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journal homepage: www.elsevier.com/locate/apjtbOriginal article <http://dx.doi.org/10.1016/j.apjtb.2017.01.024>Antibacterial activity of marine bacteria isolated from sponge *Xestospongia testudinaria* from Sorong, PapuaYatnita Parama Cita^{1*}, Achmad Suhermanto², Ocky Karna Radjasa³, Pratiwi Sudharmono¹¹Department of Microbiology, Faculty of Medicine Universitas Indonesia, Jl. Salemba Raya no 6, Jakarta, 10430, Indonesia²Maritime and Fishery Polytechnic of Sorong, KKD-BP Kesehatan Ikan dan Lingkungan, Jl. K. Pattimura, Tanjung Kasuari, P.O. Box 109, Sorong, 98410, Papua Barat, Indonesia³Department of Marine Science, Diponegoro University, Semarang, 50275, Indonesia

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ABSTRACT

Objective: To explore secondary metabolite of bacteria-associated *Xestospongia testudinaria* from Tanjung Kasuari, Sorong, Papua.**Methods:** The antimicrobial activities of extracts against two Gram-positive bacteria (*Staphylococcus aureus*) and three Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhi*) were determined by disk diffusion dilution method.**Results:** The test showed that of 15 isolates of symbiont bacteria, 6 isolates were successfully isolated and coded, namely, Xp 4.1, Xp 4.2, Xp 4.3, Xp 4.4, Xp 4.5 and Xp 4.6. Of the six bacterial isolates, isolated Xp 4.2 was found to have more powerful antibacterial activity than any other isolates of symbiont bacteria. Antibacterial activity assay for the *n*-hexane soluble fractions, ethyl-acetate soluble fractions, and *n*-buthanol soluble fractions revealed more powerful anti-bacterial activity than any other soluble fractions. Phytochemical screening showed alkaloid and steroid/triterpenoid, while identification for isolate of Xp 4.2 bacterial showed bacteria.**Conclusions:** Metabolites of bacterial associated with marine sponge *Xestospongia testudinaria* promise to be developed into antibacterial agents.

1. Introduction

Widespread use of antibiotics has led to faster and wider multidrug resistance. A 2010 report showed that 79% of *Escherichia coli* strains are resistant to ampicillin (which had been used since 1961), while 30% of the strains were resistant to ciprofloxacin (which have been used since 1987). Between 1999 and 2000 in the America, 43% of *Staphylococcus aureus* infections were found to be resistant to meticillin. Resistance of bacteria to antibiotics had led to the re-increase of mortality rate because of infectious diseases [1]. In the developing countries, including Indonesia, infectious diseases remain the greatest contributor for morbidity and mortality rates.

Around 40%–50% of the drugs now on the market were formulated from natural chemical products, including marine

microorganisms. During the last few decades, many studies have focused on the bioactive compounds of marine products, including marine microorganism [2]. Bacteria live in the nutrient-poor waters by having association with marine organisms, such as sponge [3].

Sponge is a marine biota, which is potential to have secondary metabolite and bioactive properties. This is evident from more than 6 000 bioactive substances (lead compound) that have been isolated from marine biota during the last three decades, and 40% of them were isolated from sponges [4,5]. Sponges belong to Animalia and filum Porifera kingdom, and most of porifera structures are porous. The sponge has two layers of structure, namely, epidermis and endodermis. Epidermis consists of thin epithelium cells (pinacocyte) while endodermis consists of flagella cells, which serve for food digestion, and funnels that are called neck cells or choanocytes [6]. All of the marine biota, including sponges, produce primary and secondary metabolites, which are the results of metabolic process inside the organisms. Production of secondary metabolite is influenced by the environment. Therefore, it is assumed that under different environment (despite similar species), the resulting metabolites will be different [7].

