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Bioactivity of compounds secreted by symbiont bacteria of Nudibranchs from Indonesia

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The aim of this work was to isolate bacterial symbionts from nudibranchs and subsequently to determine anti-Methicillin resistant *Staphylococcus aureus* (MRSA), cytotoxicity and anti-HSV-1 activities of bio-compounds. Fifteen species of nudibranchs were collected from Karimunjawa and five species from Bali, respectively. A total of 245 bacteria isolates were obtained. The anti-MRSA activity screening activity indicated 2 isolates of active bacteria. Ethyl acetate extracts from supernatants, indicating secreted compounds, showed an inhibition zone against MRSA at concentrations of 500-1000 µg/ml. DNA sequence analysis showed that the strain KJB-07 from *Phyllidia coelestis* was closely related to *Pseudoalteromonas rubra*, the strain NP31-01 from *Phyllidia varicosa* was closely related to *Virgibacillus salarius*. The extract of *P. rubra* was cytotoxic to Vero cells at a concentration of 75 µg/ml. The extract of *V. salarius* presented no cytotoxicity at concentrations of 5-1000 µg/ml. No anti-HSV-1 was observed. This is the first reported study describing research on anti-MRSA, cytotoxicity and anti-HSV-1 activity of bacterial symbionts from the viscera of nudibranch. Compounds produced and secreted by *Pseudoalteromonas rubra* and *Virgibacillus salarius*, symbionts of Nudibranch, had potential anti-MRSA activity. Extracts from *P. rubra* showed cytotoxic effects on Vero cells, whereas extracts from *V. salarius* did not show cytotoxic effects. Three compounds were identified by LC/MS after purification from culture supernatant.

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Abstract

The aim of this work was to isolate bacterial symbionts from nudibranchs and subsequently to determine anti-Methicillin resistant *Staphylococcus aureus* (MRSA), cytotoxicity and anti-HSV-1 activities of bio compounds. Fifteen species of nudibranchs were collected from Karimunjawa and five species from Bali, respectively. A total of 245 bacteria isolates were obtained. The anti-MRSA activity screening activity indicated 2 isolates of active bacteria. Ethyl acetate extracts from supernatants, indicating secreted compounds, showed an inhibition zone against MRSA at concentrations of 500-1000 µg/ml. DNA sequence analysis showed that the strain KJB-07 from *Phyllidia coelestis* was closely related to *Pseudoalteromonas rubra*, the strain NP31-01 from *Phyllidia varicosa* was closely related to *Virgibacillus salarius*. The extract of *P. rubra* was cytotoxic to Vero cells at a concentration of 75 µg/ml. The extract of *V. salarius* presented no cytotoxicity at concentrations of 5-1000 µg/ml. No anti HSV-1 was observed. This is the first reported study describing research on anti-MRSA, cytotoxicity and anti HSV-1 activity of bacterial symbionts from the viscera of nudibranch. Compounds produced and secreted by *Pseudoalteromonas rubra* and *Virgibacillus salarius*, symbionts of Nudibranch, had potential anti-MRSA activity. Extracts from *P. rubra* showed cytotoxic effects on Vero cells, whereas extracts from *V. salarius* did not show cytotoxic effects. Three compounds were identified by LC/MS after purification from culture supernatant.

Keywords: Nudibranch; *Virgibacillus salarius*; *Pseudoalteromonas rubra*; Anti-MRSA activity; HSV-1; antibiotic

1. Introduction

Emergence or resurgence of numerous infectious diseases is strongly rising up year by year between 1990 and 2013 (*Chen et al., 2019*). Infectious diseases are caused by four main kinds of germs, which are bacteria, viruses, fungi and parasites. Bacteria are the first cause of diseases. During the past four decades, bacterial resistance to antibiotics has become a growing problem. One type of bacteria, for example, has evolved from a controllable nuisance into a serious public

health concern. This bacterium is known as methicillin resistant *Staphylococcus aureus*, or MRSA. Approximately one-third of the world population has *S. aureus* bacteria on their bodies at any given time, primarily in the nose and on the skin. The bacteria can be present without causing an active infection. According to Centers for Disease Control and Prevention (CDC) approximately 1% of people has MRSA. There are two ways in which the infection can be acquired hospital-acquired MRSA (HA-MRSA) or community-associated MRSA (CA-MRSA). The MRSA infections caused higher morbidity and mortality compared to non-resistant strains. (Akhi et al., 2017). The rapid emergence of MRSA infection is becoming crucial, as the effectiveness of treatment of MRSA infection decreases due to the ability of bacteria to respond to antibiotics (Guzmán-Blanco et al., 2009). In 2017, according to the World Health Organization (WHO), the resistance to first line drugs to treat infections caused by *S. aureus* was widespread. People with MRSA are estimated to be 64% more likely to die than people with a non-resistant form of the infection.

