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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 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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2

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22 Abstract

23

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.
26 Fifteen species of nudibranchs were collected from Karimunjava and five species from Bali, respectively. A total of
27 245 bacteria isolates were obtained. The anti-MRSA activity screening activity indicated 2 isolates of active
28 bacteria. Ethyl acetate extracts from supernatants, indicating secreted compounds, showed an inhibition zone against
29 MRSA at concentrations of 500-1000 µg/ml. DNA sequence analysis showed that the strain KJB-07 from *Phyllidia*
30 *coelestis* was closely related to *Pseudoalteromonas rubra*, the strain NP31-01 from *Phyllidia varicosa* was closely
31 related to *Virgibacillus salarius*. The extract of *P. rubra* was cytotoxic to Vero cells at a concentration of 75 µg/ml.
32 The extract of *V. salarius* presented no cytotoxicity at concentrations of 5-1000 µg/ml. No anti HSV-1 was
33 observed. This is the first reported study describing research on anti-MRSA, cytotoxicity and anti HSV-1 activity of
34 bacterial symbionts from the viscera of nudibranch. Compounds produced and secreted by *Pseudoalteromonas*
35 *rubra* and *Virgibacillus salarius*, symbionts of Nudibranch, had potential anti-MRSA activity. Extracts from *P.*
36 *rubra* showed cytotoxic effects on Vero cells, whereas extracts from *V. salarius* did not show cytotoxic effects.
37 Three compounds were identified by LC/MS after purification from culture supernatant.

38

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

41

42 1. Introduction

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

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

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

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
73 bacterial symbionts from Indonesian marine invertebrates, such as coral (*Radjasa, et al., 2007*),
74 soft coral (Seyed 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*
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 (5⁰47'21.6''S 110⁰30'41.9''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)(*Sabdonno 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

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
126 media and incubated at 37 °C for 24 h (Radjasa, et al., 2007). The pathogenic bacterium
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
134 there has been antibacterial activity (Defer et al., 2009). Isolates that exhibited anti-MRSA
135 activity were chosen for further characterization of bioactive compounds, molecular
136 identification, cytotoxicity and anti-HSV activity.

137 **Bioactive compound extraction.** Active isolates of bacterial symbionts of nudibranchs against
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 °C 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 °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 (ϕ 6 mm; Advantec. Japan) containing 15
148 μ l 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 μ l), primer 27 F (1 μ l), primer 1492 R (1 μ l),
159 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 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

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 °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 (SpectraCount™, France, Packard). The 50% cytotoxic concentration (CC₅₀) 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. CC₅₀ 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 (3,5x10⁵ cells/ml) infected with HSV-1 at a multiplicity of
197 infection (MOI) of 0.001 ID₅₀/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 CO₂ atmosphere
200 (5% CO₂). 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 (EC₅₀). 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). EC₅₀ values were determined as the percentage of cell protection (%P): $[(OD_t \text{ virus}$
205 $- OD_c \text{ virus}) / (OD_c \text{ MOCK} - OD_c \text{ virus})] \times 100$. OD_c and OD_t were the OD values of the virus
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 1.8 μ (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.

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
240 (1400 bp) and strain KJB-07 (1376 bp) were obtained. The sequence homology of 99% in
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
243 closely related to *Virgibacillus salarius* (Figure 4). This sequence has been deposited to the
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
246 of normal cell morphology was observed and the viability assay showed destruction of a cell
247 layer for extract from *Pseudoalteromonas rubra*. This extract was cytotoxic on the Vero cell in
248 the entire range of concentration assayed (5-1000 µg/ml) as shown in table 3. At concentrations
249 of 1000 µg/ml, 71% of cell destruction was observed. At MOI 0.001 ID₅₀/cells, no anti HSV-1
250 was performed after 72 h.

251 The compounds from *Virgibacillus salarius* present no cytotoxicity in the entire range of
252 concentrations assayed (5-1000 µg/ml) as shown in table 3. At concentrations of 1000 µg/ml
253 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,

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
283 from predators (*Fajardo et al., 2014*), however, the specific function of these bacteria is still
284 under discussion. No molecular identification of bacteria has been reported to date. In the
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
287 were found to have 99% sequence identity with *Pseudoalteromonas rubra* (KJB-07) and
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 (*Isnansetyo 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.