Genus *Xestospongia* is widely distributed around the world, from Indo-Pacific to Caribbea. It is very common and varied in the

*Corresponding author: Yatnita Parama Cita, Department of Microbiology, Faculty of Medicine Universitas Indonesia, Jl. Salemba Raya no 6, Jakarta, 10430, Indonesia.

Tel: +62 (021) 31930373, 31930371, 3912477

Fax: +62 (021) 3912477

E-mail: nitatafshilaa@yahoo.com

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Northwestern Australia, Great Barrie Reef, Papua New Guinea, Solomon, West-Central Pacific, Thailand Bay, and Indonesia [8], even though the genus *Xestospongia* is also found in different locations. Since microorganisms, which associate with sponges, can vary widely, the secondary metabolites will be much different [9]. Sponge biodiversity is very potential to produce novel antibacteria, since almost 200 new metabolite compounds are identified every year [10]. The research aims at exploring secondary metabolite of *Xestospongia testudinaria* that is associated with marine bacteria from Sorong, Papua.

2. Materials and methods

2.1. Sampling technique

The research used purposive sampling technique, in which the sample was taken at Tanjung Kasuari water area, Sorong (Papua). The sample was taken at the shore water area in the depth of 15–20 m by means of SCUBA Diving. Immediately after being taken, the sample was put into a plastic container, which had been filled with sterile salt water, and then brought to the laboratory for preparation of bacterial isolates.

2.2. Isolation of sponge-associated bacteria

The principle of microbial isolation is separating a type of microbe from any others. This can be done by growing the isolated microbe in a certain media, in which the microbial cells will form a cellular colony at the specified location. After the collection of sponge colonies was put into the sterile plastic bag (Whirl-Pak, Nasco, USA), the sponges were washed using sterile salt water and 1 cm² of the sponge's tissue was cut out using a sterile knife. The tissue was then diluted using a the specified solvent series with a power of 1/2 ZoBell 2216E salt agar media and then incubated under room temperature for 48 h. The colonies were randomly taken and purified based on the morphological characteristics [11].

2.3. Fermentation and extraction of metabolite compound

The active isolate was used for pre-culture in a 500 mL Erlenmeyer Flash, which contains liquid medium of ZoBell 2216E and incubated under a temperature of 30 °C for 11 days in a shuffled position after being shuffled at a rate of 150 r/min. After 11 days of fermentation, the microbial growth medium was filtered to separate the biomass from fermentation solution. The fermentation solution was then extracted twice using organic solvent of *n*-hexane, ethylacetate and *n*-buthanol to obtain the required extract. The extract was then evaporated in an evaporator to produce thick extract and then stored in a desiccator for further assay [12].

2.4. Anti-bacterial activity assay

Anti-bacterial activity assay was conducted using agar diffusion method, which includes culturing the test bacteria for 1 day in nutrient broth medium under a temperature of 37 °C. The bacterial culture was prepared in a density of 25%T using a spectrophotometer or a density of 10⁹ cells/mL at logarithmic phase and inoculated in a sterile Petri dish, on which the culture was evenly distributed, added with Mueller-Hinton agar medium, homogenated and then let to condense in sterile condition for 20 min in LAF. A filter paper disc (8 mm; Advantec, Toyo Roshi, Ltd,

Japan) was sprayed with drops of 10 µL of test solution (which contains sponge-associated bacteria) under a concentration of 1 mg/mL. The dish was then incubated under a temperature of 35 °C for 2 × 24 h. Anti-bacterial activity was determined based on the formation of inhibition zone > 9 mm around the disc [13].

2.5. Identification of bacterial strain

The isolate which possessed highest inhibition against the pathogen, both in crude and fraction form was subjected for identification using 16s rRNA sequencing. The DNA sample of the bacteria, which had been amplified using primary gene 16S rRNA was then sequenced to produce gene sequence of 16S rRNA. The resulting sequence was then analyzed using computer program BLAST and compared with nucleotide on the database "GenBank" (NCBI) [11,12].