The second case is virus infection. Herpes simplex virus (HSV), a DNA virus, is a common human pathogen with between 60 to 95% of certain populations infected with Herpes simplex virus type 1 (HSV-1), and between 6 to 50% infected with Herpes simplex virus type 2 (HSV-2). Primary and recurrent herpes virus infections in human represent major risk factors for acquisition of primary HIV-1 infection (Sassi et al., 2008). Acyclovir is the antiviral treatment of choice, but resistance to acyclovir has been reported due to the *UL23* gene mutations (Mitterreiter et al., 2016). There is a need to develop new natural source of agents for the management of HSV infections.

The Indonesian archipelago is a hotspot for biodiversity in the world (Gastropoda et al., 2018) and a very rich marine environment. The studies regarding screening of secondary

metabolites-producing bacterial symbionts are important for understanding their biotechnological potential (*Radjasa et al., 2011*). A few reports have been documented on the potential of bacterial symbionts from Indonesian marine invertebrates, such as coral (*Radjasa, et al., 2007*), soft coral (Seyed Vahid Shetab-Boushehri, 2012) and sponge (*Radjasa, et al., 2007*).

Since marine tropical diversity also reflects chemical diversity, the isolation of the under-exploited bacterial symbionts from Indonesian nudibranchs offers a great opportunity for discovering novel bio compounds, based on screening against various disease targets. Natural products from cultured symbionts of marine invertebrates present an opportunity to be developed in ways that circumvent environmental and supply problems of pharmaceutical substances. Nudibranchs, belonging to gastropods, are of great interest for research and development of bioactive natural metabolites. These invertebrates are exposed to predators and to surface colonization by microorganisms. For them, the only hope of surviving and fighting effectively against this is to produce compounds that are toxic for infectious bacteria. These compounds have actually been found in a number of organisms and it has been suggested that they represent secondary metabolites from symbiont bacteria (*Radjasa et al., 2011*).

There is one report of symbiotic bacteria in the vestibular gland, associated with the female reproductive system, and in the egg masses of the nudibranch *Dendrodoris nigra* (Klussmann-Kolb and Brodie, 1999). In 2012, Rod-shaped gram-negative bacteria were revealed in the epithelial cells of the notum and the mantle edge as well as in the adjoining glycocalix near the outer membrane of the epithelial cells (*Zhukova and Eliseikina, 2012*) and these bacteria were enclosed in secondary vacuoles in the epithelial cells in *D. nigra*. Several drugs with interesting pharmaceutical properties are currently in clinical trials (*Chand and Karuso, 2017*). For example, Serpin serine protease produced by *Octopus ocellatus* has potential antibacterial activity (*Wei et*

94 *al.*, 2015), two gastropods *Cerastoderma edule* and *Ostrea edulis* present potential antibacterial
95 and antiviral molecules (*Defer et al.*, 2009), tambjamine D an alkaloid isolated from the *Tambja*
96 *eliora*, has been reported to have cytotoxic and genotoxic activity (*De Oliveira et al.*, 2007),
97 furthermore the bioactive alkaloid ergopeptine has been extracted from *Pleurobranchus forskalii*
98 (*Wakimoto et al.*, 2013). Overall, secondary metabolites have only been investigated from a
99 small proportion (<1%) of Mollusca species (*Benkendorff*, 2010).

100 In the present study, we explore the possibility of finding anti-MRSA and antiviral
101 molecules from nudibranch's symbiotic bacteria. Twenty nudibranchs species have been
102 collected in Bali and the national park of Karimunjawa, Jepara, in the Java Sea. Symbiont
103 bacteria collections have been isolated from stomach tissues and research of biological activities
104 was performed on supernatants of lysed bacteria. We investigate the anti-MRSA and antiviral
105 activity that can be derived from symbiont bacteria after culture of the bacteria.

106

107 2. Materials and Methods

108 **Sampling.** This research used samples that were collected from Lovina, Bali and the National
109 Park of Karimunjawa (5°47'21.6''S 110°30'41.9''E) (March 2017), Jepara Indonesia by SCUBA
110 diving 15-20 m in depth, at 18-25 °C . The samples of nudibranchs were put into sterile plastic
111 bags, filled with seawater and stored in a cool box (*He et al.*, 2014)(*Sabdon et al.*, 2015), and
112 immediately brought to the laboratory to be identified, studied and to isolate the bacteria.

113 **Symbiont bacteria collection and isolation.** The nudibranchs were washed three times, using
114 sterile seawater, to remove the impurities still attached to the nudibranchs body. The contents of
115 the stomach called viscera and the body were separated by using a sterile knife and were put in a
116 conical tube that contained sterile seawater. Bacterial isolation was done using the dilution

method (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}) (Anand et al., 2006). The bacterial inoculation was performed on the ZoBell 2216E agar media [peptone 15 g/L (Oxoid Laboratories); yeast (Himedia Laboratories); agar 15 g/L (Oxoid Laboratories)] and was incubated at a temperature of 37 °C for 5 days. Bacterial colonies were grown on a plate, and each colony was separated according to shape, elevation and colour. Isolation and purification processes were done in the Tropical Marine Biotechnology Laboratory at Diponegoro University. The pure colonies were maintained in slant cultures at -20 °C.

