Antibacterial Compound from Aspergillus elegans SweF9 an Endophytic Fungus from Macroalgae Euchema sp.

Hani Mulyani1,2, Rizna Triana Dewi2*, Chaidir1

1. Pharmacy Faculty, Pancasila University, Jl. Raya Lenteng Agung Timur No.56-80, Serang, Serang, Banten 15314.
2. Research Center for Chemistry-Indonesian Institute of Sciences, Kawasan PUSPITEK, Serpong, Tangerang Selatan, Banten 15314.

Submitted: 25-02-2019 Revised: 11-06-2019 Accepted: 19-09-2019

*Corresponding author
Rizna Triana Dewi
Email: riznati@gmail.com

ABSTRACT

The antibiotic resistance of pathogenic bacteria has become a serious health concern and encouragement to search for novel and efficient antimicrobial metabolites. On the other hand, endophytic fungi have great potential as a natural source for antimicrobial agents. The objective of this study was to isolate antibacterial compound from endophytic fungi A. elegans SweF9. The fungus was stationarily cultured at 30°C for 12 days in potato malt peptone (PMP) medium, then the filtrate was extracted with ethyl acetate. The antibacterial activities of the extract were evaluated by agar diffusion method against Gram-positive (Bacillus subtilis and Staphylococcus aureus) and Gram-negative (Escherichia coli) bacterial strains. The broth extract at a concentration of 1% was able to inhibit the growth of E. coli, S. aureus and B. subtilis with an index of antibacterial activity 84.6%, 91.6%, and 90% compared to streptomycin sulfate at the same concentration. The active compound (1) was purified to yield amorphous white and identified using FTIR, NMR, and EI-MS analyses, revealed identified as (+) - epi-Epoformin. The compound showed an antibacterial activity index of against E. coli, S. aureus and B. subtilis bacterial were 48%, 45%, and 47%, respectively, at concentration 1%.

Keywords: Aspergillus elegans SweF9, antibacteria, (+)-epi- Epoformin.

INTRODUCTION

Resistance to antimicrobial (AMR) agents is a relevant problem faced by health services and has become a serious concern around the world (Elisha et al., 2017; Santos et al., 2015). Natural products such as fungi from terrestrial, marine, and endophytic are considered an essential source for novel antibacterial compounds because of their abundant species diversity, rich in secondary metabolites and the improvements in their genetic breeding and is it relevant with natural products as an essential source for novel antibacterial compounds (Calvo et al., 2002). Endophytic fungi have proved to be a rich source of bioactive secondary metabolites, and recently several novel bioactive substances have been isolated from these microorganisms such as Epipolysulfanyl-dioxopiperazines (derived of leptosins), were extracted from the endophytic fungus Leptosphaeria sp. isolated from the brown alga Sargassum tortile showed the cytotoxicity against P-388 leukemia, and A. wentii pt-1 of marine red alga Gymnogongrus flabelliformis gifted another set of new xanthone derivatives, yicatin A C, exhibiting inhibitory activity against E. coli, S. aureus, and C. lagenarium (Sarasan et al., 2017).

The antimicrobial activities of an increasing number of fungi living in unique environments have been investigated for the discovery of new antibacterial compounds, such as endophytic fungi from wild plants and marine fungi. In the last decade, many novel bioactive natural products from marine fungi have been discovering that possess cytotoxic, anticancer, antiviral, antibacterial or antifungal activities (Thomas et al., 2010; Rateb et al., 2011). Although the active constituents may occur in lower concentrations, endophytic fungi may be the better sources of new and active antibacterial compounds than synthetic drugs to combat infectious diseases (Kalyanasundaram et al., 2015). It is due to their characteristic properties regarding temperature,
nutrients, competition, and salinity, they have developed specific or unique secondary metabolites which have different chemical structure compared with terrestrial fungi and synthetic compounds (Hasan et al., 2015).

