Antifungal activity of marine sponges (Class Demospongiae) collected from Biak, Indonesia

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Abstract. Sponges produce secondary metabolites that have potential resource for medicine. Sponges have anti-inflammatory and antimicrobial activity. This study aimed to determine the antifungal activities of marine sponges Fascaplysinopsis sp. and Haliclona sp. against Candida albicans and Aspergilus flavus, and to identify its bioactive compounds. The research procedure includes extraction, thin layer chromatography, open column chromatography, antifungal test, phytochemical test and GC-MS. The result of the study revealed that the ethyl acetate extracts of Fascaplysinopsis sp. and Haliclona sp. have an antifungal activity against C. albicans and A. flavus. Fascaplysinopsis sp. exhibited strong inhibitory activity (22.71±0.44 mm / 200 µg/disk) against Candida albicans and the rest of the extracts show moderate inhibitory activity (Fascaplysinopsis sp. to A. flavus 10.50±0.52 mm, Haliclona sp. to C. albicans and A. flavus, 10.76±0.84 mm and 8.71±0.53 mm). Phytochemical screening assays showed Haliclonaspa sp. contained alkaloid and steroid, while GC-MS analysis showed fraction 5 extract Fascaplysinopsis sp. consisted of 39 compounds, one of them is napthalene which is known to have an antifungal activity.

1. Introduction

Chemical medicines usage to avoid fungal infection can impact the human body. The long-term usage will accumulate those chemical things inside the body. In addition, a continuous and an uncontrolled use of the antibiotic will cause resistance [1]. The impact of antibiotic utilization will cause a new problem, namely a side effect and the appearance of pathogenic fungi which is resistant to antibiotic [2,3]. For that matter, it needs to find new antifungal ingredients whose side effect small. One of the sources of antifungal natural ingredients is a sponge because it produces secondary metabolites having a potential for antimicrobial, anti-inflammatory, and cytotoxic [4,5,6].

Several sea resources can be utilized as antifungal, among other things, sponge. Several compounds isolated from the sponge and been known to function as antifungal are meridine, discobahamins, eryloside A, hamacanthin, cycloepsipeptide, isonaamidine D, bengamides, bengazoles, swinholide and theopederins [6]. This research aims to determine an antifungal activity of Fascaplysinopsis sp. and Haliclona sp. against Candida albicans and Aspergilus flavus, as well as to identify bioactive compound contained within.
2. Methodology

2.1 Material

Sponge sample, namely *Fascaplysinopsis* sp. and *Haliclona* sp., was brought from Biak, Papua. The type of fungi used was *Candida albicans* and *Aspergillus flavus*. This research procedure includes extraction, fractionation, antifungal test, thin layer chromatography, open column chromatography, chemical phytotest, and GCMS test.

2.2 Extraction

The sponge was cleaned and cut into ± 1 cm. Each sample of *Fascaplysinopsis* sp. (400 gr) and *Fascaplysinopsis* sp. (400 gr) was soaked in 500 ml and 800 ml methanol in an erlenmeyer. The filtrate was concentrated at a temperature of 40 ºC. Extract solution of *Fascaplysinopsis* sp. and *Haliclona* sp. was put into a separatory funnel and added the ethyl acetate with ratio 1:2. Those two formed layers were separated and concentrated at the temperature of 40 ºC.

2.3 Antifungal Test Activity of Sponge *Fascaplysinopsis* sp. and *Haliclona* sp. against *Candida Albicans* and *Aspergillus flavus*

Quantitative preliminary test of fungal sensitivity was carried out in ethyl acetate extract and methanol extract sponge against *C. albicans* and *A. flavus* through the method of disk diffusion. The concentration used was 200 µg/disk and 100 µg/disk. After 24 hours, the diameter of the formed obstacles was observed and measured.