3. Results

The sample was taken at Sorong, Papue. Bacterial isolation was conducted at Maritime Laboratory of Fishery Academy of Sorong, and identification was conducted at Zoology Laboratory, Faculty of Mathematics and Sciences, Sepuluh November Institute of Technology, Surabaya.

3.1. Identification of sponge genus *X. testudinaria*

Genus *Xestospongia* is a marine sponge that is widely distributed around the world, from Indo-Pacific to Caribbea. *Xestospongia* is known to live and thrive under various substrates, such as sand, rock, and even dead coral rubble [13,14]. *Xestospongia* is mostly found in depth of more than 10 m from the sea surface (Figure 1).

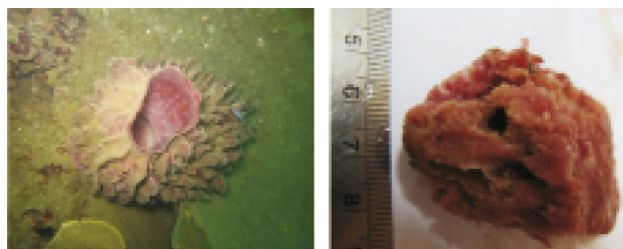


Figure 1. *Xestospongia testudinaria*.

The genus is characterized with light blue color at the external part and grayish yellow at the internal part as long as it is still alive. When taken, it will turn into yellowish brown (Figure 2).

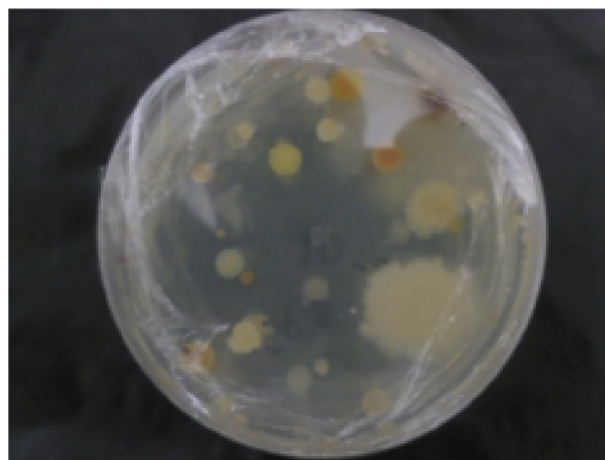


Figure 2. Morphology of symbiotic bacterial isolate *Xestospongia testudinaria* from Sorong, Papua.

3.2. Isolation of sponge-associated bacteria

Bacterial isolates, whose solvent had been determined, were purified using scratch method. Isolation of bacteria from the sponges was the first step of inoculation to produce non-purified bacterial isolate. According to Lay (1994), the number of colonies in the Petri dish adequately represent the number and composition of bacterial cells [15].

The figure above shows that dominant shape of the bacterial colonies was round, while the dominant color was snowy white. Each colony of bacteria that grew well were classified based on color, size, and colon shape, and then purified using the similar media. Observation on the morphology of symbiont bacterial colony of the sponges *X. testudinaria*, 15 isolates were found at Sorong, and only 6 bacterial isolates of them were successfully purified and coded Xp 4.1, Xp 4.2, Xp 4.3, Xp 4.4, Xp 4.5 and Xp 4.6.

3.3. Anti-bacterial activity assay

Capability of the symbiont bacterial isolate against pathogen bacteria was characterized with a transparent zone formed around the bacterial scratches. This showed that there was no growth on the cultured pathogen bacteria. The result of anti-bacterial activity assay for the symbiont bacteria against the pathogen bacteria is presented in Table 1 and Figure 3.

Table 1

The result of symbiont anti-microbial activity test was classified into some pathogen bacteria.