The resource of marine fungal species is abundant, and the antibacterial compounds from marine fungi have quickly increased since 2010, for example, the marine fungus *F. nypae* had isolated 2,2,7-trimethyl-2H-chromen-5-ol an antimicrobial activity for *E. coli*, *B. subtilis*, *S. aureus*, *C. albicans*, and *S. pombe* (Zainuddin et al., 2010). Endophytic from macroalgae offer a wide range of potential therapeutic compounds as antibacterial, antifungal, antiviral, antioxidant, antitoxic, and anti-inflammatory (Fuji et al., 2011). Endophytic *A. wentii* pt-1 of marine red alga *Gymnogongrus flabelliformis* gifted another set of new xanthone derivatives, yicathin A-C, exhibiting inhibitory activity against *E. coli*, *S. aureus*, and *C. lagernarium* (San et al., 2013). Felicio et al. 2015, has isolated forty-five endophytic microorganism strains were isolated from Brazilian marine seaweed *B. tenella* (J.V.Lamouroux) JAgardh and resulted active compound cytochalasin D a known antitumor and antibiotic compound from Xylaria sp. strain. Chemical investigation of various algal endophytes yielded a handful of metabolites inhibiting *S. aureus* as well as *E. coli*; namely, phenalenone derivatives and scleroderolide isolated from marine-derived fungus Coniothyrium cereal (Elsebai et al. 2011); dicerandrol C from the endophytic fungus *Phomopsis longicolla* isolated from the tropical red seaweed *Bostrychia dicans* (Erbert et al. 2012); brevianamide M, 6,8-di-O-methylaververufin, and 6-O-methylaververufin secondary metabolites from an algalicous *A. versicolor* strain (Miao, 2012).

Although the association of macroalgae and fungi has been described regarding pharmacological issue, there is a lack of studies about Indonesian marine seaweed endophytic fungi. Sriandace and Andayani (2015), have reported six endophytic fungi from marine Euchema sp, namely SF1 (coral reef), SF3 (sponge), SwF5 (sea worm), SweF9 (seaweed), SweF10 (seaweed), SweF11 showed antibacterial activity. Furthermore, extract of SweF9 (*A. elegans*) showed potential antibacterial activity with inhibition zones of 20-25mm at concentrations of 0.313mg/mL and 2.50mg/mL, compared to other ethyl acetate extract of isolated endophytic fungi. Therefore, the objective of this research was to isolate the antibacterial compound from endophytic fungi *A. elegans* SweF9. Bioautography-TLC and agar diffusion method were conduct to run antibacterial activity against gram-negative and gram-positive pathogen bacteria.

**MATERIALS AND METHODS**

**Materials**

The fungal *A. elegans* SweF9 culture collection of the Research Center for chemistry – LIPI and test microorganisms used were *S. aureus* (InaCC-B4), *B. subtilis* (InaCC-B334), and *E. coli* (InaCC-B5) bacteria collected of Indonesian Culture Collection of the Research enter for Biology – LIPI. was obtained from the Indonesian. Culture media PD, PDB, malt extract, peptone, Czapek-Dox broth were purchased from Difco. Streptomycin sulphate from Sigma. Silica gel 70-230 mesh and TLC plate were obtained from eMerck. All solvents used were analytical grade and distilled before use.

UV Spectra were recorded in MeOH on an Agilent Techn. Carry 60 spectrophotometer. The 1H NMR spectra were recorded in DCl on Agilent 500MHz with DD2 console system, while 13C NMR at 125MHz. The chemical shift values (δ) are given in parts per million (ppm) and coupling constant (+) (J) in Hz. ESI-MS analysis was conducted by LCMS/MS Xevo G2-XS QTOF (Waters). Column chromatography was performed on silica gel (E. Merck, 70–230 mesh). TLC separation were performed on precoated plates (Silica gel 60, PF254, 0.2mm, E. Merck) and spots were detected under UV light.

**Optimization of Culture Medium**

The strains were prepared on PDA plates and incubated at 30°C for seven days in the dark. *A. elegans* SweF9 was cultivation in three different media: potato dextrose broth (PDB), potato malt peptone (PMP) and Czapek-Dox (CDB) incubated for ten days in room temperature at static condition. The culture media and mycelium were separated by filtration. The mycelium were filtrate, and filtrate was extracted with EtOAc. Each of organic extract was dried over anhydrous Na2SO4, evaporated under reduced pressure to afford a brown oily gummy residue. All extracts were tested for their antibacterial activity.

