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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 500-1000µg/ml. DNA sequence analysis showed that the strainKJB-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 byPseudoalteromonas 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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22 Abstract

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24	The aim of this work was to isolate bacterial symbionts from nudibranchs and subsequently to determine anti-
25	Methicillin resistant Staphylococcus aureus (MRSA), cytotoxicity and anti-HSV-1 activities of bio compounds.
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 38 39 40 41 42 43 44 45 46 	Keywords: Nudibranch; Virgibacillus salarius; Pseudoalteromonas rubra; Anti-MRSA activity; HSV-1; antibiotic I. 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.

48 health concern. This bacterium is known as methicillin resistant *Staphylococcus aureus*, or 49 MRSA. Approximately one-third of the world population has S. aureus bacteria on their bodies 50 at any given time, primarily in the nose and on the skin. The bacteria can be present without 51 causing an active infection. According to Centers for Disease Control and Prevention (CDC) 52 approximately 1% of people has MRSA. There are two ways in which the infection can be 53 acquired hospital-acquired MRSA (HA-MRSA) or community-associated MRSA (CA-MRSA). 54 The MRSA infections caused higher morbidity and mortality compared to non-resistant strains. 55 (Akhi et al., 2017). The rapid emergence of MRSA infection is becoming crucial, as the 56 effectiveness of treatment of MRSA infection decreases due to the ability of bacteria to respond 57 to antibiotics (Guzmán-Blanco et al., 2009). In 2017, according to the World Health 58 Organization (WHO), the resistance to first line drugs to treat infections caused by S. aureus was 59 widespread. People with MRSA are estimated to be 64% more likely to die than people with a 60 non-resistant form of the infection. 61 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

69 The Indonesian archipelago is a hotspot for biodiversity in the world (*Gastropoda et al.*,
70 2018) and a very rich marine environment. The studies regarding screening of secondary

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71 metabolites-producing bacterial symbionts are important for understanding their biotechnological 72 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), 73 74 soft coral (Seved Vahid Shetab-Boushehri, 2012) and sponge (Radjasa, et al., 2007). 75 Since marine tropical diversity also reflects chemical diversity, the isolation of the under-76 exploited bacterial symbionts from Indonesian nudibranchs offers a great opportunity for 77 discovering novel bio compounds, based on screening against various disease targets. Natural 78 products from cultured symbionts of marine invertebrates present an opportunity to be developed 79 in ways that circumvent environmental and supply problems of pharmaceutical substances. 80 Nudibranchs, belonging to gastropods, are of great interest for research and development of 81 bioactive natural metabolites. These invertebrates are exposed to predators and to surface 82 colonization by microorganisms. For them, the only hope of surviving and fighting effectively 83 against this is to produce compounds that are toxic for infectious bacteria. These compounds 84 have actually been found in a number of organisms and it has been suggested that they represent 85 secondary metabolites from symbiont bacteria (Radjasa et al., 2011). 86 There is one report of symbiotic bacteria in the vestibular gland, associated with the female 87 reproductive system, and in the egg masses of the nudibranch *Dendrodoris nigra* (Klussmann-88 Kolb and Brodie, 1999). In 2012, Rod-shaped gram-negative bacteria were revealed in the 89 epithelial cells of the notum and the mantle edge as well as in the adjoining glycocalix near the 90 outer membrane of the epithelial cells (Zhukova and Eliseikina, 2012) and these bacteria were 91 enclosed in secondary vacuoles in the epithelial cells in *D. nigra*. Several drugs with interesting 92 pharmaceutical properties are currently in clinical trials (Chand and Karuso, 2017). For example, 93 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 eliora, has been reported to have cytotoxic and genotoxic activity (De Oliveira et al., 2007). 96 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 Park of Karimunjawa (5º47'21.6''S 110º30'41.9''E) (March 2017), Jepara Indonesia by SCUBA 109 diving 15-20 m in depth, at 18-25 °C. The samples of nudibranchs were put into sterile plastic 110 bags, filled with seawater and stored in a cool box (He et al., 2014)(Sabdono et al., 2015), and 111 112 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 sterile seawater, to remove the impurities still attached to the nudibranchs body. The contents of 114 115 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