Bacterial isolate	<i>S. aureus</i>	<i>E. coli</i>	<i>B. subtilis</i>	<i>K. pneumonia</i>
Xp 4.1	–	19	–	8
Xp 4.2	–	24	12	22
Xp 4.3	–	–	6	16
Xp 4.4	–	23	8	15
Xp 4.5	–	18	17	14
Xp 4.6	–	–	–	16

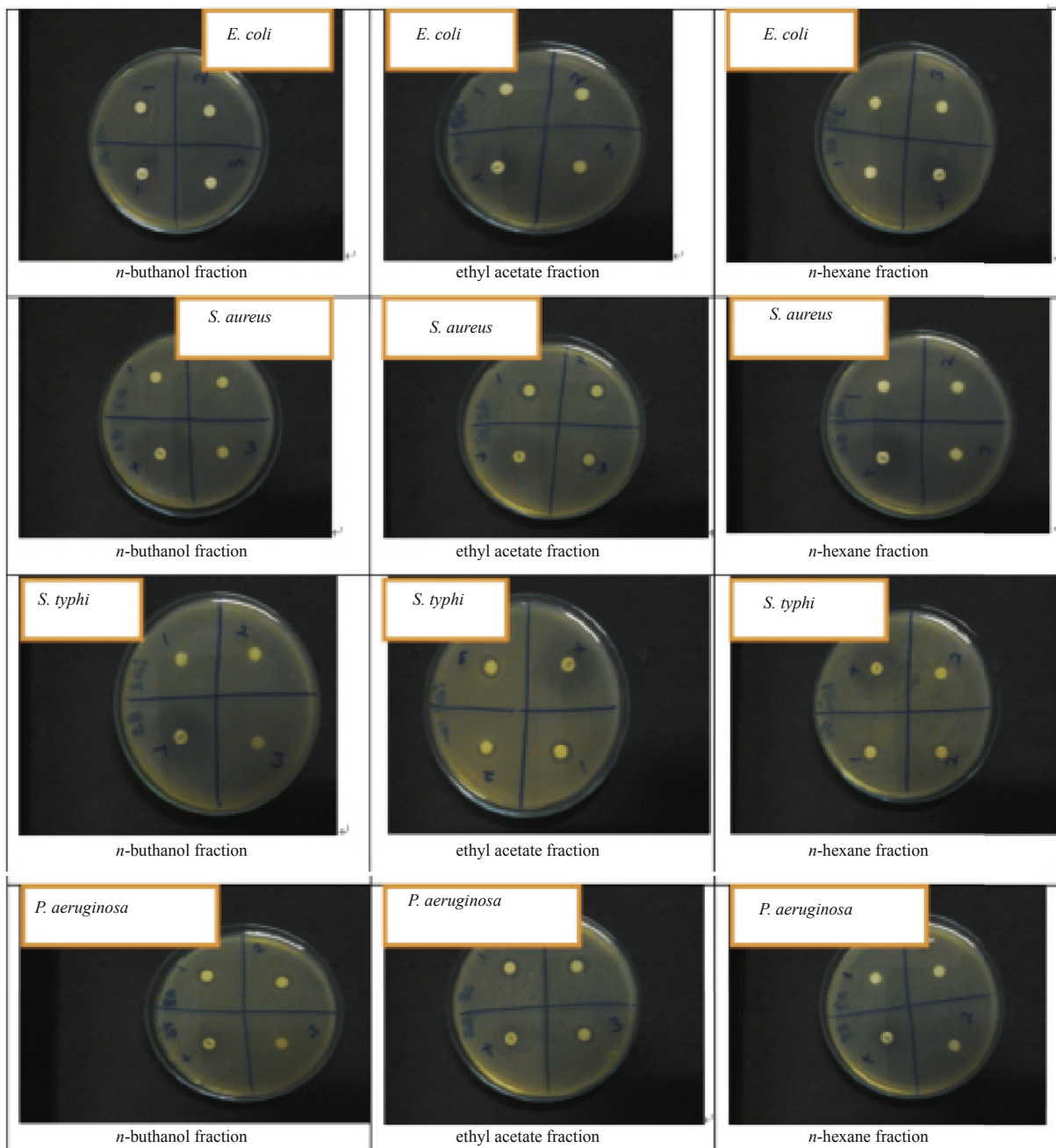


Figure 3. Results of anti-bacterial activity observation for the *n*-hexane, ethyl acetate, and *n*-buthanolic soluble fractions of the isolate of Xp 4.2 bacterial isolates.

