Secondary Metabolite Analysis and Anti-Bacterial and Fungal Activities of Marine Sponge Methanol Extract Based on Coral Cover

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Abstract. Conservation of coral reefs and marine life is important to maintain population and biodiversity. The aim of the study was to determine the relationship between the inhibition of sponge extract on the growth of bacteria and fungi to analyze the relationship formation of bioactive metabolic substances produced by sponges and their relationship with coral cover conditions.Methods of coral cover observation, habitat analysis and secondary metabolite content, anti-bacterial and fungal activity tests and sponge methanol extract. Results: Three types of sponges, each *Clathria reinwardri* (CR) lived on medium coral reef cover, live coral and partly on the abiotic component. *Hyrtios erectus* (HE) and *Callyspongia aeresuza* (CA) were obtained from damaged coral cover, some sandy live coral and abiotic components.Inhibition of CR > CA > HE sponge methanol extract against the growth of fungi and bacteria. The inhibition of sponge methanol extract against the growth of Escherichia coli (EC) and Staphylococcus aureus (SA) and Malassezia furfur (MF) fungi was the largest by CR sponge methanol extract, but did not have inhibitory activity against Candida albicans (CA). The growth and characterization of the metabolites produced by sponges are influenced by the environmental conditions of coral reef growth and cover.

INTRODUCTION

Indonesian marine waters have various types of sponges, some of which contain potential bioactive compounds as basic ingredients in the pharmaceutical industry [1]. Polar and non-polar extracts of several types of sponges are known to have anti-bacterial and anti-fungal functions, this is an early indication of the potential for the use of active sponge substances in the pharmaceutical and cosmetic industries [2]. Most of the compounds produced by sponges are antibacterial, antifungal, antitumor/ anticancer, antiviral, cytotoxic, and certain antienzymes [3]-[4]. Some sponges are also known as biological indicators for monitoring marine pollution, such as Spongia officinalis, Spongia agaricina, and Spongia nitens from measurements of heavy metal content such as Fe, Pb, Cd, Zn, Ni, Mn, Cu, V, Hg, and several pesticide compounds, like DDT and PCB [5]-[7].The Investigating diversity of sponges that exist in a number of marine areas in Indonesia, it is known that approximately 850 species of sponge contain active substances in the form of chemical components produced as primary and secondary metabolite products, and as

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many as 200 species of them are found along the waters of the Spermonde Islands [8].

Sponges are marine invertebrates that produce secondary metabolites whose further development is the largest source of bioactive compounds among other marine invertebrates. In the 2000s, it was reported that 50% of the bioactive compounds found in marine invertebrates originated from the phylum of sponges [9]. In maintaining its life, sponges adapt morphologically, anatomically, physiologically and chemically by producing enzyme-acting substances that cover the surface of the sponge's body.The production of secondary metabolites by sponges is a form of resistance against predators that threaten their life, carried out by interacting with the biotic, abiotic environment and as a chemical weapon to ward off predator attacks [1],[10]. The types of chemical compounds produced by sponges, such as terpenes, polyketides and alkaloids, are due to competition with corals and prevent infection with pathogenic bacteria. The ability of sponges to produce chemical compounds is very much determined by physical, chemical and biological factors of their habitat, while ecological factors affect the shape and growth of sponges, namely water depth, basic structure, current, temperature, nutrient content, pH, salinity and sedimentation rate [11].Secondary metabolites for marine sponges play a role in food search, introduction to their population, habitat determination, and suitable symbiotic pairs. Besides chemical compounds produced by marine invertebrates, in response to competition with the environment [12].