297 To evaluate the anti-bacterial activity, we showed that the ethyl acetate extract of the
298 supernatant of the bacteria caused an inhibition zone in MRSA cultures as indicated in Table 2,
299 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. The
303 inhibition zone of *P. rubra* increased from 8.15 - 9.23 mm at concentrations of 500 – 1000
304 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 (Isnansetyo *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
320 compound that is active against the MRSA or not.

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

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
327 *Cystodytes dellechiaiei* (*Martínez-García et al., 2007*) and marine flatworms (*Lin et al., 2017*).
328 *García, M.M., et. al.*, reported the role of the bacterial community associated with *C. dellechiaiei*
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 α 8 α -Epidioxyergosta-6,9,22-trien-3 β -ol, are not novel. Prodigiosin has been shown to have
344 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)-5 α 8 α -Epidioxyergosta-6,9,22-trien-3 β -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 **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.

382

383 **9. References.** O.K.R., . A.S., . J., . E.Z., 2007. Richness of Secondary Metabolite-Producing

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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 ($\mu\text{g/ml}$)	Diameter of Clear Zone (mm) of Anti-MRSA				
		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

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*

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

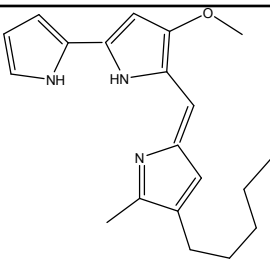
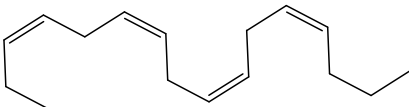
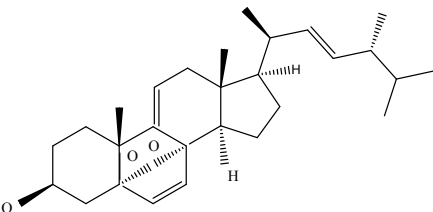
Extract of Bacteria species	CC ₅₀ (µg/ml)	EC ₅₀ (µg/ml)
<i>Pseudoalteromonas rubra</i>	75	-
<i>Virgibacillus salarius</i>	>1000	-
Acyclovir	>5	0.5

3

Table 4 (on next page)

Identification of compound in *P. rubra*

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

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)-5 α 8 α - Epidioxysterosta-6,9,22-trien- 3 β -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.

