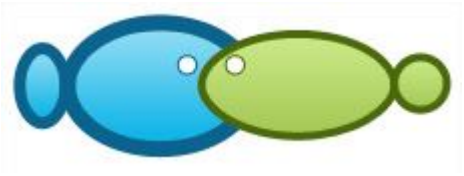

Antibacterial activity of EtOAc extract from marine-derived fungus *Aspergillus nomiae* A12-RF against clinical pathogen bacteria, *Staphylococcus aureus*

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Abstract. Sponge-derived fungi are a potential source for obtaining bioactive secondary metabolites. The aim of the study was to evaluate the *in vitro* antibacterial activity extract from sponge-derived fungi that could inhibit clinical pathogenic bacteria. In this study, nine isolated fungi were selected from deposite of Integrated Laboratory of Innovation and Technology Center, Lampung University. All isolates were maintained in malt extract media. Based on phylogenetic sequencing results, isolate 18A12RF was *Aspergillus nomius* (603 bp) using ITS1-5.8-ITS2. The isolate A12RF was cultivated and co-cultivated on rice solid media in 4 L Erlenmeyer flask to obtain 4.2 g of ethyl acetate extract (EtOAc). After that, the extract was subjected to several chromatographic steps based on bioassay-guided separation. The results of the fractionation of 2.4 g of EtOAc extract obtained 33.8 mg of the active fraction of A12RFBF3. The minimum inhibition concentration (MIC) test for the A12RFBF3 fraction showed inhibition of the growth of clinical bacteria *Staphylococcus aureus* at a concentration of 6.25 µg mL^{-1}. The findings of this study concluded that the crude extract prepared from the A12RF has antibacterial properties against clinical bacteria. This study is an important work as initial information for further studies in the search for new bioactive compounds.

Key Words: antibacterial agent, *Aspergillus nomiae*, bacterial pathogen, marine fungi.

Introduction. The decline in the quality of marine ecosystems due to human activities has serious ecological, social and economic implications. Over the past 4 decades, there has been a 58% decline in the global population of vertebrate species and 31% of marine fauna (WWF 2016). This decrease affects the quality of ecosystem diversity and endangers the sustainability of existing ecosystems. On the other hand, marine ecosystems are a potential source for substances with pharmacological properties (Rateb & Ebel 2011). However, the data that has been reported has not been comparable to the potential that should have been obtained based on the existing biological wealth. Therefore, it is necessary to make more intensive efforts to obtain information on bioactive compounds contained in marine organisms before damage occurs to marine ecosystems.

Based on the results of studies since 1985, more than 15,000 new secondary metabolites have been identified from marine organisms, many of which having interesting bioactivities (Blunt et al 2009; Al-Dhabi et al 2019). Sponges are the most studied animals among marine invertebrates as a source of diverse bioactive compounds (Varijakzhan et al 2021). However, problems arise in obtaining bioactive compounds in marine biota such as sponges, one of which is a difficult cultivation process. So that a search for bioactive compounds was carried out through the microorganisms found in the sponge. Sponges are famous for their filter feeder properties so there are various kinds

of microorganisms that are diverse and specific (Kiran et al 2018). Marine microorganisms have been reported to be able to produce new compounds and are easier to culture than marine macroorganisms (Blunt et al 2015). The utilization of marine microorganisms as a new source of bioactive compounds is able to avoid overexploitation of marine resources and the practice of over-taking marine biota (Romano et al 2017). Furthermore, the large amount of fungal biodiversity derived from sponges has not been fully investigated. This indicates that there are still many undiscovered metabolites.

Sponges are invertebrate animals that are filter feeders so they can contain various kinds of microorganisms. As much as 40% of the sponge's volume consists of microorganisms (Vacelet & Donadey 1977). The presence of microorganisms in the body of a sponge can be varied and specific. This is a consideration for the development of the search for bioactive compounds through marine microorganisms. As recently reported, epidithiodiketopiperazine DC1149B which has cytotoxic activity was obtained from the sponge-derived fungus *Trichoderma lixii* (Tang et al 2020). However, new problems arise in the fungi cultivation process to get bioactive compounds such as dereplication and loss of fungi ability to produce target compounds. To overcome this problem, it is necessary to carry out a fungal co-cultivation process with different microorganisms. Co-cultivation technique is a simple method that aims to increase the production of bioactive secondary metabolites. As has been successfully reported by Jomori et al (2020), the co-culture of *Aspergillus niger* originating from the sea with *Mycobacterium smegmatis* produced malforming C and TMC-256A1 which have cytotoxic properties.