2.4 Open Column Chromatography

Open column chromatography was conducted by using 50-cm-long burette with 0.5-cm-diameter consisted of eluent and silica gel. Each *Fascaplysinopsis* sp. and *Haliclona* sp. received 7.5 gr and 5 gr silical gel. An amount of 0.3 gr ethyl acetate fraction of *Fascaplysinopsis* sp. and 0.2 gr ethyl acetate fraction of *Haliclona* sp. was put in a column. Each ethyl acetate fraction was streamed by eluent consisted of 200 ml ethyl acetate and 100 ml n-hexane with the rate of flow ±20 dip/minute. Eluate was accommodated into 10 vials which every vial contains 10 ml. 10 eluate tubes obtained was analyzed using thin layer chromatography. The fraction having similar total stain and Rf was grouped into one fraction and then concentrated.

2.5 Quantitative Test of Fungal Sensitivity

Sensitivity quantitative test of *Candida albicans* and *Aspergillus flavus* against *Fascaplysinopsis* sp. was done to the fractions obtained from the fractionation of open column chromatography. Sponge extract was diluted with the concentration of 20 µg/disk, 10 µg/disk, and 5 µg/disk and then dropped into disk paper that has been grown on a disk media by 10 µl. The observation was carried out after 24 hours, the diameter would be measured if there was an obstacle zone. The observation and measurement were conducted after 24 hours.

While the *Haliclona* sp. was diluted at the dose of 400 µg/disk and 200 µg/disk. The dilution result was ten dropped on the paper disk by using micropipette 10 µg/µl. Antifungal bioactivity main test of fractions from open column chromatography was carried out at the dose of 200 g/disk, 100 g/disk, and 50 g/disk. Each treatment (extract concentration) was repeated three times. The obstacles zone measurement was done after 24 hours.

2.6 Gas Chromatography – Mass Spectrometry of *Fascaplysinopsis* sp. and Phytochemical Test of *Haliclona* sp.

GC-MS analysis of fraction 5 of *Fascaplysinopsis* sp. extract was carried out on Rtx-5MS column (30-m-long, 2.25-mm-diameter) with helium gas carrier, the temperature of column 100 ºC, the temperature of injector 300 ºC, early temperature 100ºC for 5 minutes and then increased by 10 ºC/minute until 290 ºC.
Phytochemical test of *Haliclona* sp. was conducted by using an active fraction isolate, namely fraction 3 by identifying the compound group contained within. Several methods used to conduct phytochemical test of *Haliclona* sp. were flavonoid test, tannin test, quinone test, steroid / triterpenoid, saponin, and alkaloid.

### 3. Result and discussion

#### 3.1 Extraction result of *Fascaplysinopsis* sp. and *Haliclona* sp.

The extraction result of *Fascaplysinopsis* sp. and *Haliclona* sp. can be seen in Table 1.

#### Table 1. Liquid extraction result of *Fascaplysinopsis* sp. and *Haliclona* sp.

| No | Type of Sponge     | Type of Extract        | Extract Weight (gr) |
|----|--------------------|------------------------|---------------------|
| 1  | *Fascaplysinopsis* sp. | Methanol Extract       | 53.497              |
|    |                    | Ethyl acetate extract  | 16.545              |
| 2  | *Haliclona* sp.    | Methanol Extract       | 7.23                |
|    |                    | Ethyl acetate extract  | 1.6                 |

#### 3.2 Sensitivity Quantitative Preliminary Test Result of *C. albicans* and *A. flavus* against the Extract of Sponge *Fascaplysinopsis* sp. and *Haliclona* sp.

Concentration of *Fascaplysinopsis* sp. and *Haliclona* sp. ethyl acetate used in sensitivity quantitative preliminary test is 100 µg/disk and 200 µg/disk. Ethyl acetate extract from both sponges shows the existence of an obstacle zone against both fungal in both concentrations, while the extract of methanol from those two types of sponge does not show an obstacle zone. It is expected that antifungal compound from the solvent was not dissolved in the polar compound like methanol but dissolved in the semi-polar compound like ethyl acetate. It shows that non-polar compound will easily penetrate the cell membrane so that the compound dissolved in ethyl acetate is more bioactive since easier to penetrate the membrane of the fungal cell compared to the compound dissolved in polar methanol. [10].