Anti-MRSA screening. The antibacterial test was conducted using the overlay method, colony-forming units of symbiont bacteria of nudibranchs were cultured in ZoBell 2216E agar media and incubated at 37 °C for 24 h (Radjasa, et al., 2007). The pathogenic bacterium *Staphylococcus aureus* (Strain MRSA from Kariadi Hospital in Semarang, Indonesia, resistant for oxacillin, gentamicin, benzylpenicillin, ciprofloxacin, levofloxacin, tetracycline) was cultured in Muller-Hinton broth (Difco Laboratories) with a shaker condition of 150 rpm at 37 °C for 24 h. The antibacterial test was conducted by mixing *S. aureus* with 0.5 McFarland standard in soft agar media and pouring into the petri dishes containing the marine bacterial colonies. All screening was performed in triplicate experiments. Clear zones were observed following overnight incubation at 37 °C. The clear zone around bacterial colonies, was an indicator that there has been antibacterial activity (Defer et al., 2009). Isolates that exhibited anti-MRSA activity were chosen for further characterization of bioactive compounds, molecular identification, cytotoxicity and anti-HSV activity.

Bioactive compound extraction. Active isolates of bacterial symbionts of nudibranchs against MRSA were cultured in 3 L of nutrient broth (Oxoid Laboratories) with seawater-based medium for 6 days (Singh et al., 2014). The bacterial suspension was centrifuged at 5000 rpm, at 4 °C for

10 minutes. The supernatant was extracted with ethyl acetate. The bacterial pellet was extracted with methanol. The ratio of solvent to culture was 1:1 (v/v) (Kontiza *et al.*, 2008). Ethyl acetate and methanol fractions were evaporated, the mass was determined and the crude extracts were kept at -20 °C.

Antibacterial assay. The antibacterial activity was evaluated using the Disc Diffusion Method (Montalvão *et al.*, 2014), according to the Clinical and Laboratory Standards Institute (CLSI, 2017). *S. aureus* was streaked on to Muller-Hinton agar medium (Sigma-Aldrich) with the total amount of standard (0.5 Mc Farland). The paper disk (φ 6 mm; Advantec. Japan) containing 15 µl of crude extract was placed on the surface of the agar plate culture. The concentrations of crude extract were 50 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml. Ethyl acetate and methanol were used as negative control. Vancomycin was chosen as positive control (Sigma-Aldrich). The culture plates were incubated overnight at 37 °C. Active isolates were shown by the clear zone around the disk (Redwan *et al.*, 2016).

PCR (Polymerase Chain Reaction) amplification. DNA extraction was done by chelex method (de Lamballerie *et al.*, 1992). This method is a modification used for marine bacteria (Lee *et al.*, 2003). The DNA template was used for PCR amplification. The primers selected to amplify the 16S rRNA gene segment were: 27f (5'-AGAGTTT-GATCMTGGCTCAG-3') and 1492r (5'-TACGGY- TACCTTGTTACGACTT-3') (Weisburg *et al.*, 1991). The PCR mixture contained GoTaq®Green Master Mix Promega (12.5 µl), primer 27 F (1 µl), primer 1492 R (1 µl), template DNA (1 µl) and ddH₂O (9.5 µl), so that the total volume was 25 µl. The primer concentration was 10pmol/µL. PCR condition was following by denaturation at 95 °C for 3 minutes, annealing at 53,9 °C for 1 minute, extension 72 °C for 1 minute for 30 cycles. The PCR products were examined using 1 % agarose gel electrophoresis and the result was visualized by

163 using UVIDoc HD5 (UVITEC Cambridge).

164 **DNA sequencing and phylogenetic analysis.** DNA sequencing was carried out in the PT.

165 Genetica Science (Jakarta, Indonesia). The gene sequences were analyzed using Basic Local

166 Alignment Search Tool (BLAST) (*Altschul et al., 1997*). To identify different species,

167 phylogenetic trees were constructed using MEGA 7 with the 1000x bootstrap test. The results of

168 BLAST Homology were deposited to the DNA Data Bank of Japan (DDBJ, www.ddbj.nig.ac.jp)

169 in order to obtain the accession number.

170 **Cells and Viruses.** African green monkey kidney cells (Vero cell line n^o ATCC zccl81) were

171 grown in Eagle's minimum essential medium (MEM, Laboratory Eurobio) supplemented with

172 8% fetal calf serum (FCS), to which was added 1% of PCS (penicillin 10,000 IU/ml, colimycin

173 25,000 IU/ml, streptomycin 10 mg/ml; Sigma). Cells were routinely passaged every 3 days.

174 A virus stock of *Herpes simplex* virus type 1, wild 27 strain ACV^s PFA^s was obtained from

175 Pr. Henri Agut, Laboratoire de Virologie de la Pitié Salpêtrière, Paris, France. Virus stock was

176 prepared by incubating Vero monolayers (75 cm² culture flasks seeded with 3,5x10⁵ cells/ml) at

177 low multiplicity and incubating at 37 °C, in a 95% air, 5% CO₂ (v,v) atmosphere. Three days

178 after infection, the cultures were frozen and thawed twice, before clearing the preparation by

179 centrifugation at a low speed to remove cell debris. The resulting supernatant aliquot was stored

180 at -70 °C until used. Virus titrations were performed by the Red and Muench dilution method,

181 using 10 wells on 96-wells microtiter plates per dilution. The virus titre was estimated from

182 cytopathogenicity and expressed as 50% infectious doses per millilitre (ID₅₀/ml).