In this study, we aimed to evaluate the *in vitro* antibacterial activity of EtOAc extract from sponge-derive fungi that could inhibit clinical pathogenic bacteria with resistance to several antibiotics. Moreover, we report a new method for the efficient production of antibacterial extracts from fungi cultured using solid fermentation technique using rice media. This initial information is very important for further development of the search for new antibiotic compounds that can inhibit the growth of resistant bacteria.

Material and Method

Isolation of fungi from marine sponges. Marine organisms, especially sponges, were selected for fungal isolation. Sponge samples were collected from Singaraja, Buleleng, Bali, Indonesia in August 2018. Samples were collected along the sea coast (8°07'20.9"S 114°34'03.8"E) at depths of 5-20 m by SCUBA diving. A small piece of sponge was rinsed and homogenized in sterile seawater. The homogenate was submitted to serial dilution and spread on plates of Malt Extract (ME) (105397, Merck kGAa Germany) and Trypthic Soy Broth (TSB) (105459, Merck kGAa Germany) agar prepared with 50% (v/v) seawater and cultured at 28^{o}C for 7 days. Fungi were isolated and purified on ME TSB agar medium prepared with 50% (v/v) seawater. The purified isolate was streaked on slants of ME TSB agar at 4^{o}C and in glycerol 20% (v/v) suspensions at -20^{o}C.

Cultivation and co-cultivation

Cultivation. Fungal isolates were grown on ME media and TSB (10 mL) and then cultivated for 2-4 days with shaking at 100 rpm. After the fungus grows, the fungi inoculum was poured into the rice medium consisting of 100 g of rice in 110 mL of sterile artificial seawater (ASW). After that, it was cultivated at 30^{o}C for 1 week under static conditions.

Co-cultivation. For co-cultivation, 10 mL inoculum bacterial broth (1.0 x 10^{8} CFU mL^{-1} Mc Farland) was prepared in TSB (Tryptic Soy Broth) (Merck, KGaA) medium and incubated overnight at 37^{o}C. Then, *S. aureus* inoculum was added to the fourth day of fungal cultivation. Co-cultivation was carried out at 30^{o}C for 14 days (Frank et al 2019; Jomori et al 2020). The cultivation and co-cultivation processes were repeated three times. The fungal biomass from cultivation and co-cultivation was then extracted with EtOAc. The extraction results were then concentrated under reduced pressure using a vacuum rotary evaporator (Büchii RA 210 with vacuum pump V-100, Switzerland) to obtain the crude EtOAc extract for further experiments.

Clinical pathogenic multidrug resistance (MDR) bacteria. The clinical pathogenic *S. aureus* and *Pseudomonas aeruginosa* which were used in this study were collected from General Hospital Abdoel Moeloek, Bandar Lampung. For inoculum preparation, *S. aureus* and *P. aeruginosa* were re-cultured on nutrient agar (105450 Merck KGaA, Germany) at 37^{o}C for 18 h, and then inoculated in TSB. Turbidity was adjusted to 0.5 standard McFarland (equal to 5 x 10^{8} CFU mL^{-1}). The disc diffusion method (Kwasny & Opperman 2010) was used to determine the sustainability of *S. aureus* and *P. aeruginosa* to several commercial antibiotics. According to the CLSI guidelines (2017), test results showed *S. aureus* was resistant to amoxicillin (25 µg), ciprofloxacin (5 µg), and clindamycin (2 µg), while *P. aeruginosa* was resistant to amoxicillin (25 µg), ciprofloxacin (5 µg), clindamycin (2 µg), and chloramphenicol (30 µg).