Coral reefs are a living habitat for various tropical marine life, including sponges. The diversity of biota species that interact and associate with coral reefs is directly proportional to the various shapes and color variations. Association of various types of biota on coral reefs, sponges occupy a very specific ecological niche, namely the bottom of clear waters that still get enough sunlight [13]-[15]. The phenomenon of coral cover, secondary metabolic and inhibition of sponge extracts against the growth of fungi and bacteria that guided this research was carried out for the purpose of predictive analysis and dynamics and the effect of inhibition of sponge methanol extract on the growth of fungi and tested bacteria. The types of secondary metabolic and bioative substances produced by sponges, are also thought to be influenced by the condition of coral reefs, indicating that there is a reciprocal relationship in the formation of secondary metabolics and an effect on the condition of coral cover in the habitat for sponge growth [16]-[17].

DATA AND METHOD

Materials

Research samples in the form of sponges and coral reefs were obtained around the coast on three different islands, namely Cambang-Cambang Island, Cangkeh Island and Gondong Island Bali, these three islands are included in the administrative area of Pangkajene and Islands Regencies (Figure 1).

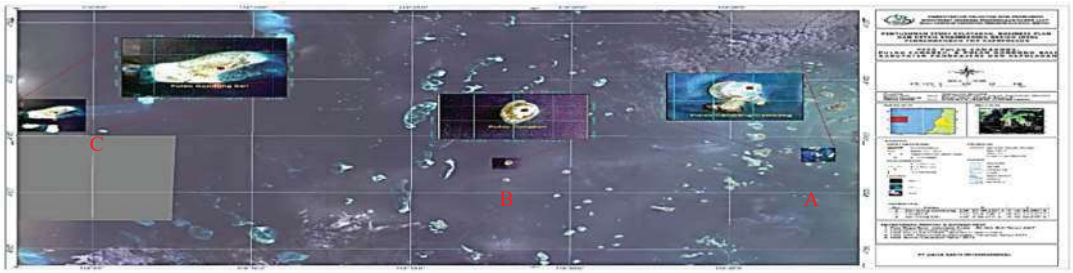

FIGURE 1. Map shows the point of sponge sampling and coral reef observation. Point A is the surrounding water Cambang-Cambang Island, point B is Cangkeh Island and point C is Gondong Bali Island, these three islands are included in the administrative area Pangkajene Regency and Islands, Spermonde Archipelago Cluster

Three islands in the sampling location are small islands that are included in the Spermonde Archipelago Cluster. There are three sponge sampling points in the coral reef area, each with the coordinates of point A (Cambang-Cambang island): 119^0 27'49.156 "E and 04^0 46 '31.291"S; point B Cangkeh Island: 119^0 18 '04.194" E and 04^0 47' 19.131" S and point C (Gondong Island Bali): 119^0 03' 50.277" E and 04^0 42' 58.974" S. The average depth of the sponge sampling points of each point A: 3 m; point B: 6 m and point C: 11 m. The determination of the depth of the sponge sampling point is based on the common habitat of sponge species. *Clathria reinwardri* (CR) sponges are

generally found at a depth of 3-6 m; *Hyrtios erectus* (HE) at a depth of 5-10 m and *Callyspongia aeresuza* (CA) at a depth of 9-15 m [2,17]. The research materials consisted of three types of sponges, namely CR; HE and CA, while there are two types of tested fungi, namely Candida albicans (CA) and Malassezia furfur (MF) and also two types of test bacteria Escherichia coli ATCC 25923 (EC) and Staphylococcus aureus ATCC 25922 (SA).

Sampling Technique

Field data collection includes observations of coral reefs, observations of sponges and habitats. Observation of the condition of coral reefs at the sponge sampling site was carried out using the line transect method [18]-[19]. The transect method is carried out by placing the transect tool ± 50 m long, using a diving tool and writing equipment in the water, the biota on the transect line is recorded and measured with accuracy up to units of cm. The criteria for live coral cover are said to be very good if it is in the range of 75 – 100 % (damaged level 4), good if it reaches 50 - 74.9 % (damaged level 3), moderate if coral cover is in the range 25 - 49.9 % (damaged level 2) and is heavily damaged if it is in the range 0 - 24.9% (damaged level 1) [20,21]. Observation of the condition of the sponge habitat was carried out by observing the dominant condition of the habitat in which the sponge was grown and carried out sampling, then determining the condition of the habitat. Sponge sampling points were carried out at three different points based on different depths and environmental conditions from the dive.SCUBA (Self Contained Underwater Breathing Apparatus), at a point monitored by a GPS device. The sponge samples obtained were cleaned of garbage, washed with sea water, then preserved by placing them in a container filled with methanol until the samples were completely submerged during transportation [3],[22].