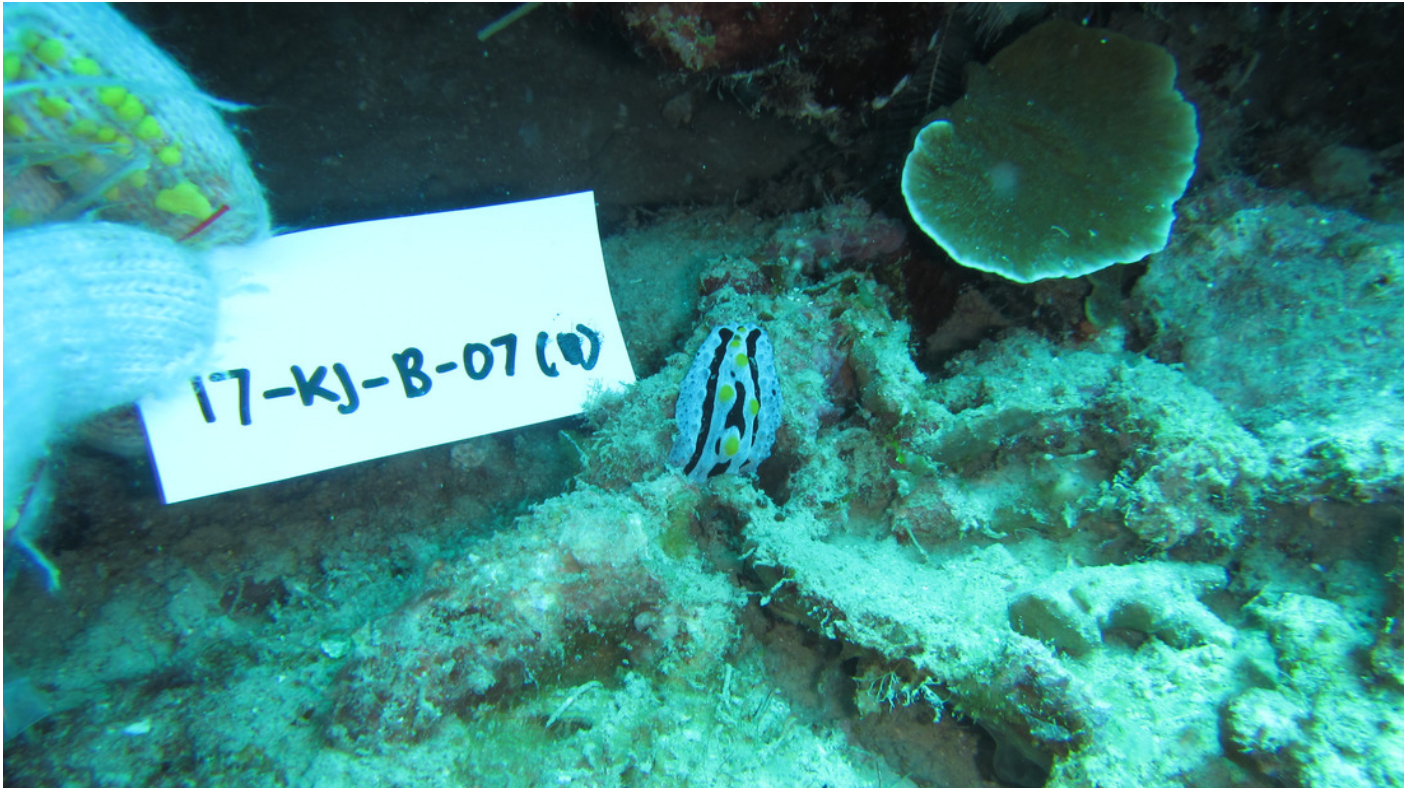


Figure 3 (on next page)

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

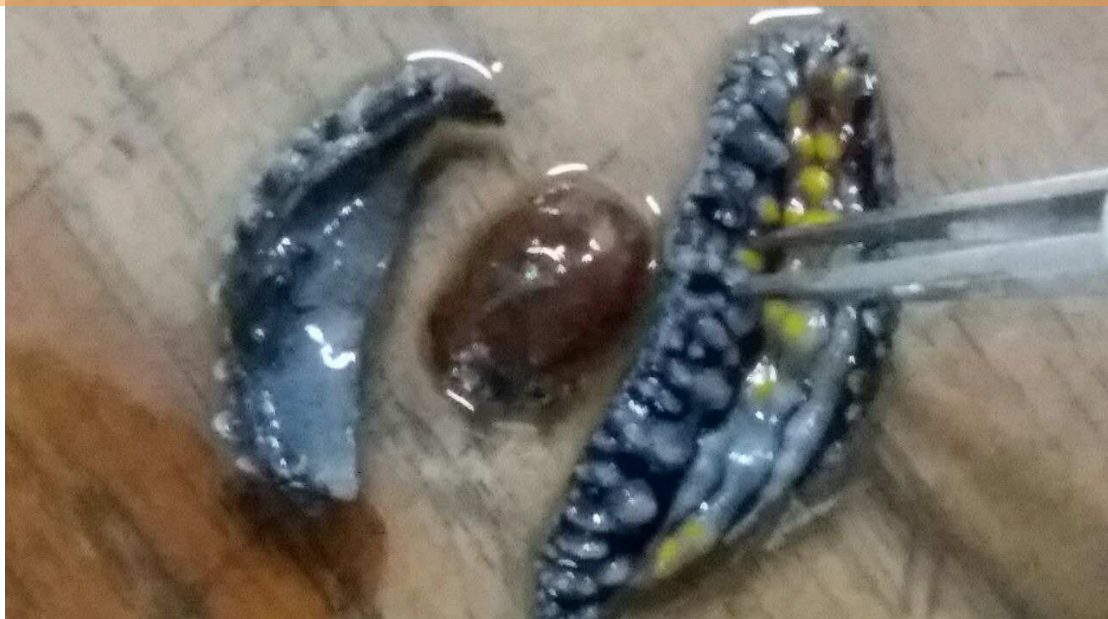


Figure 4 (on next page)