117 method (10⁻¹, 10⁻², 10⁻³, 10⁻⁴)(Anand et al., 2006). The bacterial inoculation was performed on 118 the ZoBell 2216E agar media [peptone 15 g/L (Oxoid Laboratories); yeast (Himedia 119 Laboratories); agar 15 g/L (Oxoid Laboratories)] and was incubated at a temperature of 37 °C for 120 5 days. Bacterial colonies were grown on a plate, and each colony was separated according to 121 shape, elevation and colour. Isolation and purification processes were done in the Tropical 122 Marine Biotechnology Laboratory at Diponegoro University. The pure colonies were maintained 123 in slant cultures at -20 °C. 124 Anti-MRSA screening. The antibacterial test was conducted using the overlay method, 245 125 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 126 127 Staphylococcus aureus (Strain MRSA from Kariadi Hospital in Semarang, Indonesia, resistant 128 for oxacillin, gentamicin, benzylpenicillin, ciprofloxacin, levofloxacin, tetracycline) was cultured 129 in Muller-Hinton broth (Difco Laboratories) with a shaker condition of 150 rpm at 37 °C for 24 130 h. The antibacterial test was conducted by mixing S. aureus with 0.5 McFarland standard in soft 131 agar media and pouring into the petri dishes containing the marine bacterial colonies. All 132 screening was performed in triplicate experiments. Clear zones were observed following 133 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 pexhibited anti-MRSA 134 135 activity were chosen for further characterization of bioactive compounds, molecular 136 identification, cytotoxicity and anti-HSV activity. *Bioactive compound extraction*. Active isolates of bacterial symbionts of nudibranchs against 137

138 MRSA were cultured in 3 L of nutrient broth (Oxoid Laboratories) with seawater-based medium

139 for 6 days (*Singh et al., 2014*). The bacterial suspension was centrifuged at 5000 rpm, at 4 ^oC for

140 10 minutes. The supernatant was extracted with ethyl acetate. The bacterial pellet was extracted 141 with methanol. The ratio of solvent to culture was 1:1 (v/v) *(Kontiza et al., 2008)*. Ethyl acetate 142 and methanol fractions were evaporated, the mass was determined and the crude extracts were 143 kept at -20 $^{\circ}$ C.

144 Antibacterial assay. The antibacterial activity was evaluated using the Disc Diffusion Method

145 (Montalvão et al., 2014), according to the Clinical and Laboratory Standards Institute (CLSI,

146 2017). S. aureus was streaked on to Muller-Hinton agar medium (Sigma-Aldrich) with the total

147 amount of standard (0.5 Mc Farland). The paper disk (\$\$\phi\$ 6 mm; Advantec. Japan) containing 15

148 µl of crude extract was placed on the surface of the agar plate culture. The concentrations of

149 crude extract were 50 μ g/ml, 250 μ g/ml, 500 μ g/ml and 1000 μ g/ml. Ethyl acetate and methanol

150 were used as negative control. Vancomycin was chosen as positive control (Sigma-Aldrich). The

151 culture plates were incubated overnight at 37 °C. Active isolates were shown by the clear zone

152 around the disk (*Redwan et al., 2016*).

153 PCR (Polymerase Chain Reaction) amplification. DNA extraction was done by chelex method

154 (de Lamballerie et al., 1992). This method is a modification used for marine bacteria (Lee et al.,

155 2003). The DNA template was used for PCR amplification. The primers selected to amplify the

156 16S rRNA gene segment were: 27f (5'-AGAGTTT-GATCMTGGCTCAG-3') and 1492r (5'-

157 TACGGY- TACCTTGTTACGACTT-3') (Weisburg et al., 1991). The PCR mixture contained

158 GoTaq®Green Master Mix Promega (12.5 μl), primer 27 F (1 μl), primer 1492 R (1 μl),

template DNA (1 μ l) and ddH2O (9.5 μ l), so that the total volume was 25 μ l. The primer

160 concentration was 10pmol/ μ L. PCR condition was following by denaturation at 95 °C for 3

161 minutes, annealing at 53,9 °C for 1 minute, extension 72 °C for 1 minute for 30 cycles. The PCR

162 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 n⁰ 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

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 (ID_{50}/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 μ g/ml in Eagle's MEM with a total volume of 200 μ l were