#### 3.3 Open column chromatography test results of *Fascaplysinopsis* sp. and *Haliclona* sp.

Open column chromatography test was done to separate compound component in the extract. Open column chromatography test of *Fascaplysinopsis* sp. generated 10 vials with 10 ml volume and then analyzed using silica plate thin layer chromatography with solvent ratio 3:1.

#### 3.4 Sensitivity quantitative test result of *Candida albicans* and *Aspergillus flavus* against sponge *Fascaplysinopsis* sp. and *Haliclona* sp. extract

The 6 fractions of open column chromatography results of *Fascaplysinopsis* sp. were tested to the fungus *C. albicans* and *A. flavus* to find out fraction which is able to inhibit fungus growth. Sensitivity quantitative test result of the *C. albicans* can be seen in Table 2.

#### Table 2. Sensitivity quantitative test result of *C. albicans* with the extract of *Fascaplysinopsis* sp.

| Fraction | Concentration (µg/disk) | Inhibition zone (mm) 24 hours |
|----------|-------------------------|-------------------------------|
|          | 5                       | 13.278±0.419                 |
|          | 10                      | 13.278±0.419                 |
|          | 20                      | 20.067±0.058                 |
|          | 5                       | 12.433±0.361                 |
|          | 10                      | 13.300±0.379                 |
|          | 20                      | 20.767±0.240                 |
|          | 5                       | 13.800±0.376                 |
6 10 16.722±0.299
20 18.489±0.084

Description:
- The value explained above is the average ± SD (n=3)
- SD is Standard Deviation

Fractions of open column chromatography result were given a *Haliclona* sp. antifungal test against *Candida albicans* and *Aspergillus flavus*. The antifungal test result of open column chromatography fractions against *Candida albicans* and *Aspergillus flavus* is shown in Table 3 and 4.

Table 3. Sensitivity quantitative test result of *C. albicans* with the extract of *Haliclona* sp.

| Fraction | Concentration (µg/disk) | Incubation | 24 hours | 48 hours |
|----------|-------------------------|------------|----------|----------|
|          |                         |            | Diameter (mm) |        |
| 1        | 200                     | 8.74±0.26  | 8.83±0.2 |
|          | 100                     | 7.92±0.36  | 8±0.29   |
|          | 50                      | 7.54±0.44  | 7.52±0.4 |
| 2        | 200                     | 8.38±0.21  | 8.47±0.28|
|          | 100                     | 7.75±0.18  | 7.76±0.25|
|          | 50                      | 7.31±0.3   | 7.33±0.3 |
| 3        | 200                     | 9.36±0.65  | 9.41±0.60|
|          | 100                     | 8.77±0.10  | 8.8±0.12 |
|          | 50                      | 8.13±0.1   | 8.16±0.08|

Table 4. Sensitivity quantitative test result of *Aspergillus flavus* with the extract of *Haliclona* sp.

| Fraction | Concentration (µg/disk) | Incubation | 24 hours | 48 hours |
|----------|-------------------------|------------|----------|----------|
|          |                         |            | Diameter (mm) |        |
| 1        | 200                     | 10.7±0.61  | 10.7±0.51|
|          | 100                     | 9.55±0.53  | 9.81±0.15|
|          | 50                      | 8.41±0.63  | 8.48±0.71|
|          | 200                     | 10.61±0.30 | 10.67±0.30|
| 2        | 100                     | 9.81±0.24  | 9.86±0.23|
|          | 50                      | 8.51±0.72  | 8.54±0.81|
|          | 200                     | 11.74±0.13 | 11.83±0.12|
| 3        | 100                     | 10.65±0.24 | 10.75±0.19|
|          | 50                      | 9.07±0.25  | 9.18±0.32|