**Determination of the incubation period**

The strain *A. elegans* SweF9 was inoculated into PMP medium, incubated up to 20 days in stationary condition at room temperature. Their growth and secondary metabolite production were determined every two days by comparing mycelial weight and antibacterial activity.
Extraction and Isolation

Secondary metabolites extraction was carried out by culturing A. elegans SweF9 in PMP (10 L) incubated for 12 days at room temperature at static condition. The culture were filtered to separate (filtrate) and mycelia (residual cake). The filtrate (F) was extracted with EtOAc (4x5L) to give a brown gum (6.35g). The F extract (4.5g) was subjected to column chromatography (CC) on silica gel (70-230 mesh) using a stepwise gradient n-hexane: EtOAc, to EtOAc: MeOH to obtain twelve fractions (F1-F12) based on TLC profile analysis. The fraction F2 showed highest inhibition zone on bacterial tests and Furthermore, TLC analysis of F2, showed major spot with 2 minor other spots. For isolation of antibacterial compounds, semi preparative TLC was used. To obtain bigger amounts of fractions, 150µL of F2 in a 15cm band was applied on TLC plate and developed to an 8cm distance with phase mobil of n-hexane: EtOAc (2:1). Separated fractions was scraped off together with silica gel, then was extracted with MeOH and concentrated. Recrystallization of the extract from methanol and n-hexane to yield F2.1 (20mg) as an active compound (1).

Antimicrobial activities assay

Agar disk-diffusion method

Extracts, fractions and isolated compound were tested by disc diffusion method (Kalyanasundaram et al., 2015). The sterile nutrient agar (NA) media plates were prepared and inoculated with the test organism. Ten microliters of samples (1%) was added on to a sterile 6 mm paper disc using a micropipette and allowed to dry. Discs containing compounds were placed on the surface of the medium. The experiment was carried out in triplicates. Streptomycin sulphate (1%) was used as positive control and 10µL of DMSO was used as negative controls. The plates were incubated at ±35 for 12-24h. The diameter of the inhibition zone (mm) around the disc was measured by using the scale. The value of activity was adjusted to Activity Index= (Zone of inhibition of extract/Zone of inhibition of antibiotic) x 100%.

Thin layer chromatography (TLC)-bioautography

The procedure autobiographic method is similar with agar diffusion method. TLC plate (Merck Silica Gel 60 F254) was loaded with 10µL of fraction F2. The solvent system used was n-hexane: EtOAc (2:1). The chromatogram was kept for evaporation of the solvent then placed on the bacterial inoculated agar surface for a few minutes to allow diffusion. Next, the plate is removed and the agar plate was incubated. The zone of inhibition growth appears in the places, where the antimicrobial compounds were in contact with the agar layer (Choma et al., 2015). The antimicrobial activity was observed by inhibition zone and the Rf value was defined.

RESULTS AND DISCUSSION

Optimization of culture condition

To examine the effect of culture medium on antibacterial activity of secondary metabolite, the strain A. elegans SweF9 was cultured in three different mediums, PDB, PMP, and CzB under static condition for ten days at room temperature. all extracts obtained were tested for antibacterial activity (Table I). Based on the result, the antibacterial activity of extracts of PDB and PMP does not differ significantly. However, extract (F) PMP shows stronger antibacterial activity, where the inhibition zone at the lowest concentration (1%) was wider than inhibition zones of the other extracts.
Antibacterial Compound from Aspergillus elegans SweF9

The result showed that antibacterial compounds were efficiently produced when the fungus *A. elegans* SweF9 was fermented in PMP medium. The activity index of PMP filtrate extract (1%) on *E. coli*, *S. aureus*, and *B. subtilis* to streptomycin sulfate as standard were 74, 80, and 78%, respectively. Therefore, the PMP culture media was chosen for scale up of antibacterial compound from *A. elegans* SweF9. Further studies to determinant the optimum incubation period of *A. elegan* SweF9, revealed that incubation period for 12 days was observed to be optimum for maximum inhibition against *E. coli*, *S. aureus*, and *B. subtilis* with inhibition zone 22, 22, and 2mm, respectively. The activity index for this extract was 84.6%, 91.6% and 90% (Figure 1). Based on that result, *A. elegan* SweF9 could produced antibacterial agent after the fungus reached its stationary phase and decreased up to 12 days of incubation and remaining almost constant from 14 to 20 days’ incubation. Based on these results, for scale up production to isolation antibacterial agent, *A. elegan* SweF9 was cultured in PMP medium (10L) at the stationary condition for 12 days.

