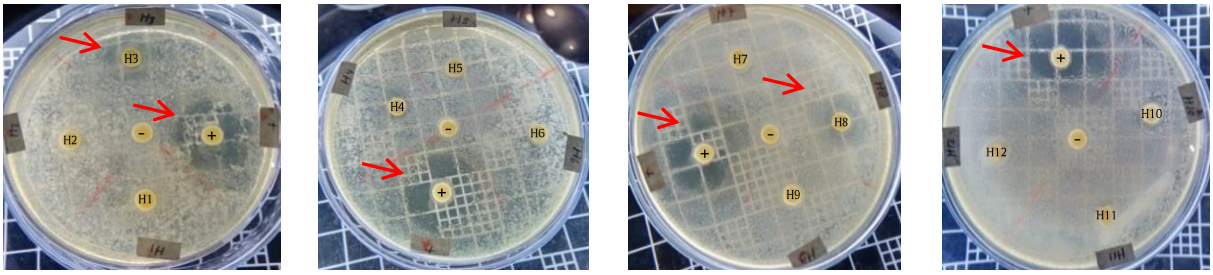

Figure 2. The results of the inhibition zone test of *Haliclona* sp. symbiont bacteria in inhibiting MRSA. Bacteria Isolates H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, + (positive control), - (negative control).

The inhibition test results for *Haliclona* sp. symbiont bacteria againts ESBL revealed the symbiont bacteria's capability to inhibit the growth of ESBL *Escherichia coli*-producing bacteria. In terms of inhibition zone diameter measurements, the positive control (gentamicin) exhibited smaller zones than those formed around the paper disc extract of sponge *Haliclona* sp. Specifically, the inhibition zone diameter measurements for ESBL *E. coli*-producing bacteria, including isolates H3 (13.70 mm), H8 (10.80 mm) and positive control (13.25 mm) are presented in Table 3.

Comparison of the inhibition zone diameters of negative control (aquadest), positive control (gentamicin), and sponge extract of *Haliclona* sp. against ESBL Escherichia coli-producing bacteria is presented in the following Figure 3.

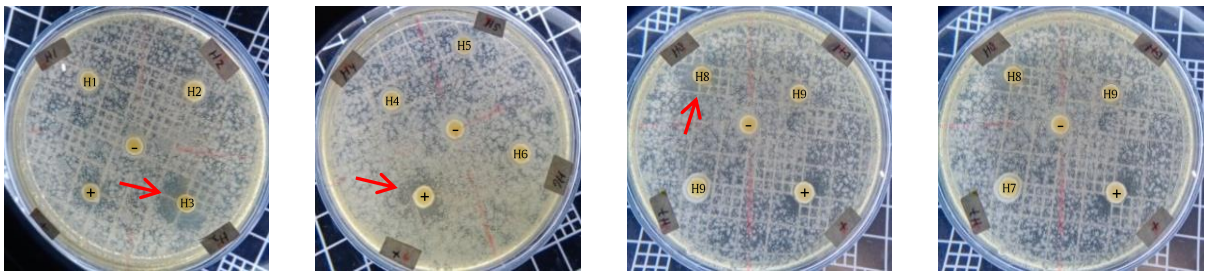

Figure 3. The results of the inhibition zone test of *Haliclona* sp. symbiont bacteria against ESBL. Isolates H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, + (positive control), - (negative control).

The results presented in Tables 2 show the results of the inhibition tests performed on 12 successfully purified bacterial isolates. All isolates were tested for activity against and ESBL *E. coli* pathogens using secondary metabolites using the paper plate method. According to the results of qualitative screening tests, four strains were prepared, the strain codes were H3, H4, H8 and H10, which had inhibitory activity against MRSA pathogenic bacteria. Additionally, two bacterial isolates (H3 and H8) exhibited inhibitory activity against ESBL-*E. coli* pathogenic bacteria, as determined through qualitative screening tests. It suggests that the symbiont bacteria may produce compounds with antimicrobial properties against MRSA.

Based on the results of the sensitivity test, as depicted in Table 2, the qualitative screening revealed isolates with the highest inhibition against MRSA pathogenic bacteria. Specifically, isolates H3 (12.30 milimeter), H8 (10.25 milimeter), H10 (8.20 milimeter), and H4 (6.76 milimeter) demonstrated significant inhibition zone. Notably, isolates H3 and H8 exhibited the largest inhibition zone areas after the positive control with erythromycin (19.25mm). Erythromycin is a macrolide class of antibiotics produced by Streptomyces erythreus. The mechanism of action of erythromycin is to inhibit protein synthesis by binding to the 50S ribosomal RNA subunit of bacteria. Erythromycin is active against Gram-positive bacteria such as MRSA. This one form of antibiotic resistance by MRSA bacteria is resistance to the erythromycin class of antibiotics (18,19).

3.3. Results of Characterization of Potential Symbiotic Bacterial Isolates

Symbiotic Bacterial Isolates which have a strong inhibitory zone and have antibacterial potential is carried out based on the instructions of Bergey's Manual Determinative Bacteriology. This involved the observation of cell shape, Gram staining of bacteria, biochemistry test. Characterization data are shown in Table 3.

Based on the results of the sensitivity test, isolates H3 (13.70 milimeter) and H8 (10.80 milimeter) demonstrated the highest inhibition against ESBL *E. coli* pathogenic bacteria. Isolate H3 is the isolate with a larger inhibition zone zona than the gentamicin positive control (13.25 milimeter). Gentamicin, an aminoglycoside class of antibiotics widely used to treat infectious diseases, is effective against Gram-negative bacteria, including *Pseudomonas* sp., *E. coli*, and Enterobacter (20).