Description:
- Average ± SD (n=3)
- SD = Standard Deviation
- Control = 0.00±0.00 mm

3.6 Analysis of bioactive compound content of *Fascaplysinopsis* sp. and *Haliclona* sp. extract

GC-MS analysis aims to identify a compound in the extract. The analysis result of fraction 5 gas chromatography of *Fascaplysinopsis* sp. ethyl acetate extract results in 39 peaks detected so that expected at least there are 39 compounds in fraction 5. The five highest peaks were then analyzed using mass spectrometry so that obtained the compound estimation which can be seen in Table 5.
Fascaplysinopsis addition, several matters, among other things, *A. flavus* is not sensitive to the extract of *Fijian is able to*. A. flavus by a compound is due to every compound will work or react specifically on its target [1] two fungi test *Candida albicans* chromatograph shows that the size of obstacle zone average of *Silica gel is an acidid stationary phase and tends to tie polar compounds* [1] chromatography result eluate of *The solvent used in extraction method is methanol since it can dissolve almost all polar or non-polar organic compounds* [7]. In addition, methanol is also able to dissolve all secondary metabolites groups [8] so that expected to dissolve in methanol maximally. Liquid-liquid extraction aims to separate compound based on its non-polar characteristics. The solvent used in liquid extraction consisted of two types of not-mixed solution. Ethyl acetate is a semi-polar solvent so that semi-polar and a non-polar compound is expected to be dissolved in ethyl acetate and the rest is methanol phase compound [9].

Eluate column chromatography 1 has the lowest level of polarity which then increases into the next eluate. Polar solvent will tie polar compound more strongly than non-polar solvent and non-polar compound will be tied by non-polar solvent [10]. A polar silica gel stationary phase causes the polar compound will be strongly tied by silica gel while the non-polar compound will come out together with eluate so that the first eluate coming out has a lowest polarity. The ten eluate tubes generated will then be grouped by using thin layer chromatography.

Open column chromatography test result of *Haliclona sp.* resulted in 110 ml eluates accommodated in 9 vials with 12 ml in size. Based on the test result of tin layer chromatography test, open column chromatography result eluate of *Haliclona sp.* extract acetate ethyl fraction is grouped into 3 fractions. Silica gel is an acidid stationary phase and tends to tie polar compounds [12]. Fractions 2 and 3 are evaluated to be nonpolar, and fraction 1 is thought to be semipolar.

The antifungal test result of the three active fractions of *Haliclona sp.* open columns chromatograph shows that the size of obstacle zone average of *Aspergillus flavus* is bigger than *Candida albicans*. Fraction 3 of *Haliclona sp.* extract results in the largest obstacle zone towards those two fungi test so that fraction 3 is the most active fraction. The difference of toxic activity generated by a compound is due to every compound will work or react specifically on its target [17,4].

Based on the research result, the six fractions of *Fascaplysinopsis* sp. did not hamper the growth of *A. flavus*, but fraction 4, 5, and 6 hampers the growth of fungus *C. albicans*. This result is in accordance with the result which has been recorded that the type of *Fascaplysinopsis bergquist* collected from Fijian is able to hamper the growth of *C. albicans* [13] Based on that result is expected that the *A. flavus* is not sensitive to the extract of *Fascaplysinopsis* sp. at that concentration. It can be caused by several matters, among other things, *A. flavus* is a fungus that grows quickly on disk media [14]. In addition, *A. flavus* has high resistency [15], so it is thought to need a higher extract concentration of *Fascaplysinopsis* sp. to be able to hamper the growth of *A. flavus*.