183 **Cytotoxicity and antiviral test.** Using the Vero cell/HSV-1 model, 100 µl of cellular suspension

184 (3,5x10⁵ cells/ml) in Eagle's MEM containing 8% FCS and dilution of potential extract (NP31-

185 01, KJB-07) at concentration 5-1000 µg/ml in Eagle's MEM with a total volume of 200 µl were

grown in 96 well plates. Vero cells were placed in Eagle's MEM with a final volume of 200 μ l in each well and were used as positive controls. The well plates were incubated at 37 °C for 72 hours. Cytotoxic activity was observed by microscope and cells were tested using the neutral red dye method. Optical density was measured at 540 nm using a spectrophotometer (SpectraCount™, France, Packard). The 50% cytotoxic concentration (CC₅₀) of the test compound was defined as the concentration that reduced the absorbance of mock-infected cells to 50% of that of controls. CC₅₀ values were determined as the percentage of destruction (%D): $[(OD_c)C - (OD_c)MOCK / (OD_c)C] \times 100$. (OD_c)C – (OD_c)MOCK were the OD values of the untreated cells and treated cells (*Langlois et al., 1986*).

A serial dilution of the extracts was prepared in MEM media, 50 μ l of extract, 50 μ l MEM and 100 μ l of cellular suspension (3,5x10⁵ cells/ml) infected with HSV-1 at a multiplicity of infection (MOI) of 0.001 ID₅₀/ml were added in 96 well plates. Acyclovir (9-(2-hydroxyethoxymethyl) was used as a control positive against HSV-1 ranging from 0.05 – 5 μ g/ml. Cultures were grown in incubation at 37 °C for 72 hours in a humidified CO₂ atmosphere (5% CO₂). Antiviral activity was observed by microscope and cells were tested using the neutral red dye method. The protection of the extract from virus-infected cells was expressed by 50% effective antiviral extract concentration (EC₅₀). OD was measured at 540 nm. The OD was related directly to the percentage of viable cells, which was inversely related to the cytopathic effect (CPE). EC₅₀ values were determined as the percentage of cell protection (%P): $[(OD_t \text{ virus} - OD_c \text{ virus}) / (OD_c \text{ MOCK} - OD_c \text{ virus})] \times 100$. OD_c and OD_t were the OD values of the virus control and test sample, OD_c MOCK was the OD of mock-infected control (*Langlois et al., 1986*). Interpretation of the data was presented using a system of linear regression equations.

208 **TLC Direct-bio autography and LC-MS/MS analysis.** The compounds were analyzed using
 209 Thin Layer Chromatography (TLC) with silica gel on aluminum sheets (20x20 cm) F₂₅₄ KgaA).
 210 Gradient: hexane and ethyl acetate (7:3, 1:1, 3:7). Compound bands were detected by UV
 211 absorbance and fluorescence. The compounds were characterized by LCMS/MS (*Choma and*
 212 *Jesionek, 2015*). LC-MS/MS analyses were done on a UNIFI chromatographic instrument, with
 213 an acquity UPLC® HSS T3 1.8μ (2.1 x 100 mm) column. Mobile phase A was 0.1%formic
 214 acid/water and mobile phase B was acetonitrile + 0.1 formic acid with gradient A/B = 95/5,
 215 60/40, 0/100 and 95/5 in 10 minutes.

216

217 3. Results

218 **Nudibranchs identification.** Nudibranchs are widely spread in the seas around the world,
 219 especially in Indonesia (<http://www.marinespecies.org/imis.php?persid=7211>). Fifteen
 220 nudibranchs species were collected from the National Park of Karimunjawa, Jepara and five
 221 from Bali. Several nudibranchs have been found on various basic substrates as shown in Table 1.
 222 Most of them feed on sponges, tunicates, hydroids and bryozoans (*Avila, 2006*). The species
 223 were identified based on colour, shape of the body and surface texture of the body as shown in
 224 Table 1 (*Behrens, 2005*)(*Coleman, 2001*). The identification results showed that 20 nudibranch
 225 species collected from Karimunjawa and Bali seawaters belonged to four genera, three
 226 nudibranch Phyllidiidae, Chromodorididae, Flabellinidae and Plankobranchidae (Sacoglossa)
 227 (Table 1). Examples are shown in figure 1.

228 **Isolation of symbiont bacteria.** Preliminary isolation of bacterial symbionts from the surface and
 229 the viscera of nudibranchs (figure 2) were determined by the growth of bacteria in Zobell 2216E
 230 agar media. The numbers of isolates were determined and are presented in Table 1.