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

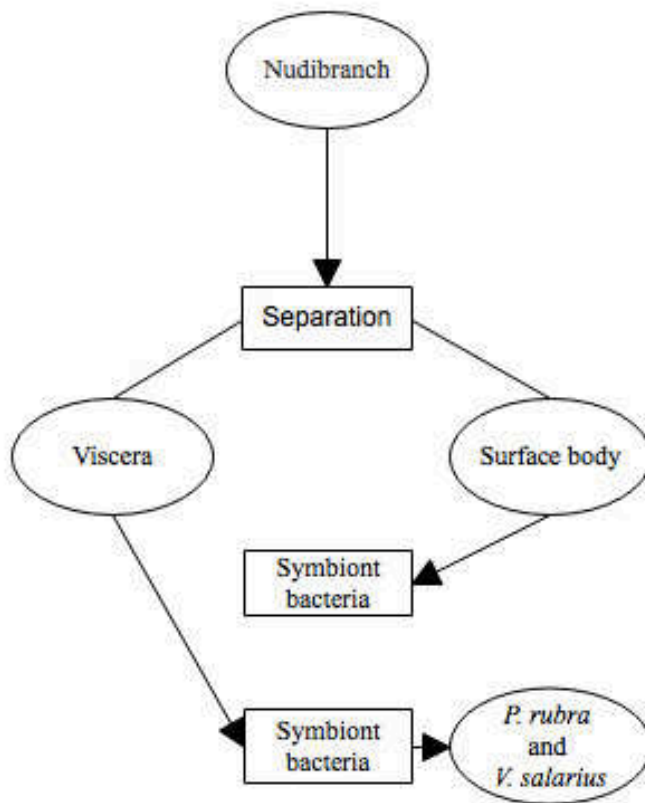


Figure 5

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