Screening antibacterial activity against clinical pathogen. The fungi extract was screened using a 96-well plate assay according to CLSI guidelines (CLSI 2017). Briefly, serial two-fold dilutions of the extract were prepared by dissolving 2 mg of fungi extract in 1 mL of 12.5% methanol (range, 500 to 3.9 µg mL^{-1}) and 50 µL of extract solution and 25 µL of bacterial suspension added to each well. The bacterial suspension was prepared from 12 h pure colonies of *S. aureus* and *P. aeruginosa*. Suspensions were adjusted to 0.5 McFarland standard turbidity (10^{6} CFU mL^{-1}) and subsequently incubated for 18-24 h at 37°C. Wells with 12.5% MeOH were used as solvent control and wells without bacteria were used as contamination control. An extract control was also included. The plates were prepared in triplicates, and placed in an incubator for 18 hours at 37^{o}C. After that, resazurin was added and incubated for 8 hours, and the absorbance (Abs) values were measured at 630 nm using Hospitex reader (Italy). Furthermore, the minimum inhibition concentration (MIC) test was carried out on only the active extract (Elsikh et al 2016).

Morphology of fungus A. nomiae. Fungi were spotted on slides and covered with coverslips and then were identified morphologically as fungi with light microscopy, using an Observer A1 Zeiss microscope. ME and TSB agar was poured on sterile slides and allowed to solidify. Then organisms were streaked on it and incubated at 37^{o}C for 2-3 days (Goodfellow et al 2012). Scanning Electron Microscopy (SEM) was performed to study the mycelial and spore arrangements of isolated fungi. Fungi were grown on standard media after 4 days, and a small part of the coverslip was cut off using a microtome SLEE Disposable Blades to obtain a piece of 0.5 cm x 0.5 cm with a thickness of 0.1 cm. Prepared samples were placed on stubs, which were fixed with carbon adhesive tabs. Prepared samples were placed on stubs, which fixed with carbon adhesive tabs. The upper surface of each stub was then coated, under vacuum, with a film of gold. The gold coating (Quorum Q150R ES, Germany) process was completed in ~ 20 min. The gold-coated metal stubs were viewed on the SEM (Zeiss EVO MA10, Germany) at an accelerating voltage of 20 kV.

DNA extraction and PCR amplification. Fungal genomic DNA was extracted as previously described by Landum et al (2016), in accordance to the manufacturer's instructions, using the QIAamp DNA Minikit (Qiagen, Germany). The nuclear ribosomal DNA internal transcribed spacer (ITS) of the fungal isolates was amplified using the forward primer, ITS1-F (5′-TCCGTAGGTGAACCTGCGG-3′) and the reverse primer, ITS4-R (5′-TCCTCCGCTTATTGATATGC-3′) (White et al 1990). The final reaction volume was 20.5 µL, containing 10 µL of PCR kit NEXpro TM (PCR Biosystems, UK), 0.25 µL of forward, 0.25 µL reverse primers, 5 µL ddH_2O, and 5 µL of genomic DNA template. For negative control, the DNA was replaced with distilled water to verify the absence of contamination. PCR was carried out using Sensoquest Sensodirect Gradient Termo block 96 (SensoQuest, Germany), programmed for 5 min at 94^{o}C; 35 cycles for 1 min at 94^{o}C, 1 min at 52^{o}C, and 1 min at 72^{o}C; and a final 5 min extension at 72^{o}C. The PCR products were separated using 2% agarose gel in 1X TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0), stained with ethidium bromide (0.5 µg mL^{-1}) and documented using Qiaxcell Advanced (Qiagen, Germany). PCR products were sent for direct bi-directionally sequencing using ABIPRISM3730 × 1 Genetic Analyzer (Applied Biosystems, USA) at the First BASE Laboratory Sdn. Bhd., Selangor, Malaysia.

Sequence and phylogenetic analysis. The resulting DNA sequences were aligned using MUSCLE software embedded in MEGA 7 (Kumar et al 2016), manually trimmed and edited to obtain complete sequences. Homology searches were carried out using the BLASTn program against the NCBI *GenBank* database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The appropriate DNA substitution models for the ITS gene was assessed using the "find best DNA/Protein Models" function embedded in the software MEGA 7. Futhermore the maximum likelihood (ML) statistical method was used to test the goodness of fit against various models of evolution. According to the estimated values of all parameters for each model, the model best fitting to the dataset from the ITS sequences was general time reversible (GTR) model and gamma distributed (+G) with invariant sites (+I) (=GTR+G+I) model. ML tree was constructed using MEGA 7 with all positions containing gaps and missing data were included for analysis. Clade supports were calculated based on 1,000 bootstrap resamplings.