Sample Preparation

The sponge sample preserved in methanol is cut into small pieces, put in a maceration container, then methanol is added, left for 24 hours, the liquid is replaced with new methanol. The extraction was carried out for 3 cycles with the same amount of liquid. The dilute methanol extract was collected and concentrated using a rotary evaporator, then the evaporation was continued over a water bath until a thick methanol extract was obtained. Then the weight is determined. Thick extract was used to determine its activity against fungi and microbes [9,23].

The test bacteria were selected which had been rejuvenated for 18-24 hours. Selected types of test mushrooms that have been rejuvenated in SDA medium were suspended using NaCl solution, 0.9% physiological. he preparation of CA and MF test mushrooms was obtained from pure culture. CA fungi were cultured by taking one ose, then inoculated by streaking on slant PDA medium then incubated for 1 x 24 hours at room temperature, while for MF culture the same medium was used, but the incubation time was 3 to 5 days at room temperature [24,25,26]. he suspensions of EC and SA bacteria and AC and MF fungi, respectively, were diluted and homogenized until the turbidity was the same as the Mac Forland standard 0.5 tube or the test bacteria and fungi suspension had 25% transmittance to the sterile 0.9% NaCl blank, at wave 580 nm using a 13 nm diameter cuvette.

The positive control solution used was Ampicillin solution for antibacterial test with a concentration of 30 ppm. A total of 0.03 g of Ampicillin was dissolved in 100 mL of distilled water (a solution with a concentration of 300 ppm was obtained). The solution was pipetted as much as 1 mL then distilled water was added to the solution volume of 10 mL, while for the anti-fungal test used Nystatin and salicylic acid. The negative control solution used was DMSO [27,28].

Inhibition test of sponge bioactive substances against the growth of EC and SA bacteria using the agar diffusion method with iron cylinder iron with an inner diameter of 6 mm, an outer diameter of 8 mm and a height of 10 mm. The sterile MHA (Muller Hinton Agar) medium was cooled at 40^0 C - 45^0 C, then poured aseptically into a 20 mL volume petri dish and allowed to solidify as a base layer. After solidifying, put the test bacterial suspension each as much as 1 mL into 10 mL of MHA medium then homogenized and poured over the base layer and left half solid as the seed layer.A total of 7 anchors were placed aseptically using sterile tweezers on the surface of the seed layer. Each cultivator in one petri dish each filled with 250 µL of crude extract, DMSO (negative control) and Ampicillin (positive control). Petri dishes are labeled to differentiate the samples tested. Subsequently incubated for 24-48 hours at a temperature of 37^0 C [29,30].

Determination of the anti-fungal activity of the sponge methanol extract, begins by pouring the Saboroud Dextrose Agar (SDA) medium on the two types of tested mushrooms into a 15 mL volume petri dish, allowed to freeze (base layer), then pouring sterile SDA medium mixed with 10 mL of mushroom suspension CA test into

sterile petri dishes, allowed to half solidify. The same method was carried out on the MF test fungal culture using sterile SDA medium. Place the 7 fruit on the seed layer. Each cultivator in one petri dish was filled with 250 µg of crude extract, Bovin Serum Albumin (BSA) as a negative control and anti-fungal Nystatin and salicylic acid as a positive control.Incubated at room temperature for 1 x 24 hours for CA and MF for two to three days in room temperature incubators. The zone of inhibition of the sponge methanol extract was determined to measure the diameter of the resistance against the tested bacteria and fungi, carried out three times each using a shear ruler [7],[31].

RESULT AND DISCUSSION

Observation parameters of spongy habitat or coral reef cover and other types of coral include live coral cover, dead coral cover, abiotic coral cover and other coral conditions, are presented in Table 1.