186	grown in 96 well plates. Vero cells were placed in Eagle's MEM with a final volume of 200 μ l in
187	each well and were used as positive controls. The well plates were incubated at 37 0 C for 72
188	hours. Cytotoxic activity was observed by microscope and cells were tested using the neutral red
189	dye method. Optical density was measured at 540 nm using a spectrophotometer
190	(SpectraCountTM, France, Packard). The 50% cytotoxic concentration (CC_{50}) of the test
191	compound was defined as the concentration that reduced the absorbance of mock-infected cells
192	to 50% of that of controls. CC_{50} values were determined as the percentage of destruction (%D):
193	$[(OD_c)C - OD_c)MOCK/(OD_c)C] \times 100. (OD_c)C - (OD_c)MOCK$ were the OD values of the
194	untreated cells and treated cells (Langlois et al., 1986).
195	A serial dilution of the extracts was prepared in MEM media, 50 μ l of extract, 50 μ l MEM
196	and 100 μ l of cellular suspension (3,5x10 ⁵ cells/ml) infected with HSV-1 at a multiplicity of
197	infection (MOI) of 0.001 ID ₅₀ /ml were added in 96 well plates. Acyclovir (9-(2-
198	hydroxyethoxymethyl) was used as a control positive against HSV-1 ranging from $0.05 - 5$
199	μ g/ml. Cultures were grown in incubation at 37 °C for 72 hours in a humidified CO ₂ atmosphere
200	(5% CO ₂). Antiviral activity was observed by microscope and cells were tested using the neutral
201	red dye method. The protection of the extract from virus-infected cells was expressed by 50%
202	effective antiviral extract concentration (EC ₅₀). OD was measured at 540 nm. The OD was
203	related directly to the percentage of viable cells, which was inversely related to the cytopathic
204	effect (CPE). EC ₅₀ values were determined as the percentage of cell protection (%P): [(ODt virus
205	$- OD_c virus)/(OD_c MOCK- OD_c virus)] x 100. OD_c and OD_t were the OD values of the virus$
206	control and test sample, OD _c MOCK was the OD of mock-infected control (Langlois et al.,
207	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 (<u>http://www.marinespecies.org/imis.php?persid=7211</u>). Fifteen

220 nudibranchs species were collected from the National Park of Karimunjawa, Jepara and five

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

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

the viscera of nudibranchs (figure 2) were determined by the growth of bacteria in Zobell 2216E

agar media. The numbers of isolates were determined and are presented in Table 1.

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was performed after 72 h.

231 Anti-MRSA screening and crude extract assay. The results of the antibacterial activity are 232 presented in Figure 3. There is one active symbiont isolate of Phyllidia coelestis (KJB-07) from 233 Karimunjawa and one active symbiont isolate of Phyllidia varicosa (NP31-01) from Bali. The 234 strain KJB-07 has stronger activity than the strain NP31-01 based on the diameter of the clear 235 zone. The total mass of crude extract from supernatant of KJB-07 and NP31-01 was 1 g/3L. The 236 ethyl acetate extract was found to be active against MRSA at concentrations of 500 µg/ml and 237 1000 µg/ml as shown in Table 2. The methanol extract from the bacterial pellet was found to be 238 inactive against MRSA. 239 **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 240 241 phylogenetic tree showed that the strain KJB-07 from Phyllidia coelestis was closely related to 242 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 243 244 Genbank. The sequences have received the accession number LC328972.1 and MH016561. 245 Cytotoxic and antiviral activity. After three days of treatment, microscopically visible alteration

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

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 μ g/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

TLC and LC-MS/MS data. The results showed that the three compounds were well separated on
Thin Layer Chromatography (Figure 5). Of the three compounds produced by *P. rubra* in LCMS/MS (Figure 6), compound 1, prodigiosin showed ions at *m/z* 324.29; compound 2,
stearidonic acid showed ions at *m/z* 277.21; and compound 3, (22E,24R)-5α8α-Epidioxyergosta6,9,22-trien-3β-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 surce 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 *Phyllididae*, whereas in 270 Bali a wide range of species was found from different families. Previous work reported that 271 *Phyllididae* 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,