Fermentation process was conducted for the isolate of Xp 4.2 in a marine broth medium under a temperature of 30 °C in shuffled position, after being shuffled at a rate of 150 r/min for 11 days. Then, the results of fermentation were filtered and the supernatants were extracted using *n*-hexane, ethyl acetate, and *n*-buthanol solvents. Organic solvent that is ordinarily used in isolation of marine biota is associated with polarity of the compounds to be isolated. Fractions resulting from isolation using organic solvent were subjected to anti-microbial activity assay (Table 2).

Table 2

Results of phytochemical screening of different extractives of secondary metabolite from bacteria symbiont Xp 4.2.

Test	<i>n</i> -Buthanolic soluble fraction	Ethyl acetate soluble fraction	<i>n</i> -Hexane soluble fraction
Alkaloid	–	+	–
Tanin	–	–	–
Saponin	–	–	–
Steroid/ Triterpenoid	+/+	+/+	–/–
Phenols	+	+	–
Cumarine	–	–	–

+: Presence of phytoconstituents; –: Absence of phytoconstituents.

4. Discussion

Inhibitory interactions among sponge-associated bacteria that occur within the sponge surface are of great interest to search for secondary metabolite-producing bacteria. Isolation and screening for secondary metabolite-producing bacteria sponges have been strongly neglected until now in comparison with the invertebrate parts [12]. The present study indicated that among 6 marine bacteria associated with sponges showed growth inhibition against indicator microorganism.

Table 1 shows that isolate of symbiont bacterium Xp 4.2 had an inhibition activity against Gram-negative bacteria (*E. coli* and *Klebsiella pneumoniae*). This inhibition was more powerful than Gram-positive bacteria (*Bacillus subtilis*). The strength of the activity was classified as strong for inhibition zone diameters (i.d.) > 16.0 mm, moderate (good) for diameters ranging from 11 to 16 mm, weak for diameters 7–11 mm and no activity for diameters < 7 mm [16,17]. In another research conducted by Ponce *et al.*, active compounds with anti-bacterial activity were successfully isolated from the isolates of bacterium *Micrococcus luteus*, which is associated with *Xestospongia* sp sponge [18]. While the sponge *Xestospongia* sp, which was taken from the Philippines water area, which had been found to have a strong anti-bacterial activity against *B. subtilis* with inhibition zone of 15 mm and *S. aureus* of 17 mm [13].

Phytochemical screening showed that *n*-buthanolic fraction and ethyl acetate fraction contained compound groups of phenols, steroid, and triterpenoid, while the ethyl acetate fraction also contained alkaloid compounds. Iwagawa *et al.* found some alkaloid compounds that are also found on *Xestospongia exigua* [13,19], while Umeyama and coworkers first isolated xestobergsterols from the Okinawan marine sponge, *Xestospongia bergquistia* [20].

Antimicrobial activity was observed in both *n*-buthanolic soluble fraction as well as the ethyl acetate soluble fraction of the secondary metabolite from bacteria symbiont Xp 4.2; however, higher inhibition was exhibited by the ethyl acetate soluble

fraction. Sponge bacterium Xp 4.2 showed high homology to *M. luteus* MB-26 (98%), as the extract of the sponge-associated bacterial strain *M. luteus* was found to exhibit potent antimicrobial activity [18].

Phytochemical screening and anti-microbial activity test for ethyl acetate fraction found alkaloid and steroid compounds, which had antibacterial activity. A recent research isolated compounds from ethyl acetate fractions, which had antibacterial activity.

Conflict of interest statement

We declare that we have no conflict of interest.

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