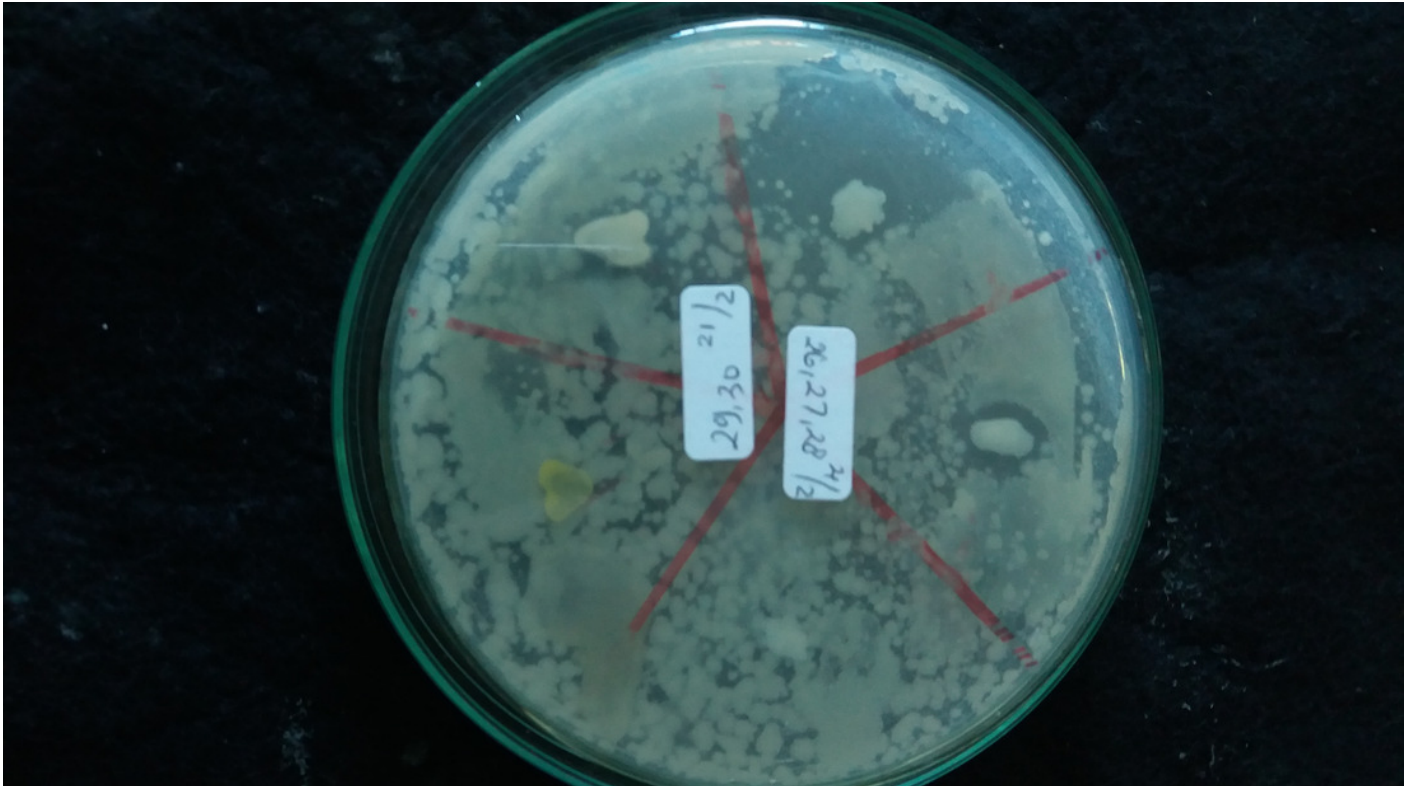


Figure 6

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

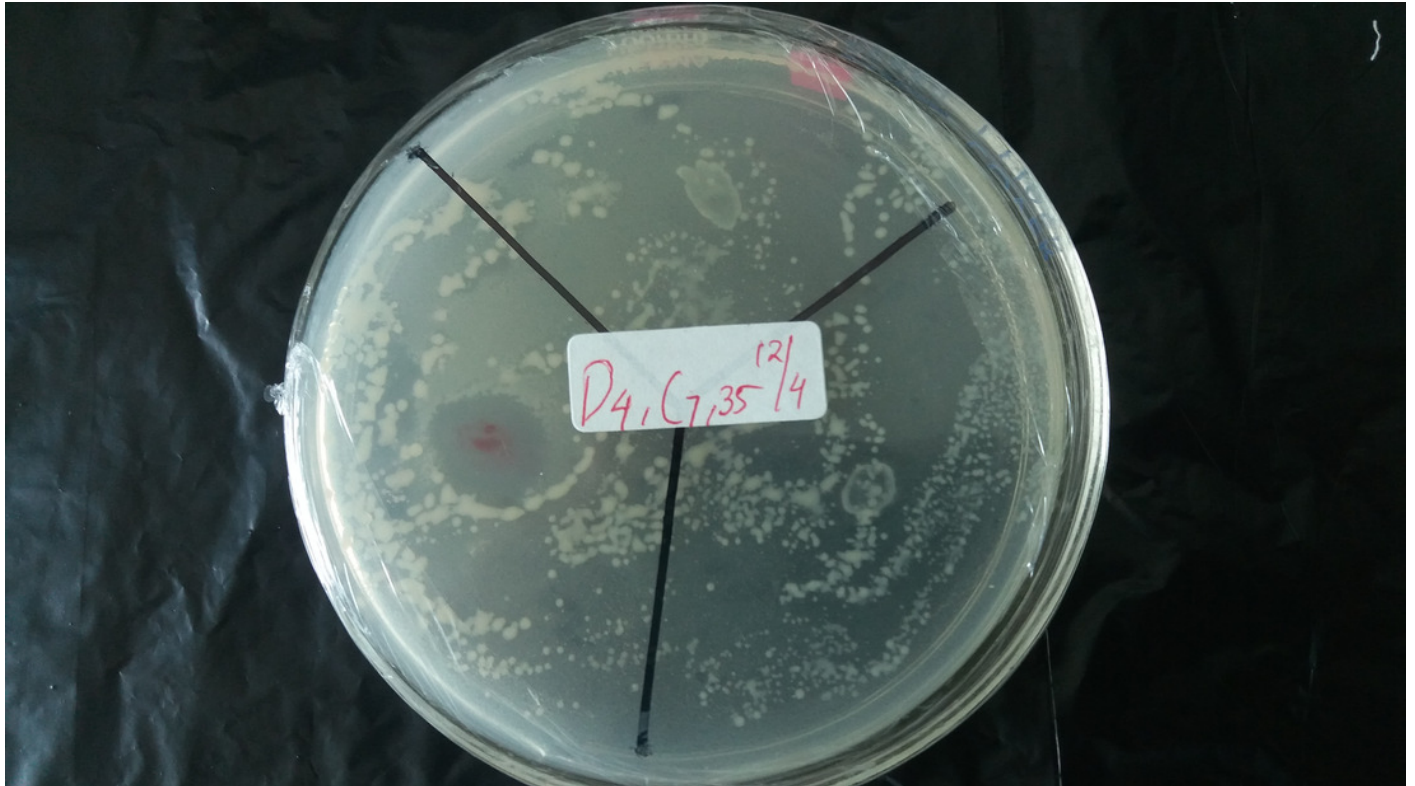


Figure 7 (on next page)