Anti-MRSA screening and crude extract assay. The results of the antibacterial activity are presented in Figure 3. There is one active symbiont isolate of *Phyllidia coelestis* (KJB-07) from Karimunjawa and one active symbiont isolate of *Phyllidia varicosa* (NP31-01) from Bali. The strain KJB-07 has stronger activity than the strain NP31-01 based on the diameter of the clear zone. The total mass of crude extract from supernatant of KJB-07 and NP31-01 was 1 g/3L. The ethyl acetate extract was found to be active against MRSA at concentrations of 500 µg/ml and 1000 µg/ml as shown in Table 2. The methanol extract from the bacterial pellet was found to be inactive against MRSA.

DNA sequencing and phylogenetic tree. The complete 16S rDNA sequences of strain NP31-01 (1400 bp) and strain KJB-07 (1376 bp) were obtained. The sequence homology of 99% in phylogenetic tree showed that the strain KJB-07 from *Phyllidia coelestis* was closely related to that of *Pseudoalteromonas rubra* (Figure 4) and the strain NP31-01 from *Phyllidia varicosa* was closely related to *Virgibacillus salarius* (Figure 4). This sequence has been deposited to the Genbank. The sequences have received the accession number LC328972.1 and MH016561.

Cytotoxic and antiviral activity. After three days of treatment, microscopically visible alteration of normal cell morphology was observed and the viability assay showed destruction of a cell layer for extract from *Pseudoalteromonas rubra*. This extract was cytotoxic on the Vero cell in the entire range of concentration assayed (5-1000 µg/ml) as shown in table 3. At concentrations of 1000 µg/ml, 71% of cell destruction was observed. At MOI 0.001 ID₅₀/cells, no anti HSV-1 was performed after 72 h.

The compounds from *Virgibacillus salarius* present no cytotoxicity in the entire range of concentrations assayed (5-1000 µg/ml) as shown in table 3. At concentrations of 1000 µg/ml only 10% of cell destruction was shown but no anti HSV-1 was present.

254 **TLC and LC-MS/MS data.** The results showed that the three compounds were well separated on
 255 Thin Layer Chromatography (Figure 5). Of the three compounds produced by *P. rubra* in LC-
 256 MS/MS (Figure 6), compound 1, prodigiosin showed ions at m/z 324.29; compound 2,
 257 stearidonic acid showed ions at m/z 277.21; and compound 3, (22E,24R)-5 α 8 α -Epidioxyergosta-
 258 6,9,22-trien-3 β -ol showed ions at m/z 449.31. The chemical structures are given in Table 4.

259

260 4. Discussion

261 In this paper, we present the first study on anti-MRSA, cytotoxicity, and antiviral activities
 262 from crude extracts of symbiotic bacteria in the Heterobranchia. Nudibranchs, are one of many
 263 marine invertebrate groups that feed on marine sponges, a source of bioactive compounds. The
 264 bioactive compounds found in nudibranchs are greatly influenced by food and symbiotic bacteria
 265 (Fisch *et al.*, 2017). Nudibranchs have a wide variety of bacteria that are affected by location and
 266 food. In previous studies, the different habitats and conditions have been shown to affect the
 267 biodiversity of symbiotic bacteria and bioactive compounds (Zhukova, 2014). We found different
 268 activities of two potential symbiotic bacteria of nudibranchs from the two locations studied.
 269 Many species we found in Karimunjawa are from the same family, the *Phyllidiidae*, whereas in
 270 Bali a wide range of species was found from different families. Previous work reported that
 271 *Phyllidiidae* commonly produced new sesquiterpenoid isonitriles and several other bioactive
 272 compounds (Fisch *et al.*, 2017)(Sim *et al.*, 2017). We report here that symbiotic bacteria from the
 273 family of *Phyllidiidae* have potential anti-MRSA and cytotoxicity activity.

274 Earlier studies were limited by the fact that entire animals were used for isolation of extract
 275 (Dewi *et al.*, 2016)(Sim *et al.*, 2017)(Zhukova, 2014). In contrast, we cultured the symbiont
 276 bacteria that produce the active compounds, allowing us to produce large amounts of extract,

while conserving the marine environment by using only a very limited number of animals.

We isolated bacteria from the body surface and internal organs of nudibranchs known as viscera. We have identified two bacteria species, which show potential anti-MRSA activity.

In previous studies, bacteria have been successfully isolated from the epithelial cells of the notum, the mantle edge and vestibular gland (*Klussmann-Kolb and Brodie, 1999*)(*Zhukova and Eliseikina, 2012*). It has been suggested that the bacteria may play a role in protection or defense from predators (*Fajardo et al., 2014*), however, the specific function of these bacteria is still under discussion. No molecular identification of bacteria has been reported to date. In the screening effort of our studies, we have identified the symbiotic bacteria as capable of producing anti-MRSA compounds by the complete sequences of the 16S rRNA gene. Two bacterial isolates were found to have 99% sequence identity with *Pseudoalteromonas rubra* (KJB-07) and *Virgibacillus salarius* (NP31-01), respectively. Previous work reported that *P. rubra*, that had been successfully isolated from a small piece of marine sponge (*Mycale armata*) producing red pigment, had antibacterial activity (*Fehér et al., 2008*). Our study is comparable with previous research, in which a novel phenolic anti-MRSA compound from *Pseudoalteromonas phenolic* was isolated from sea water (*Isnansetyo and Kamei, 2003*). Another study on anti-MRSA has been explored in other bacterial symbionts of marine organisms such as Tunicata, Porifera, and marine algae (*Fedders et al., 2010*)(*Kamei and Isnansetyo, 2003*)(*Hentschel et al., 2001*). No research on anti-MRSA activity of the symbiotic bacteria of nudibranchs has been reported previously.