Fraction 4, 5, and 6 of *Fascaplysinopsis* sp. hampered the growth of *C. albicans* is shown by the existence of obstacle zone around the disk paper. Based on the observation is known that the compound on those three fractions directly proportional to its extract concentration indicated by the

### Table 5. Compound estimation of GC-MS result of *Fascaplysinopsis* sp. extract

| Peak Number | Time of Retention | The area of Peak (%) | Compound Name |
|-------------|------------------|----------------------|---------------|
| 11          | 18.649           | 10.74                | octahedron naphtalene |
| 19          | 20.817           | 7.25                 | 1-(1,1-dimethylethyl) Dihydronaphthalene |
| 29          | 24.250           | 5.14                 | 6-(2,6,6-trimethyl-1-cyclohexenyl)-4-methyl-(e)-3-hexen-1-ol |
| 36          | 27.405           | 17.55                | (2,6,6-trymethyl-cyclohex-1-enylmetan sulfonyl)-benzene |
| 37          | 27.973           | 19.17                | 2-Bromomethyl-1-isopropenyl-3-methyl-cyclopentane |

### 3.7. Discussion
The obtained extract was then tested against *Candida albicans* and *Aspergillus flavus*. The sponge extract of *Fascaplysinopsis* sp. was tested with the concentration of 100 µg/disk and 200 µg/disk whilst *Haliclona sp.* was tested by the concentration of 400 g/disk and 200 g/disk.

In the detection of toxic activity generated by every compound on *C. albicans* and *A. flavus*, we can see from the data that the extract of *Fijian* is 5 times bigger than the extract of *F. bergquist*. This is in accordance with the data of compound which is more polar and also in accordance with the data of compound which is more toxic. The amount of toxic activity generated by the compound extract of *Fijian* in *C. albicans* is bigger than *F. bergquist*. This is due to the extract of *Fijian* is more polar than extract of *F. bergquist* and also due to the amount of toxic activity generated by the compound extract of *Fijian* is bigger than extract of *F. bergquist*. This is in accordance with the data of compound which is more polar and also in accordance with the data of compound which is more toxic.
addition of obstacle zone size on the extract addition of sponge _Fascaplysinopsis_ sp., the higher the concentration, the further the rate of diffusion. The size of obstacle zone diameter is influenced by several matters, namely test microorganism sensitivity towards antibiotic agent, media suitability of microorganism growth, incubation condition, and diffusion rate from the antibiotic agent [16].

From the five peaks identified, it gets peak number 11 with a retention time of 18,649 minutes. The compound shows a similarity by 89% with octahedron naphthalene compound. The modified naphthalene compound also ever been identified from _Disidea _sp. sponge derives from Australia and _Disidea avara_ derives from Mediterranean [18]. The modified naphthalene compound is also reported to have an antifungal activity towards _Candida_ sp. and _Aspergillus niger_ [19], so that expected that one of the antifungal activities deriving from sponge _Fascaplysinopsis_ sp. is naphthalene compound.

The identification of secondary metabolites compound aims to determine the compound content qualitatively and might be quantitatively of the compound type contained in the biota tested. The identification test of the compound type of _Haliclona_ sp. extract is conducted against the most active fraction, namely, fraction 3. The result of phytochemicals test against fraction 3 of _Haliclona_ sp. shows that the compounds contained in fraction 3 categorized as the type of alkaloid and steroid compound.

4. Conclusion

Based on this research result, it can be concluded that all fraction of _Fascaplysinopsis _sp. sponge does not hamper the growth of the _A. flavus_, but fraction 4, 5 and 6 hampers the growth of the fungus _C. Albicans_. The result of antifungal 3 of _Haliclona_ sp. fraction hampers the growth of _Aspergillus flavus_ which actually want to be bigger than _Candida albicans_. The result of GCMS test of _Fascaplysinopsis_ sp. shows that 39 compounds are dominated by octahedron naphthalene. The result of phytochemicals test shows that the compound contained in _Haliclona _sp. is a type of alkaloid and steroid compound.

5. References

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