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

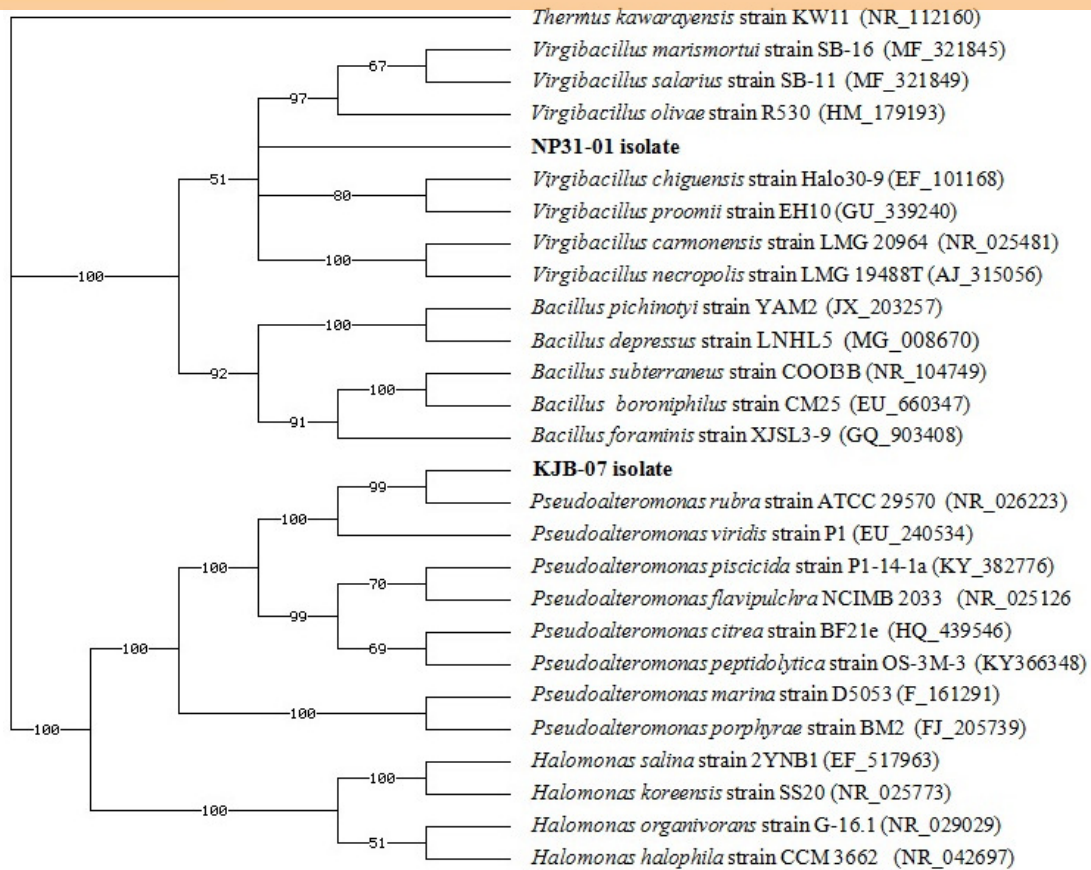


Figure 8

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.