Extraction, fractionation and MIC test. Fungi biomass was harvested using EtOAc extraction. Filtrate of the EtOAc extract was concentrated by evaporation under reduced pressure. The EtOAc extract was partitioned into a water–ButOH mixture (1:1). Fractionation was carried out on the basis of bioassay-guided separation. The MIC test was carried out on the active fraction obtained following the Elsikh et al (2016) method.

Results and Discussion

Sample collection and isolation of fungus. Marine organisms were collected at Singaraja, Buleleng, Bali, Indonesia. During a one-week collection trip, nine sponges were collected along the coast (8°07'20.9"S 114°34'03.8" E) at a depth of 5-25 meters. Identification of marine organisms based on their physical form and the presence of spicules showed that all specimens were sponges belonging to the genus *Desmospongiae*. After going through the process of enrichment and purification on agar media, nine fungi were isolated from the sample of sponges as shown in Table 1.

Table 1

Isolate fungi from marine organisms

No.	*Sponge code*	*Species*	*Isolate fungus*	*Color*
1	18A02	*Callyspongia armigera*	18A02RF	White
2	18A12	Unidentified	18A12RF	White-orange
3	18B15	*Plakina jamaicensis*	18B15RF	White
4	18B20	*Cinachyrella kuekenthali*	18B20RF	White
5	18B21	*Niphates alba*	18B21RF	White
6	18B23	*Diplastrella* sp.	18B23RF	White
7	18C24	*Batzella* sp.	18B24RF	White
8	18E41	*Niphates erecta*	18E41RF	White
9	18E42	*Aplysina cauliformis*	18E42RF	White

The presence of microorganisms in the body of the sponge is certainly related to its filter feeder nature (Müller 2003). Furthermore, the presence of fungi in marine organisms such as sea fans, corals, and macroalgae (Loque et al 2009; Amend et al 2012) has also been reported. However, studies show that the fungi originating from the sea have a relatively small number, which is about 0.6% of the total number of fungi globally. It is estimated that there are still many fungi that have not been isolated (Burgaud et al 2009). In addition, fungi have a role in marine ecosystems as saprophytes in dead organisms, and symbionts (parasites, commensals, and mutualists) in living marine organisms (Raghukumar 2017).

Screening antibacterial activities. Antibacterial screening tests have been carried out on nine extracts of fungal isolates prepared from cultivation and co-cultivation on rice media. The test results on clinical pathogenic bacteria, *S. aureus*, and *P. aeruginosa* are

shown in Figures 1 and 2. Crude extracts from cultivation and co-cultivation results show differences in antibacterial activity. The test results of fungal extracts prepared from co-cultivation against *S. aureus* (Figure 1) showed a significant increase in the inhibitory activity, especially in the 18A12RF extract. Meanwhile, the co-cultivation process had no significant effect on the other isolates.

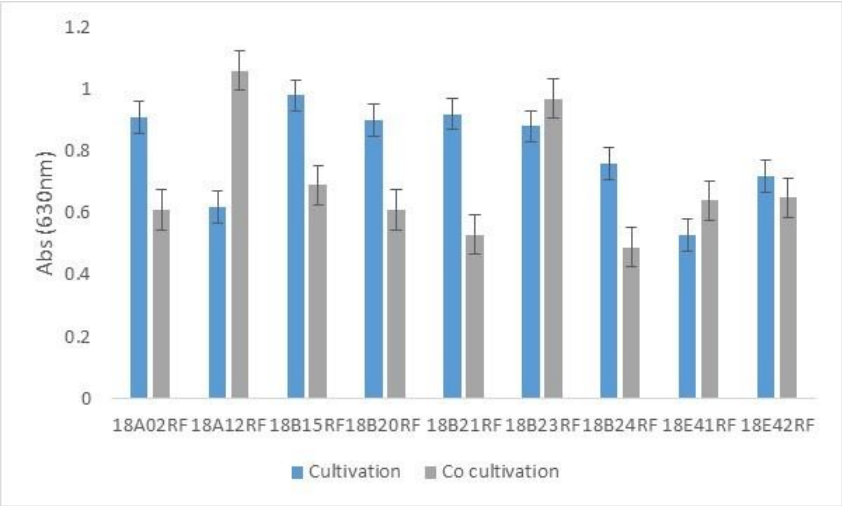

Figure 1. Screening extract EtOAc (cultivation and co cultivation) of isolate fungus against *S. aureus.*