277 while conserving the marine environment by using only a very limited number of animals. 278 We isolated bacteria from the body surface and internal organs of nudibranchs known as 279 viscera. We have identified two bacteria species, which show potential anti-MRSA activity. 280 In previous studies, bacteria have been successfully isolated from the epithelial cells of the 281 notum, the mantle edge and vestibular gland (Klussmann-Kolb and Brodie, 1999)(Zhukova and 282 *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 283 under discussion. No molecular identification of bacteria has been reported to date. In the 284 285 screening effort of our studies, we have identified the symbiotic bacteria as capable of producing 286 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 287 288 Virgibacillus salarius (NP31-01), respectively. Previous work reported that P. rubra, that had 289 been successfully isolated from a small piece of marine sponge (Mycale armata) producing red 290 pigment, had antibacterial activity (Fehér et al., 2008). Our study is comparable with previous 291 research, in which a novel phenolic anti-MRSA compound from *Pseudoalteromonas phenolic* 292 was isolated from sea water (Isnansetvo and Kamei, 2003). Another study on anti-MRSA has 293 been explored in other bacterial symbionts of marine organisms such as Tunicata, Porifera, and 294 marine algae (Fedders et al., 2010)(Kamei and Isnansetyo, 2003)(Hentschel et al., 2001). No 295 research on anti-MRSA activity of the symbiotic bacteria of nudibranchs has been reported 296 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

300 extract showed that anti-MRSA activity of P. rubra was higher than V. salarius at concentrations 301 of 500-1000 µg/ml. The diameter of an inhibition zone of V. salarius was 7.18 mm at a 302 concentration of 500 µg/ml and increased to 8.26 mm at concentration 1000 µg/ml. Thehe 303 inhibition zone of *P. rubra* increased from 8.15 - 9.23 mm at concentrations of 500 - 1000304 µg/ml. The anti-MRSA activity of crude extract was comparable with Vancomycin in that the 305 antibiotic had higher inhibition zone in 15 mm at concentrations of 30 µg/ml. 306 Table 2 shows that the two extracts had different effects on the MRSA. In this research, P. 307 rubra as a gram-negative bacteria had cell walls that were thinner than V. salarius as gram-308 positive. *Pseudoalteromonas rubra* was able to prevent the loss of intracellular proteins, and 309 reduced access of hydrolytic enzymes and some compounds produced by S. aureus (Pinto et al., 310 2017). Extract from P. rubra had a stronger activity than from V. salarius. Recent studies 311 reported that the ethyl acetate extract from *Pseudomonas* sp. was able to produce the compound 312 1-acetyl-beta-carboline, which is active against MRSA at concentrations of 32-128 µg/ml (Lee et 313 al., 2013). Isnansetyo, et. al., showed bactericidal and bacteriolytic activity of 2,4-314 diacetylphloroglucinol (DAPG) produced by Pseudomonas sp. against MRSA and VRSA 315 (Isnansetvo et al., 2003). The mechanisms of resistance affected the activity of DAPG. The 316 activity of compounds produced by marine bacteria should be explored and evaluated. 317 The cytotoxicity in the *P. rubra* extract on Vero cells was directly proportional to the 318 increase in the concentration of the extract. This may be due to production of endotoxins. We 319 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. 320 321 The V. salarius extracts had no cytotoxic effect at any of the concentrations tested.

322 Virgibacillus sp. has been known as non-cytotoxic and to be a potential source of new

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323 polysaccharide bioflocculant (Cosa et al., 2011). Cell wall of V. salarius contained meso-324 diaminopimelic acid as a major component that has been suggested to correspond to the non-325 cytotoxicity active compounds against MRSA and (Hua et al., 2008). Previous work reported the 326 mechanisms of bacteria associated marine organisms, which are bacteria associated with ascidian Cystodytes dellechiajei (Martínez-García et al., 2007) and marine flatworms (Lin et al., 2017). 327 328 García, M.M., et. al., reported the role of the bacterial community associated with C. dellechiajei 329 in production of Pyridoacidine alkaloids. This research evaluated that antiproliferative activities 330 are found in the host and the associated bacteria but the cytotoxic activities are only found in the 331 associated bacteria. The research indicated that the associated bacteria transformed the 332 compounds and it could be toxic due to environmental influences. The crude extract from 333 bacteria associated with the marine flatworm had potential anti-MRSA and toxic activity against 334 Hela-cells. Further study is necessary to separate and identify the active and cytotoxic 335 compound. 336 Recent studies have demonstrated the importance of antiviral compounds from marine

337 invertebrate; such as *Phyllocaulis boraceiensis*, which contains polyunsaturated fatty acids that

338 disturb the virus envelope (Toledo-Piza et al., 2016). Manzamine A from sponge genus

339 Acanthostrongylophora, worked by inhibiting viral replication (Palem et al., 2011). In our study,

340 crude extracts of *P. rubra* and *V. salarius* showed no activity against HSV-1.