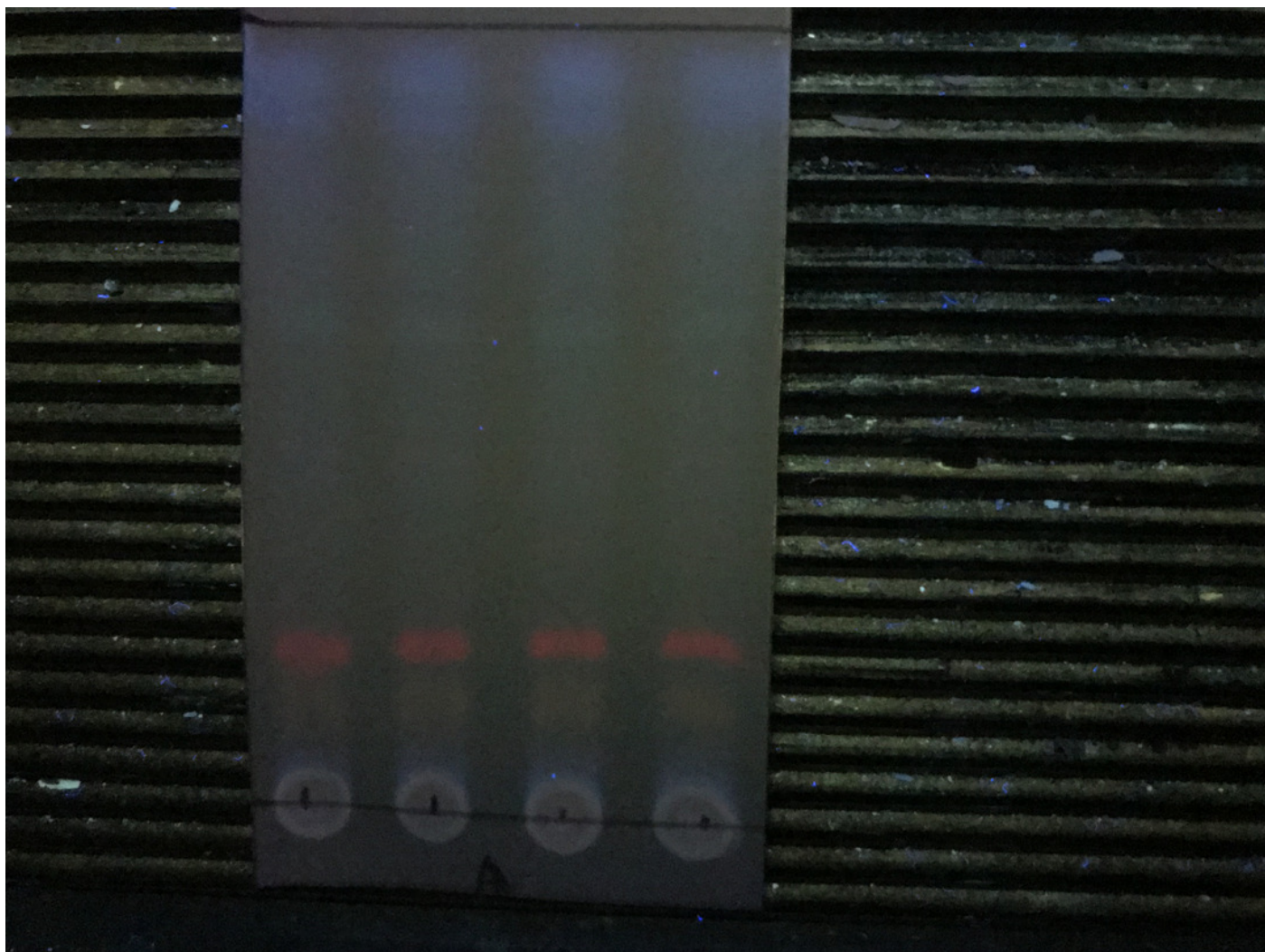
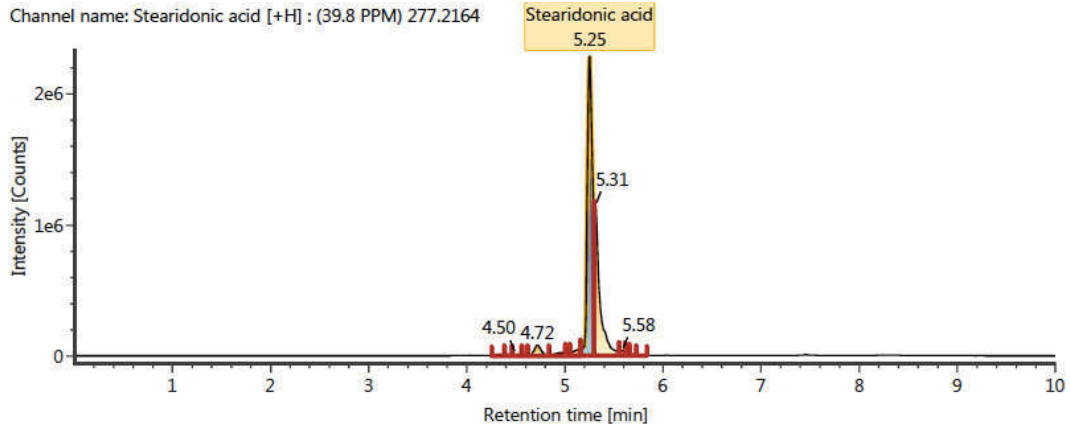