Different results also occurred in the screening tests for *P. aeruginosa*. Nine extracts of co-cultivated fungal isolates that were tested showed an increase in the ability to inhibit the growth of *P. aeruginosa* (Figure 2). The increase in antibacterial activity in the co-cultivation process could be due to competition between different microorganisms for self-defence, thereby triggering the formation of secondary metabolites through activation and transcription under stress conditions. The competition or antagonism experienced during co-cultivation was shown to lead to significantly enhanced compound production and the accumulation of undetectable cryptic compounds in axenic cultures of the producing strains. Co-cultivation proves that this experimental approach increases chemical productivity (Li et al 2011).

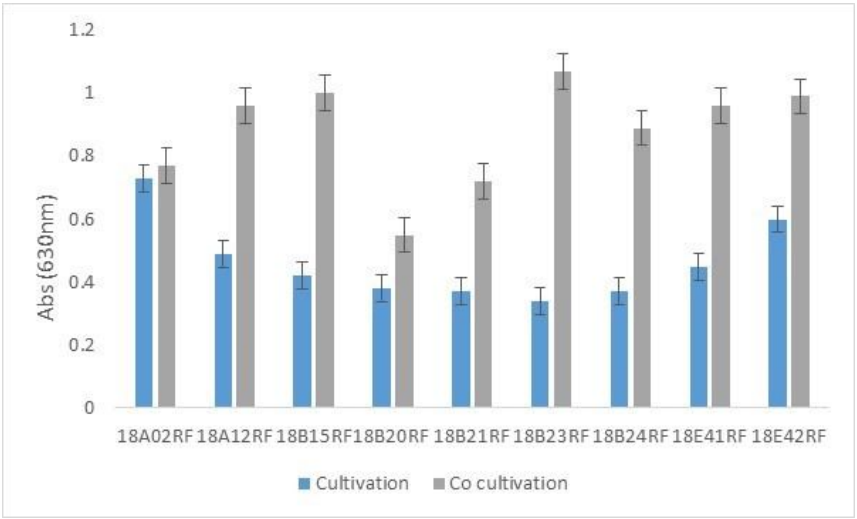

Figure 2. Screening extract EtOAc (cultivation and co cultivation) of isolate fungus against *P. aeruginosa*.

Morphology of fungus 18A12RF. Visual observation of fungi cultivated in media showed mycelia penetrating the substrate, while mycelia grew vertically at the media-air interface. Under microscopic observation, all isolated fungi had an average mycelium

diameter of ~5 microns. This is larger than many fungi which have mycelia of 1-2 microns (Toledo-Hernandez et al 2008).

The microscopic characteristics of 18A12RF are shown in Figure 3. Under the microscope, the conidiophores of 18A12RF were colourless, thick-walled, roughed, and bearing vesicles. The diameter of the conidiophores ranged from 2.5 to 3 µm. The vesicle shape of 18A12RF was globose to sub-globose. The diameter of the vesicles ranged from 20 to 30 µm. The cells were uniseriate or biseriate. In the biseriate cells, the phialides grew on the metulae whereas, in uniseriate cells, they grew on the vesicles. The metulae enclosed the vesicles surface and emitted in all directions. The conidia were globose, thin walled, slightly roughed, and oblong ranged from 2.5 to 3 µm in diameter.

(a) (b) (c)

Figure 3. (a). Isolate 18A12RF in standard media; (b) Visualization of 18A12RF, scale 400x; (c) SEM (Scanning Electron Microscope) image.