TABLE 1. The condition of coral reefs and other corals at three sampling point locations

Sampling depth (m)	Benthic lifeform (%)					Coral reefs cover condition
	HCL	DCA	Algae	OT	Abiotic	
3	47.94	0	0	08.17	41.21	broken level 2
6	16.17	40.26	1.17	39.94	05.47	broken level 1
11	22.68	0	0	12.85	55.11	broken level 1

Note: HCL = *Hard coral life;* DCA = *Dead coral with algae* and OT = other reef state

Based on Table 1, it can be seen that the value of live coral cover (HCL) ranges from ± 47.94%. The largest percentage of live coral cover (HCL) was found at a depth of 3-5 m. The percentage of live coral cover (HCL) and non-living (abiotic) ecosystem components is greater than other components. This condition can be said that the condition of the HCL coral reef with a depth of 3 m is in the damaged category level 2, which is a good place to grow sponges. Dead coral cover overgrown with algae (DCA) reached ± 40.26 % was found at a depth of 5-7 m and other corals (OT) reached ± 39.94 %. Corals in this zone are categorized as damaged level 2 [8],[32]. The presence of dominant sponge samples at three points of depth ± 3-11 m from the water surface or on the seabed, the sponge sample collection points with dominant habitat conditions for each sponge type are presented in Table 2.

TABLE 2. Identified Sponge Types and Dominant Habitats based on sampling From the water surface

Sampling depth (m)	sponges type	Dominant Habitat
3	*Clathria reinwardri (CR)*	Live coral reef cover, there are abiotic components and other coral reef conditions
6	*Hyrtios erectus (HE)*	There are other coral reefs, dead coral cover and overgrown with algae and some live coral reefs
11	*Callyspongia aeresuza (CA)*	Live coral cover (encrusting), no algae and predominantly abiotic components

According to Table 2, above shows that the dominant sponge type in the 3-5 m depth zone is *Clathria reinwardri* (CR) with the dominant habitat in the form of live coral, abiotic components, not overgrown with algae and no other coral conditions. 5-10 m depth, found sponge type *Hyrtios erectus* (HE). The dominant habitat in this zone is dead coral overgrown with algae, other corals and a few live coral reefs, and there is an apparent abiotic ecosystem. At a depth of 10-15 m identified the species *Callyspongia aeresuza* (CA). The dominant habitat in this zone is in the form of abiotic ecosystems, live coral branching and encrusting and a small part of other coral conditions. The results of the analysis concluded that at a depth of 4-6 m the sunlight was sufficient, so that there was growth of algae and hard coral species due to the influence of currents, even though the condition of coral reef cover was in the damaged category level 1 [21,30]. The physical appearance of the sponges identified at each sampling point can be seen in Figures 2-4.