To evaluate the anti-bacterial activity, we showed that the ethyl acetate extract of the supernatant of the bacteria caused an inhibition zone in MRSA cultures as indicated in Table 2, whereas the methanol extract of the centrifuged bacteria showed no activity. The ethyl acetate

extract showed that anti-MRSA activity of *P. rubra* was higher than *V. salarius* at concentrations of 500-1000 µg/ml. The diameter of an inhibition zone of *V. salarius* was 7.18 mm at a concentration of 500 µg/ml and increased to 8.26 mm at concentration 1000 µg/ml. The inhibition zone of *P. rubra* increased from 8.15 - 9.23 mm at concentrations of 500 – 1000 µg/ml. The anti-MRSA activity of crude extract was comparable with Vancomycin in that the antibiotic had higher inhibition zone in 15 mm at concentrations of 30 µg/ml.

Table 2 shows that the two extracts had different effects on the MRSA. In this research, *P. rubra* as a gram-negative bacteria had cell walls that were thinner than *V. salarius* as gram-positive. *Pseudoalteromonas rubra* was able to prevent the loss of intracellular proteins, and reduced access of hydrolytic enzymes and some compounds produced by *S. aureus* (Pinto et al., 2017). Extract from *P. rubra* had a stronger activity than from *V. salarius*. Recent studies reported that the ethyl acetate extract from *Pseudomonas* sp. was able to produce the compound 1-acetyl-beta-carboline, which is active against MRSA at concentrations of 32-128 µg/ml (Lee et al., 2013). Isnansetyo, et. al., showed bactericidal and bacteriolytic activity of 2,4-diacetylphloroglucinol (DAPG) produced by *Pseudomonas* sp. against MRSA and VRSA (Isnansetyo et al., 2003). The mechanisms of resistance affected the activity of DAPG. The activity of compounds produced by marine bacteria should be explored and evaluated.

The cytotoxicity in the *P. rubra* extract on Vero cells was directly proportional to the increase in the concentration of the extract. This may be due to production of endotoxins. We analyzed by LCMS to identified the cytotoxic effect of the *P. rubra* extract is due to the same compound that is active against the MRSA or not.

The *V. salarius* extracts had no cytotoxic effect at any of the concentrations tested. *Virgibacillus* sp. has been known as non-cytotoxic and to be a potential source of new

polysaccharide bioflocculant (*Cosa et al., 2011*). Cell wall of *V. salarius* contained meso-diaminopimelic acid as a major component that has been suggested to correspond to the non-cytotoxicity active compounds against MRSA and (*Hua et al., 2008*). Previous work reported the mechanisms of bacteria associated marine organisms, which are bacteria associated with ascidian *Cystodytes dellechiaiei* (*Martínez-García et al., 2007*) and marine flatworms (*Lin et al., 2017*). *García, M.M., et. al.*, reported the role of the bacterial community associated with *C. dellechiaiei* in production of Pyrdoacidine alkaloids. This research evaluated that antiproliferative activities are found in the host and the associated bacteria but the cytotoxic activities are only found in the associated bacteria. The research indicated that the associated bacteria transformed the compounds and it could be toxic due to environmental influences. The crude extract from bacteria associated with the marine flatworm had potential anti-MRSA and toxic activity against Hela-cells. Further study is necessary to separate and identify the active and cytotoxic compound.

Recent studies have demonstrated the importance of antiviral compounds from marine invertebrate; such as *Phyllocaulis boraceiensis*, which contains polyunsaturated fatty acids that disturb the virus envelope (*Toledo-Piza et al., 2016*). Manzamine A from sponge genus *Acanthostrongylophora*, worked by inhibiting viral replication (*Palem et al., 2011*). In our study, crude extracts of *P. rubra* and *V. salarius* showed no activity against HSV-1. We identified three compounds from *P. rubra*, that showed anti MRSA activity and also cytotoxic activity (table 4). These compounds, prodigiosin, stearidonic acid and (22E,24R)-5 α 8 α -Epidioxyergosta-6,9,22-trien-3 β -ol, are not novel. Prodigiosin has been shown to have antibacterial and cytotoxic activity (*Francisco et al., 2007*). Another study has shown that steridonic acid had synergism with amphotericin-B in inhibiting *Candida* (*Taitt, 2012*). The

compound (22E,24R)-5 α 8 α -Epidioxyergosta-6,9,22-trien-3 β -ol has been found previously in *Hypsizigus marmoreus* (Xu et al., 2007) and in the South-East Asian mushroom *Amanita subjunquillea* (Kim et al., 2008). This compound has been shown to have moderate toxicity against human tumor cells.