Scanning electron microscopy (SEM) revealed that the mycelia were composed of branched, septate, smooth-walled hyphae 1.3 to 2.7 µm wide (mean = 2.1 µm). Conidial heads radiated, normally splitting into two to four dense columns (up to 500 µm long) with age. Stipes were 69 to 242 µm long (mean = 181 µm), were 3.7 to 6.0 µm wide (mean = 5.3 µm), had rough walls, and were light orange pigmented. Vesicles were globose, 6.8 to 10.9 µm in diameter (mean = 8.9 µm), and biseriate. Metulae were smooth walled and cylindrical, measuring 1.7 to 3.1 (mean = 2.6) × 1.7 to 2.1 (mean = 1.9) µm. Phialides were smooth walled and flask shaped, measuring 4.3 to 5.7 (mean = 4.8) × 1.2 to 1.3 (mean = 1.2) µm, with 0.6 to 1.4 µm-long collula. Conidia were middle orange-yellow and globose to subglobose when mature, measuring 1.4 to 1.7 (mean = 1.5) µm (Figures 3a to 3c), and roughened. A teleomorphic state was not observed. Based on the observations and descriptions above, isolate 18A12RF is a fungus that belongs to the *Aspergillus* group.

Phylogenetic analysis. ITS1-5.8S-ITS4 sequence region (603 base pairs (bp)), deposited in GenBank with accession number LC638671) of strain 18A12RF was amplified by PCR and sequenced. A phylogenetic tree was constructed, using the maximum-likelihood method and Tamura 3-parameter model based on the similarity of a 603-bp consensus length of the ITS1-5.8S-ITS4 sequence. Thirty *Aspergillus* representatives along with *Emericella nidulans* (Acc. No. HQ026740) as an outgroup were used (Figure 4). Strain 18A12RF was found to belong to a clade related to *A. nomiae* in the tree, with sequence identities of 99% respectively. The properties of culture and morphology of strain 18A12RF were consistent with those of *A. nomiae* as previously. The ITS phylogenetic analyses confirmed that the fungus strain A12-RF belonged to *A. nomiae*, and was designated as *A. nomiae* A12-RF.

Fractionation and MIC test. An EtOAc extract 18A12RF (2.4 g) that exhibited antibacterial activity against *S. aureus* and *P. aeruginosa* at 250 µg mL^{-1} was partitioned into a ButOH-water mixture to obtain a ButOH soluble portion A12RFB (0.8 g). The ButOH soluble portion showed growth inhibition at 62.5 µg mL^{-1} against *S. aureus*. This fraction A12RFB was subjected to SiO_2 gel open column chromatography eluted with n-hexane and isopropanol, affording four fractions: 252.2 mg A12RFBF1 (eluted with n-

hexane: isopropanol 5:1), 35.4 mg A12RFBF2 (eluted with n-hexane: isopropanol 5:1), 33.8 mg A12RFBF3, and 107.9 mg A12RFBF4 (eluted with n-hexane: isopropanol 7:3). Each fraction was subjected to a MIC assay. The fractions A12RFBF3 and A12RFBF2 inhibited growth of *S. aureus* and *P. aeruginosa* most effectively at a concentration of 6.25 and 125 µg mL^{-1} respectively (Figure 5).