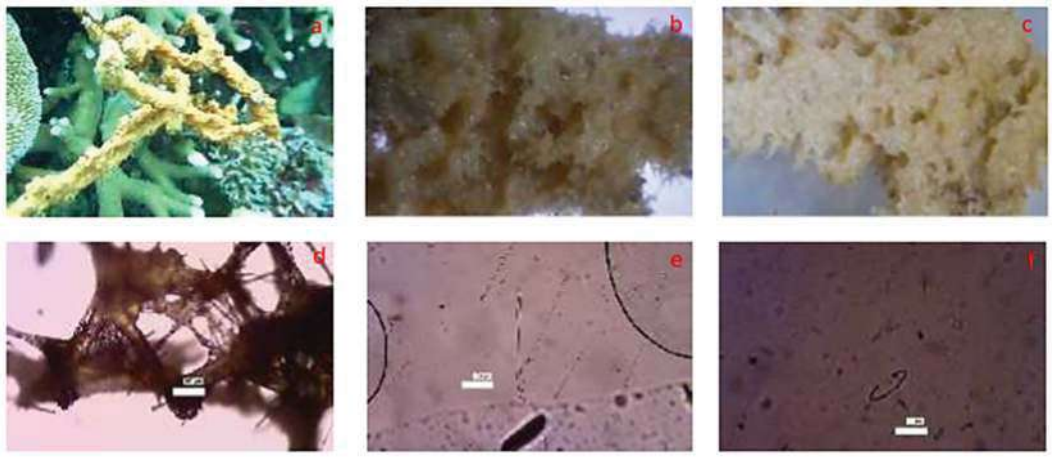

FIGURE 2. Morphology and structure of *Clathria reinwardri* (CR) sponge. a. Growth form: yellow Branching-repent sponge; b. Consistency: soft consistency, compressible sponge; c. Projection: Brittle sponge body when dried up; d. Choanosome: anastoming reticulate choanosome Skeleton; e. Megasclere: long-style megasclere; f. Microsclere: chelae microsclere

The morphology of the three types of sponges obtained at three different sampling locations and also at different depths, showed varying body structures. The body surface color of the CR type sponge is predominantly yellow, the HE type sponge looks black, while the CA type is a blue-yellow combination. Other differences are seen in the body structure and structure which has a Choanosome of varying shapes, as well as the skeleton in the form of fibers, the shape and arrangement of Megasclere and Microsclere also varies.The structure and morphology of these sponges are influenced by the habitat conditions for growth, especially the status of coral reefs, abiotic ecosystems, presence of algae, and other factors, such as depth, presence of pollutants or toxic contaminants, underscores, waves, exposure to sunlight, predatory biota and other factors [2],[10],[14]. This condition is a stimulus for the production of bioactive substances from the sponge in response to the dynamics that occur in their growing environment.

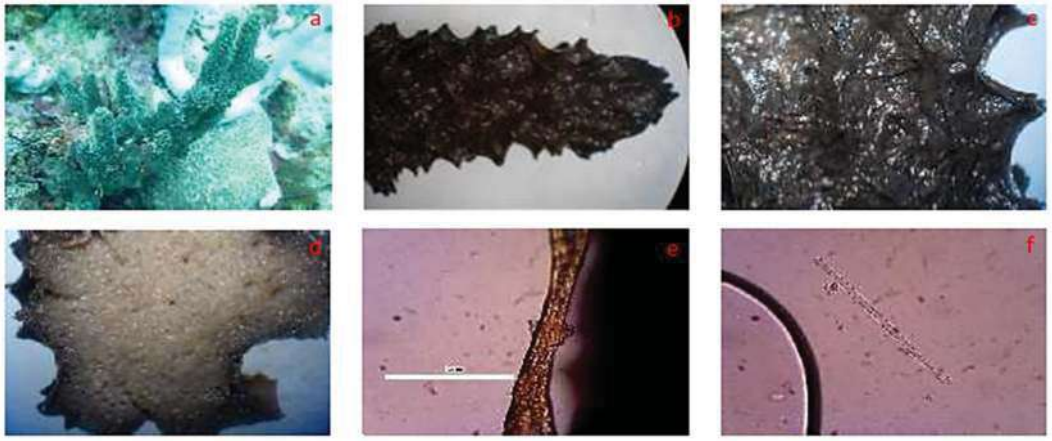

FIGURE 3. Morphology and structure of *Hyrtios erectus* (HE) sponge**.** a. Growth form: digitate Branching Sponges, black colored; b. Consistency: Unidentified oscular, firm, fleshy sponge Characteristic; c. Surface: pyramid-shaped ornament surface; d. Choanosome: Cut-section of the body. Heavily fiber body sponge; e. Skeleton: fibre pitched skeleton; f. Spicule: Substylostyle megaclera (magnification 10x), non microsclera