Table 3. Results of characterization of potential symbiotic bacterial isolates

Isolat code	Biochemistry Test								Cell Shape	Gram	Identification Result
	Carbohydrat fermentation	MR-VP	Citrat	Motilitas	TSIA	Indol	Katalase	Mannitol			
H3	+	-	+	+	-	-	+	+	Bacil	Positive	*Corynebacterium* spp.
H8	-	-	+	+	+	-	+	-	Coccus	Positive	*Micrococcus* spp.

Characterization results indicated that symbiont bacterial isolate *Haliclona* sp. H3 and H8 have the potential as anti-MRSA and anti-ESBL agents. Specifically, the H3 isolate belongs to the genus *Corynebacterium* spp., while the H8 isolate belongs to the genus *Micrococcus* spp. Additionally, *Micrococcus* sp. bacteria found in symbiosis with black sea cucumbers, have demonstrated antibacterials potential against *Bacillus cereus*. Bacterial quality test revelaed that the concentration of lactic acid from the isolates effectively inhibited the growth of *B. cereus* (21).

In this study, the results showed that the results of bacterial isolates of isolates H3 and H8 have a broad antibacterial spectrum zone because they show antibacterial activity for Gram-negative (ESBL *E. coli*) and Gram-positive (MRSA) bacteria. In contrast, isolates H4 and H10 have a narrow spectrum zone because they can only inhibit the growth of one group of bacteria, namely Gram-positive (MRSA) bacteria. These findings align with a previous study on the inhibitory test of sea sponge extract (*Callyspongia aerizusa*) on the growth of *Salmonella typhi* and *Streptococcus pyogenes* bacteria. The study revealed that Gram-positive bacteria are more susceptible to the active substances in extracts compared to Gram-negative bacteria, due to differences in the cell wall structure. Gram-negative bacteria possess a complex lipopolysaccharide structure in their cell wall, which can impede the penetration of the extract into the microbial cell. In contrast, Gram-positive bacteria have an outer membrane formed by a peptidoglycan layer which is easily penetrated by the active compounds in the test material (22).

This proves that sponge *Haliclona* sp. has bioactive compounds that can inhibit the growth of ESBL *E. coli* bacteria and MRSA bacteria. The relatively dominant inhibition zone observed the bacterial isolates are particularly noteworthy againts Gram-positive bacteria (MRSA). Among the four bacterial isolates from *Haliclona* sp., namely H3, H4, H8, and H10, each displayed clear zone around the paper discs, indicating their ability to inhibit the growth of the tested bacteria.

The width of the diameter of the inhibition zone formed around the paper discs can be used as a parameter to see the strength of the bioactive compounds contained in sponge *Haliclona* sp. The wider the inhibition zone formed, the stronger the bioactive compounds inhibit bacterial growth. The antibacterial strength of an extract is determined by the size of the inhibition zone. An extract considered to have a strong inhibitory effect if the resulting inhibition zone diameter is around 10 – 20 milimeter. If the diameter falls between 5 – 10 milimeters, the extract is categorized as having a moderate inhibitory effect. An extract is deemed to have a weak inhibitory effect if the diameter of the resulting inhibition is less than 5 milimeter (23).

The antibacterial sensitivity test showed number antimicrobial sensitivity to Haliclona sp. extract, with isolate codes H1, H2, H5, H6, H7, H9, H11, and H12. Because the antimicrobial activity is characterized by the presence of a clear zone around the paper discs which indicates the ability to inhibit the growth of the tested bacteria. Antimicrobial or antibiotic sensitivity to *Amphimedon* sp. sponge extracts and fractions. This suggests that there is no symbiotic association between *Amphimedon* sp. and the tested isolates (H1, H2, H5, H6, H7, H9, H11, and H12), leading to a lack of secondary metabolite production such as alkaloids, terpenoids, steroids, quinones, phenols, etc. Most of these compounds are known for their potential as bioactive compounds (24). Therefore, it can be stated that *Haliclona* sp. extracts, with isolate codes H1, H2, H5, H6, H7, H9, H11, and H12 are unable to produce secondary metabolites as bioactive compounds.

In this study, distilled water served as a negative control, functioning as a diluent solution in both the sponge *Haliclona* sp. extract and positive control. As a negative control in the antibacterial tests against ESBL *E. coli* and MRSA, aquadest exhibited no formation of an inhibition zone around the paper discs. The absence of an inhibition zone around the wells treated with sea sponge *Callyspongia aerizusa* extract, compared to the negative control, indicates that the diluent solution had no impact on the formation of the inhibition zone (25).

The size of the inhibition zone can be influenced by the density or viscosity of the media, the rate of diffusion of antibiotics, the concentration of antibiotics, the sensitivity of organisms to antibiotics, and the interactions of antibiotics with the media (25). Meanwhile, some microbes that live in the sea are generally difficult to culture in the laboratory. Some secondary metabolite-producing microbes can lose their ability to produce secondary metabolites when stored for a certain time in artificial media (23). This is due to unfulfilled nutritional needs in artificial media. In some cases, strains producing secondary metabolites are under stress. Loss of the ability of a bacterial strain to produce secondary metabolites can also be caused by the abiotic environment.

4. CONCLUSIONS

The results of the qualitative screening test identified four bacterial isolates, namely H3, H4, H8, and H10, with potential application as anti-MRSA agents. Additionally, two bacterial isolates, namely H3 and H8 demonstrated potential as anti-ESBL *E. coli* agents. Therefore, sponge *Haliclona* sp. exhibits promise as a microorganism capable of producing antibacterial compounds, serving as an effective againts MDR (*Multidrug-Resistant*) bacteria.

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