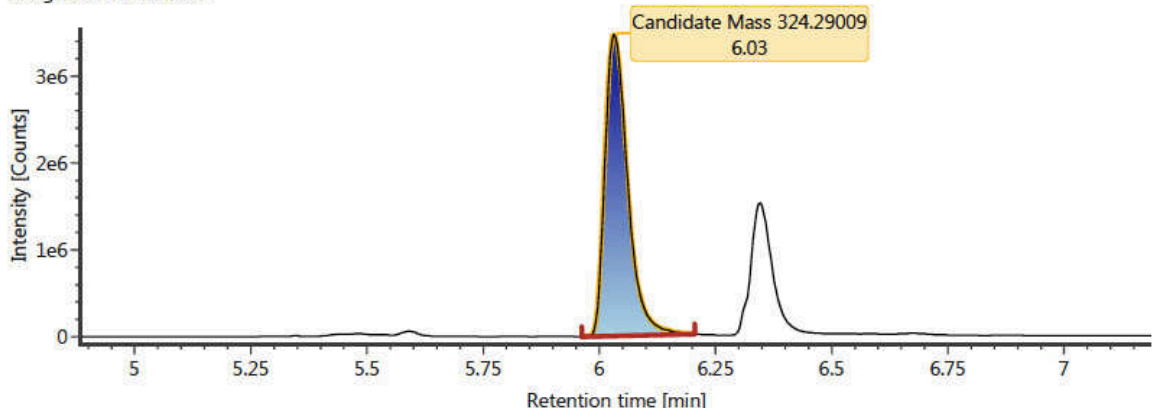
Figure 9 (on next page)

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.



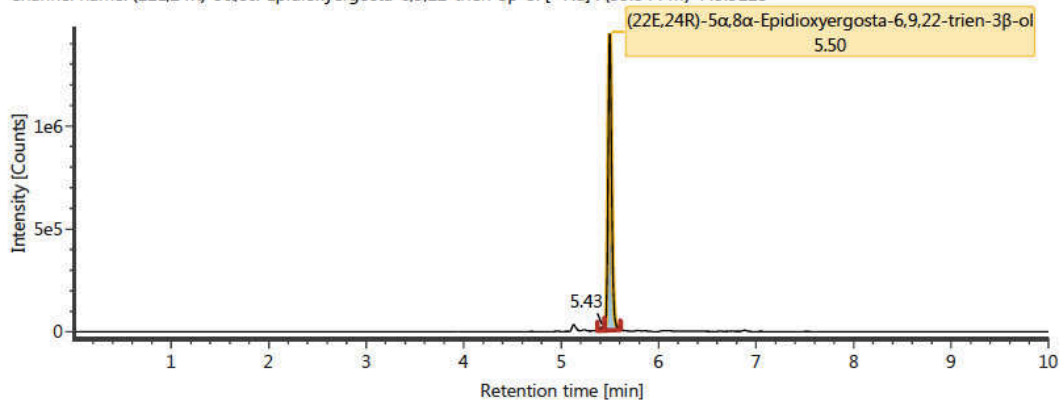
A

Channel name: 1: +324.2901 (39.8 PPM) +325.2933 (39.8 PPM) +326.2962 (39.8 PPM) : TOF MS^F (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