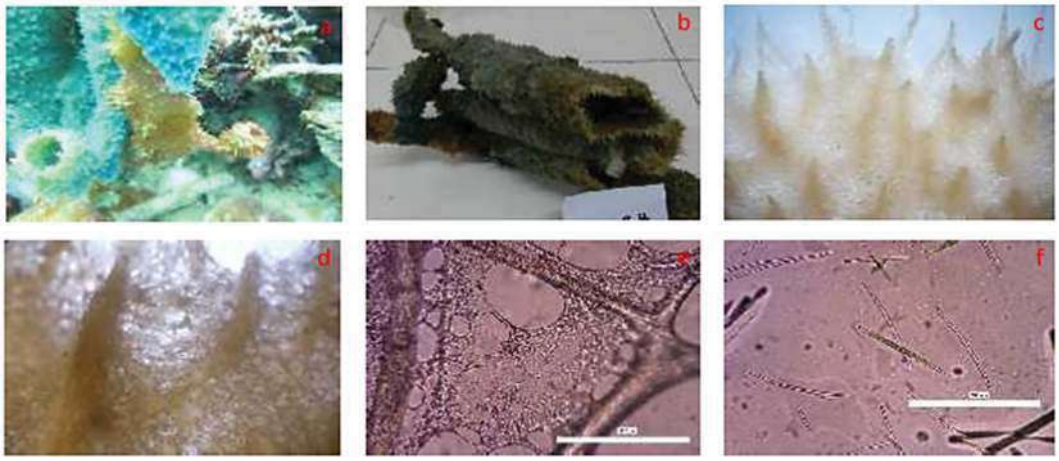

FIGURE 4. Morphology and structure of *Callyspongia aeresuza* (CA) sponge.a. Growth form: Cylindrical shape sponge, blue and Yellow colored sponge; b. Color in Etoh: the color fades After being prepared with alcohol; c. Surface: pappilate surface sponge; d. Skeleton: primary and Secondary fibre. Interconnected tract; e. Fiber: high density of fiber; f. Spicule: slender oxea (magnification 40x), no microsclera

Based on the qualitative analysis carried out, it is suspected that the bioactive types contained in the CA type sponge are compounds of the alkaloid group and the spongidae group contains Pyrrole-2-carboxylic derivatives, also thought to contain terpenes and sesquiterpenes. Methanol sponge extract type CR is thought to contain guanine and sulphonete compounds. Analysis of the zone of inhibition of methanol extract of three types of sponges obtained from locations with different characteristics. This analysis was carried out to obtain information and data on estimates of secondary metabolites content and other bioactive substances in sponges. The data in Table 3 suggests that the sponge contains several chemical compounds of secondary metabolites and systematically can be drawn a red line regarding aspects of the condition of coral reef cover (Table 1) in relation to the morphology and structure of the sponge body (Figures 2 - 4) and activity. Anti-fungal and anti-bacterial in estimating the content of secondary metabolites produced by sponges. The inhibition zone data for sponge methanol extract are presented in Table 3.

TABLE 3. Diameter of Barriers to Screening of Antimicrobial and Antifungal Activity Extracts on Sponge with a Concentration of 1000 µg/mL

Sponges type	Inhibition (mm) Methanol Sponge Extract Against Fungi and Bacteria				Status of Coral Reefs in Sponge Growth Sites
	Fungus		Bacteria		
	MF	CA	EC	SA	
Clathria reinwardri (CR)	22.14	1.15	16.68	21.17	Coral reefs are, partly alive, have an abiotic component
	20.21	0.87	18.21	20.57	
	22.37	1.04	17.52	19.83	
Hyrtios erectus (HE)	1.01	1.11	11.21	11.48	Coral reefs are damaged, dead and there are other types of coral
	1.71	1.19	9.82	12.26	
	1.21	1.23	9.46	11.74	
Callyspongia aeresuza (CA)	10.35	1.72	12.06	12.63	Coral reefs are damaged, there are abiotic components, some coral reefs are live
	10.03	1.11	13.03	13.26	
	11.27	0.89	11.87	13.37	

Note:
MF: Malassezia furfur CA: Candida albicans
EC: Escherichia coli SA: Staphylococcus aureus

Observation of the clear zone formed by measuring the diameter of the zone of resistance in each of the test methanol extract concentrations, namely: bacteria and fungi in each different environmental characteristics. Screening for antifungal and bacterial properties and zone of inhibition against fungi and bacteria can be seen in Table 3 and Figure 5.