341 We identified three compounds from *P. rubra*, that showed anti MRSA activity and also

342 cytotoxic activity (table 4). These compounds, prodigiosin, stearidonic acid and (22E,24R)-

343 $5\alpha 8\alpha$ -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

345 steridonic acid had synergism with amphotericin-B in inhibiting Candida (*Taitt, 2012*). The

346	compound (22E,24R)-5 α 8 α -Epidioxyergosta-6,9,22-trien-3 β -ol has been found previously in
347	Hypsizigus marmoreus (Xu et al., 2007) and in the South-East Asian mushroom Amanita
348	subjunquillea (Kim et al., 2008). This compound has been shown to have moderate toxicity
349	against human tumor cells.
350	
351	5. Conclusions:
352	This is the first reported study describing research on anti-MRSA, cytotoxicity and anti HSV-1
353	activity of bacterial symbionts from viscera of nudibranchs. We showed that crude extracts from
354	culture supernatants of Pseudoalteromonas rubra and Virgibacillus salarius, symbionts of these
355	nudibranchs, have anti-MRSA activity, which must be caused by compounds that are produced
356	and secreted by the bacteria. Extracts from P. rubra showed cytotoxic effects on Vero cells,
357	whereas extracts from V. salarius did not show cytotoxic effects. No anti HSV-1 activity was
358	detected in any of the bacterial extracts. Three compounds were identified: prodigiosin,
359	stearidonic acid, and (22E,24R)-5α8α-Epidioxyergosta-6,9,22-trien-3β-ol. Prodigiosin and
360	stearidonic acid have been suggested to exhibit antibacterial activity; moreover epidioxyergosta
361	has been suggested to have cytotoxic activity.
362	
363	6. Conflict of interest statement
364	We declare that we have no conflict of interest.
365	
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374 375 376	8. Author Contribution 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.
382	
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570

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.

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

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

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

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

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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*

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

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

3

Table 4(on next page)

Identification of compound in P. rubra

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



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

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Figure 4(on next page)

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

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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-07symbiotic *Phyllidia coelestis*.



Figure 7(on next page)

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

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Thermus kawarayensis strain KW11 (NR_112160) Virgibacillus marismortui strain SB-16 (MF_321845) Virgibacillus salarius strain SB-11 (MF_321849) Virgibacillus olivae strain R530 (HM_179193)

NP31-01 isolate

Virgibacillus chiguensis strain Halo30-9 (EF_101168) Virgibacillus proomii strain EH10 (GU_339240) Virgibacillus carmonensis strain LMG 20964 (NR_025481) Virgibacillus necropolis strain LMG 19488T (AJ_315056) Bacillus pichinotyi strain YAM2 (JX_203257) Bacillus depressus strain LNHL5 (MG_008670) Bacillus subterraneus strain COOIBB (NR_104749) Bacillus boroniphilus strain CM25 (EU_660347) Bacillus foraminis strain XJSL3-9 (GQ_903408) KJB-07 isolate

Pseudoalteromonas rubra strain ATCC 29570 (NR_026223) Pseudoalteromonas viridis strain P1 (EU_240534) Pseudoalteromonas piscicida strain P1-14-1a (KY_382776) Pseudoalteromonas flavipulchra NCIMB 2033 (NR_025126 Pseudoalteromonas citrea strain BF21e (HQ_439546) Pseudoalteromonas peptidolytica strain OS-3M-3 (KY366348) Pseudoalteromonas marina strain D5053 (F_161291) Pseudoalteromonas porphyrae strain BM2 (FJ_205739) Halomonas salina strain 2YNB1 (EF_517963) Halomonas koreensis strain SS20 (NR_025773) Halomonas organivorans strain G-16.1 (NR_029029) Halomonas halophila strain CCM 3662 (NR_042697)

Figure 8

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



Figure 9(on next page)

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

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







С