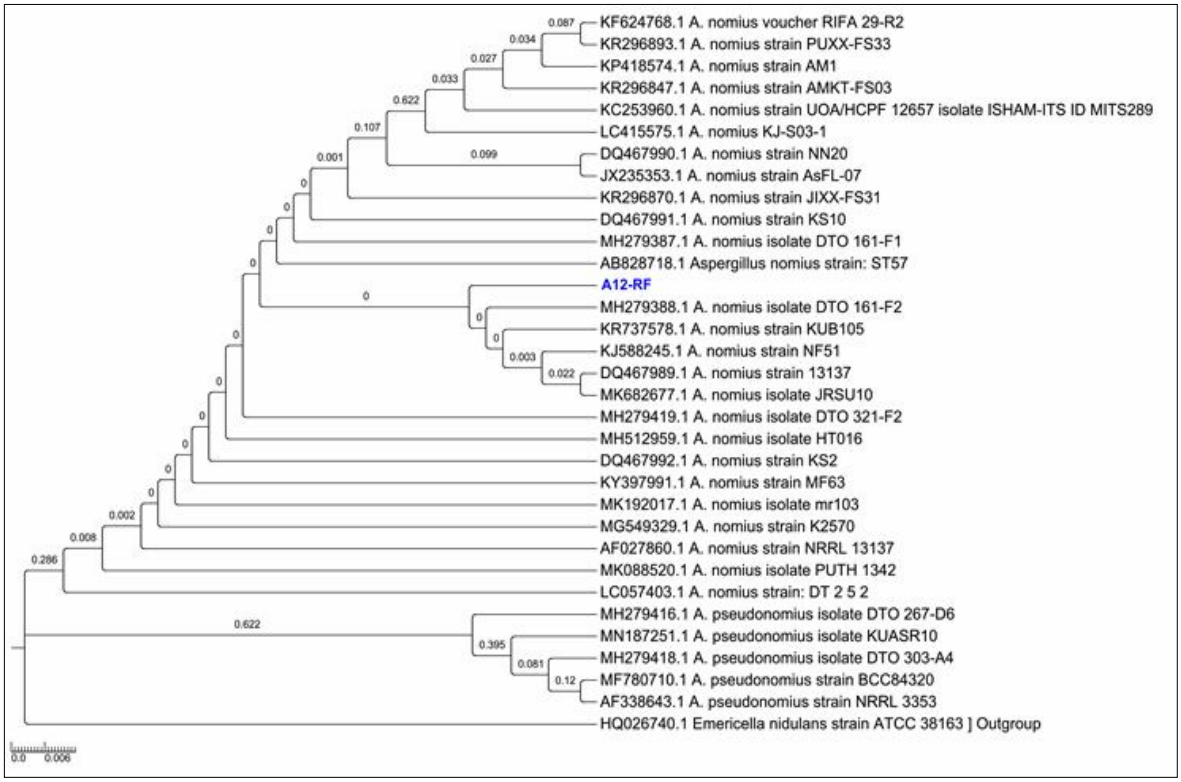

Figure 4. Phylogenetic tree strain A12-RF.

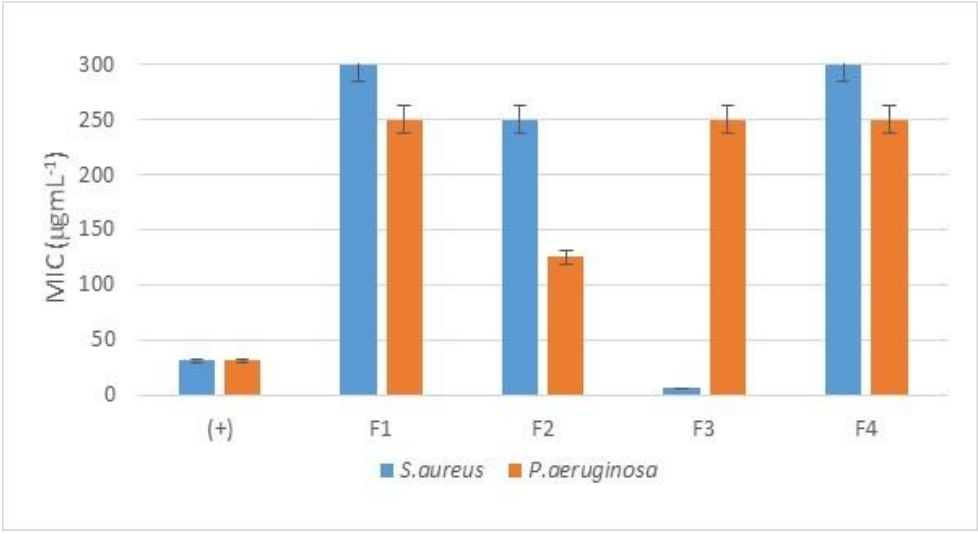

Figure 5. Minimum inhibitory concentration of fraction A12RFB (F1-F4) control (+) = chloramphenicol).

Conclusions. The finding of the present study concluded that the extract prepared from *Aspergillus nomiae* A12-RF had bioactive metabolites with antibacterial properties. The extract fraction A12-RFBF3 inhibited the growth of clinical bacteria *S. aureus* (6.25 µg mL^{-1}) and the fraction A12-RFBF2 inhibited the growth of clinical bacteria *P. aeruginosa* (125 µg mL^{-1}), both of which were resistant to several antibiotics. This study builds on the work for further studies on the development of antibacterial agents.

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Conflict of interest. The authors declare that there is no conflict of interest.

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