5. Conclusions:

This is the first reported study describing research on anti-MRSA, cytotoxicity and anti HSV-1 activity of bacterial symbionts from viscera of nudibranchs. We showed that crude extracts from culture supernatants of *Pseudoalteromonas rubra* and *Virgibacillus salarius*, symbionts of these nudibranchs, have anti-MRSA activity, which must be caused by compounds that are produced and secreted by the bacteria. Extracts from *P. rubra* showed cytotoxic effects on Vero cells, whereas extracts from *V. salarius* did not show cytotoxic effects. No anti HSV-1 activity was detected in any of the bacterial extracts. Three compounds were identified: prodigiosin, stearidonic acid, and (22E,24R)-5 α 8 α -Epidioxyergosta-6,9,22-trien-3 β -ol. Prodigiosin and stearidonic acid have been suggested to exhibit antibacterial activity; moreover epidioxyergosta has been suggested to have cytotoxic activity.

6. Conflict of interest statement

We declare that we have no conflict of interest.

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374

375 8. Author Contribution

376 R.K. collected samples, conducted bioassays, performed all these experiments and prepared the
377 manuscript; G.P., reviewed the manuscript and interpreted the results; G.B., A.S., O.K.R., N.B.,
378 and A.T., planned the experiments, supervised the experiments and reviewed the manuscript;
379 A.T., collected samples; I.W.M. identified the nudibranchs; L.T., and C.M. performed
380 cytotoxicity assay; R.K., M.A.A., and D.A performed the bacteria isolation and interpreted the
381 results; all authors have seen the final version of the manuscript and agree with the content.

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Table 1(on next page)

Samples collected and total number of symbiont bacteria in Nudibranch.

The table show about morphology identification of nudibranch

1 **Table 1.** Samples collected and total number of symbiont bacteria in Nudibranch.

Species Code	Nudibranch Species	Appearance of the Opisthobranch	Number of Isolates of Bacteria	Depth (m)	Substrate	Locality
NT1-162	<i>Hypselodoris whitei</i>	Whitish background colour with reddish purple longitudinal lines covering the mantle. The rhinophores are orange to orange-red with a distinctive white tip. The gills are similarly coloured with white on the inside and usually at the tip of each gill.	10	19	Sand	Bali
NT1-164	<i>Hypselodoris infucata</i>	Grey mantle with yellow and purple spots spread on the surface. The rhinophores are red and the gills composed by two-dimensional leaf with a red line along the internal and external edge.	11	15	Hydroid	Bali
NP31-04	<i>Goniobranchus leopardus</i>	The mantle consists of purple-brown marks, ringed with reticulate brownish background, and a purple border. There are four colour bands around the mantle edge, an outermost white, then translucent greyish purple, then white, then yellow.	12	18	Coral	Bali
NT31-03	<i>Thuridilla gracilis</i>	Dark background, fine white longitudinal lines and the foot, head and rhinophores edged with bright orange. The parapodia are bordered with bright orange tips.	8	17	Sand	Bali
NP31-01	<i>Phyllidia varicosa</i>	The mantle consists of longitudinal, tuberculate notal ridges. The ridge and bases of the tubercles are blue-grey in colour and the tubercles are capped in yellow.	10	15	Coral	Bali
KJN-17	<i>Phyllidia ocellata</i>	The dorsal pattern consists of series of white tubercles, dark background and yellow rims.	19	15	Hydroid	Karimunjawa
KJN-09	<i>Phyllidia varicosa</i>	The mantle consists of longitudinal, tuberculate notal ridges. The ridge and bases of the tubercles are blue-grey in colour and the tubercles are capped in yellow.	19	15	Sand	Karimunjawa
KJT-02	<i>Caloria indica</i>	The body is full of white cerata and the horns are white with few purple rings spread along the axes.	10	15	Sponge	Karimunjawa
KJN-13	<i>Phyllidiella nigra</i>	The black background mantle is ornamented by white clustered tubercles.	12	17	Hydroid	Karimunjawa
KJB-07	<i>Phyllidia coelestis</i>	Black background mantle with blue-grey ridges; yellow-capped mid- dorsal tubercles.	18	15	Coral	Karimunjawa
KJN-08	<i>Phyllidia varicosa</i>	The mantle consists of longitudinal, tuberculation total ridges. The ridge and bases of the tubercles are blue-grey in colour and the tubercles are capped in yellow.	19	15	Sponge	Karimunjawa
KJN-18	<i>Phyllidiopsis pipeki</i>	The rhinophores are pink with a black tip, black lines extending from pale brown mantle edges.	15	15	Sponge	Karimunjawa