The inhibition diameter of methanol extract of three types of sponges against the tested bacteria and fungi is shown in Table 3, the diameter of the inhibition zone of the methanol extract of the CR sponge against the EC test

bacteria is 17.47 mm in average and the average value of resistance for the SA test bacteria is 20, 52 mm, while the inhibition power of CA sponge methanol extract against MF fungi was 10.55 mm on average and 21.57 mm for CR sponge extract. In accordance with Table 3 and Figure 5, above the sponge methanol extract types CR, HE and CA have very little inhibition power relative to the CA function. The order of inhibition value of sponge methanol extract against MF fungi is CR > CA > HE, indicating that this type of sponge contains bioactive compounds which are strongly suspected to be Triterpenoids [17],[20],[25].

The sponge methanol extract also contains Saponin compounds which can interfere with the development of protozoa by bonding sterols on the surface of the protozoan cell membrane, causing the membrane to break and lysing cells to die. Saponins have a high level of toxicity against fungi, the mechanism of action as an anti-fungal is related to the interaction of Saponins with membrane sterols. The magnitude of the inhibition of the sponge types above against the tested bacteria and fungi is influenced by several factors such as sponge habitat, existing predators and is thought to have a relationship with exposure to waste in the sponge growth environment. The amount of metabolite content in this sponge is suspected by the number of predators, so that this type of sponge in self-defense stimulates itself to produce a shielding agent by producing chemical compounds in the form of secondary metabolites which are anti-toxic to predators around them [4],[18].

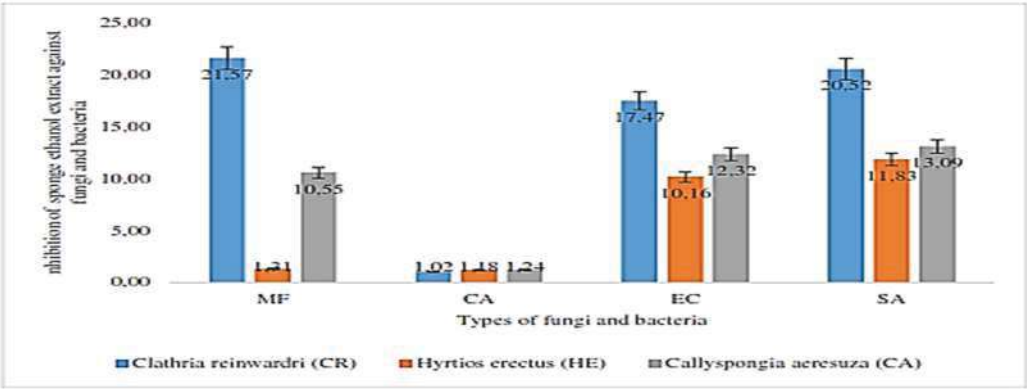

FIGURE 5. Histogram Inhibition average of Methanol Sponge Extract on fungi and bacterium

The anti-fungal test of Candida albicans using methanol extract of three types of sponge methanol extract showed very little inhibition in the range 1.01 - 1.24 mm, presumably because the Candida albicans fungus has a complex cell wall structure, with a thickness of 100-400 nm, besides that The cell wall of this fungus consists of five different layers, so it is suspected that the active components of the sponge methanol extract have difficulty penetrating the cell walls of this fungus.The MF anti-fungal test by sponge CR has the greatest inhibition power influenced by the content of sesquiterpen compounds which are anti-fungal, besides that the large bioactivity of the sponge extract is caused by the presence of chemical compounds that are immunosuppressive, neurosuppressive and anti-viral, Meanwhile, the lowest inhibition power was owned by the methanol extract of the HE type sponge [18],[21],[27].