KJN-19	<i>Phyllidiella striata</i>	White clustered tubercles and few yellow caps; black background dorsal.	8	15	Coral	Karimunjawa
KJN-47	<i>Phyllidia varicosa</i>	The mantle consists of longitudinal, tuberculate notal ridges. The ridge and bases of the tubercles are blue-grey in colour and the tubercles are capped in yellow.	12	16	Sponge	Karimunjawa
KJN-46	<i>Phyllidia varicosa</i>	The mantle consists of longitudinal, tuberculate notal ridges. The ridge and bases of the tubercles are blue-grey in colour and the tubercles are capped in yellow.	11	18	Tunicata	Karimunjawa
KJN-48	<i>Phyllidiopsis shireenae</i>	White dorsal with white tubercles; two longitudinal black lines.	11	14	Sponge	Karimunjawa
KJN-42	<i>Goniobranchus kuniei</i>	They all have large purple or purple-brown spots or marks, usually ringed with white, brownish background, and a purple border.	12	16	Coral	Karimunjawa
KJN-44	<i>Phyllidiella cooraburrama</i>	Extremely large, isolated, notal tubercles and black dorsal background.	10	18	Coral	Karimunjawa
KJN-5	<i>Phyllidiopsis shireenae</i>	White dorsal with white tubercles; two longitudinal black lines.	8	15	Bryozoa	Karimunjawa
KJN-45	<i>Phyllidiopsis pipeki</i>	The rhinophores are pink with a black tip, black lines extending from pale brown mantle edges.	10	15	Coral	Karimunjawa

Table 2 (on next page)

Anti-MRSA activity (overlay, extract, antibiotic)

1 **Table 2.** Anti-MRSA activity (overlay, extract, antibiotic)

Crude extract	Concentration (µg/ml)	Diameter of Clear Zone (mm) of Anti-MRSA				
		KJB-07	NP31-01	KJB-07	NP31-01	Vancomycin
Bacterial pellet (Methanol)	50	-	-	-	-	nt
	250	-	-	-	-	nt
	500	-	-	-	-	nt
	1000	-	-	-	-	nt
Supernatant (Ethyl acetate)	50	-	-	-	-	nt
	250	-	-	-	-	nt
	500	+	+	8.15	7.18	nt
	1000	+	+	9.23	8.26	nt
Vancomycin	30	nt	nt	nt	nt	15

2

Table 3 (on next page)

Evaluation of cytotoxic and anti-HSV-1 activity of ethyl acetate extract of supernatant from *P. rubra* and *V. salarius*

Table 3. Evaluation of cytotoxic and anti-HSV-1 activity of ethyl acetate extract of supernatant from *P. rubra* and *V. salarius*

Extract of Bacteria	CC ₅₀	EC ₅₀
species	(µg/ml)	(µg/ml)
<i>Pseudoalteromonas rubra</i>	75	-
<i>Virgibacillus salarius</i>	>1000	-
Acyclovir	>5	0.5

Table 4(on next page)

Identification of compound in *P. rubra*

1 **Table 4.** Identification of compound in *P. rubra*

Compound name	Observed RT (min)	Formula
Compound 1. prodigiosin	6.04	$C_{20}H_{37}NO_2$
Compound 2. stearidonic acid	5.26	$C_{18}H_{28}O_2$
Compound 3. (22E,24R)-5α,8α-Epidioxysteroid-6,9,22-trien-3β-ol	5.50	$C_{28}H_{42}O_3$

2

3

Figure 1

Underwater photographs of the nudibranch; (A) *Phyllidia varicosa* from Bali

Figure 2

Underwater photographs of the nudibranch; (B) *Phyllidia coelestis* from Karimunjawa, Jepara Indonesia.

Figure 3 (on next page)

Isolation of bacteria symbiont from the surface (A); viscera

Figure 4(on next page)

Isolation of bacteria symbiont from the surface (B) of *Phyllidia varicosa*; flowchart of isolation of symbiont bacteria.

Figure 5

Active isolate symbiotic bacteria of nudibranchs against MRSA; (A) NP31-01 symbiotic *Phyllidia varicosa*.

Figure 6

Active isolate symbiotic bacteria of nudibranchs against MRSA; (B) KJB-07 symbiotic *Phyllidia coelestis*.

Figure 7 (on next page)

Phylogenetic tree of bacteria associated with nudibranch isolated from Karimunjawa National Park and Bali, Indonesia. *Thermus kawayensis* KW11 was used as the out-group.

Figure 8

Typical TLC photography of ethyl acetate extract of *P.rubra*; (A) stearidonic acid, (B) Prodigiosin, (C) (22E,24R)-5 α 8 α -Epidioxyergosta-6,9,22-trien-3 β -ol.

Figure 9 (on next page)

LSMS/MS Chromatogram of three compounds (A) stearidonic acid, (B) Prodigiosin, (C) (22E,24R)-5 α 8 α -Epidioxyergosta-6,9,22-trien-3 β -ol.

A

Channel name: 1: +324.2901 (39.8 PPM) +325.2933 (39.8 PPM) +326.2962 (39.8 PPM) : TOF MS^E (100-1200) 6eV ESI+ - Low CE : Integrated : Smoothed

B

Channel name: (22E,24R)-5 α ,8 α -Epidioxysterosta-6,9,22-trien-3 β -ol [+Na] : (39.8 PPM) 449.3118

C