Figure 3 shows the results of the CR sponge methanol extract activity test against bacteria and fungi compared to other sponge types. These results provide a strong picture that the formation of secondary metabolic compounds, especially the types of compounds and levels of chemical components, is influenced by multi factors, especially the condition of coral reef cover, abiotic ecosystems, algae and other coral cover, as well as the dynamics of the growth environment and physical factors, such as predators, exposure to waste, thus influencing the status of coral reef cover. The cover status was moderately damaged, and the dead coral where the CR sponge was obtained was different from the condition of the damaged coral reef in the sponge habitat, the HE and CA sponge growth environment. This situation triggers the sponge's stimulus to immediately produce metabolic compounds as a defense response to environmental threats in order to defend itself. This results in the presumption of the formation of secondary metabolic compounds of the carboxylate, terpenes, guanine and sulphonate groups [3],[8],[30].

Other factors that affect the inhibitory activity of the methanol extract of *Clathria reinwardri* against tested bacteria and fungi, except for the fungus Candida albicans, because this type of sponge lives in a habitat condition dominated by live corals with moderate damage and abiotic components. This assumption is illustrated by the formation of variations in metabolic compounds compared to the habitat conditions of the other two sponges which

are dominated by sandy corals and there are coral fragments, dead coral and abiotic components. Comparing the weaker inhibition of the methanol extract of the sponge *Callyspongia aeresuza* and *Hyrtios erectus*, with the status of the coral reefs at the location of the growth of the two types of sponges that were damaged and in the form of sand, broken coral, indicating that the dynamics of sponge life in the growth environment lacked adequate nutrients and presumptions. The predators do not vary, so there is no strong stimulus for the sponge to produce metabolic compounds for self-defense. This is confirmed by the notion that the only types of metabolic compounds produced are pyridine and alkaloids [10],[26]. The case of the Candida albicans test fungus which has very small inhibition zone activity shows that the sponge used is not caused by the absence of metabolic compounds formed, but rather the thickness of the CA fungal cell walls, so that the active substance from the methanol sponge extract is less able to penetrate the cell walls of Candida albicans fungi on certain contact period.

The content of secondary metabolites that can be produced by sponges is strongly influenced by the condition of the sponge habitat itself which can be seen physically in the condition of the coral reef habitat for sponge growth. This phenomenon is also influenced by the ability of sponges to adapt to environmental conditions where the sponge's body structure filters food, oxygen and releases food and CO_2 [3],[6],[22]. The sponge's nutritional pattern, which is a filter feeder, allows for the filtering of thousands to millions of trapped microbes, if the concentration of microbes is very large, the sponge will be infected and sick, so that the sponge is stimulated to produce chemical compounds capable of immobilizing trapped microbes. Microbes that are resistant to these chemical compounds will survive and live in symbiosis in the sponge's body, so it can be said that the types of metabolic compounds produced by sponges are bio-indicators that provide an overview of the dynamics and mechanisms of life experienced by sponges in their growth environment [3],[14]. The development of further research that refers to the alleged data on chemical compounds and metabolite components as well as their activity on fungi and bacteria can be used as a reference for analyzing the potential of sponge extracts as a new material for the manufacture of certain drugs, including investigation of spongy symbiotic microorganisms, also has the potential for development in drug manufacturing.

CONCLUSION

The types of secondary bioactive and metabolic compounds produced by sponges are influenced by the condition of coral reefs as living habitats and other coral cover including abiotic ecosystems**.** Sponges produce secondary bioactive and metabolic substances in response to growing habitat conditions and are intended to protect themselves from various predators. The inhibition of sponge methanol extract on the growth of tested bacteria and fungi was influenced by sponge habitat conditions**.** Methanol extract of *Clathria reinwardri*(CR) sponge had the greatest inhibition against bacteria and fungi, compared to sponge samples of *Callyspongia aeresuza* (CA) and *Hyrtios erectus* (HE). Habitat conditions that were dominated by massive corals and dead corals overgrown with algae had the greatest inhibition for bacteria and fungi, except for candida albicans.

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