**Isolation and characterization of antibacterial compound**

Approximately ~ 6.5g filtrate EtOAc extract (F) was obtained from the culture broth (10L). The extract (F) was examined against pathogen bacterial by TLC-bioautographic test. The bioautographic revealed clear zones of bacterial growth inhibition for *E. coli*, *S. aureus*, and *B. subtilis* were 30, 27, 29mm (Figure 2). Bioautographic belongs to microbiological screening methods commonly used for the detection of antimicrobial activity from several organic extracts, mainly plant extract (Jesionek *et al.*, 2015).
In this study, we used agar diffusion-TLC bioautographic also known as an agar contact method. It involved the transfer of the antimicrobial agent by diffusion from TLC to an agar plate previously inoculated with microorganism test (Choma, et al., 2015). It is one of the simplest and cheapest methods for detecting antimicrobial compounds in extracts or fraction because the method is easy to run, reproducible, highly sensitive and requires less equipment (Jesionek et al., 2015).

Separation of the extract F (±5.5g) was conducted by vacuum column chromatography with gradual polarity increase to obtain 12 fractions (F1-F12) based on TLC profile. Each fraction obtained was tested for its antibacterial activity. Fraction F-2, F-3, and F-4 have wider inhibition zona than other fractions against three bacteria tests (Table II). Based on that result, F-2 and F-3 fraction were further purified, whereas F-4 was not purified due to the limited of sample (<100mg). The F3 fraction was purified by chromatography column, but no pure active compound was isolated. Preparative-TLC and recrystallization of F2 with hexane and methanol to yield powder white (20mg) referred to as compound 1. In agar diffusion assays (Table III), compound 1 was active against E. coli, S. aureus, and B. subtilis with an activity index of 48, 45 and 47%, respectively.

Spectroscopic analysis was conducted to elucidate the structure of the active compound. Compound 1 was obtained as powder white and showed FTIR absorption bands at 3336 and 1678 cm\(^{-1}\) which indicated the presence of hydroxyl (-OH) and carbonyl (C=O) groups, respectively. (Cala et al., 2018). The molecular formula was deduced to be C\(_7\)H\(_8\)O\(_3\) with two unsaturation’s from EI-MS (m/z 141).

In order to elucidate the compound’s structure, NMR measurements were carried out in CDCl\(_3\). Interpretation of \(^1\)H- and \(^13\)C-NMR spectra confirmed the presence of 6 carbon atoms and 6 proton signals, including methyl and hydroxyl groups. \(^1\)H chemical shift at 6H 6.45 (brs, 1H, H-3) shows the proton shift bound to double bonded carbon (HC=\(\equiv\)C), 6H 4.65 (brs, 1H, H-4), 3.78 (m, 1H, H-5), and 3.50 (m, 1H, H-6),

| Sample          | Inhibition Zone(mm) | Activity index (%) |
|-----------------|---------------------|--------------------|
|                 | E. coli  | S. aureus | B. subtilis | E. coli  | S. aureus | B. subtilis |
| Compound 1      | 10       | 8         | 8          | 48       | 45        | 47         |
| Streptomycin sulfate | 21       | 18        | 17         |          |           |            |

*Sample concentration: 1%
indicating the presence of 3 protons metin-ox (CH-O), and δH 1.84 (s, 3H, H-7) is a typical chemical shift for methyl (-CH3). The presence of a hydroxyl (-OH) proton was inferred from the signals at δH 2, 29.

The number of carbon atom was a measurement with 13C-NMR, spectrum revealed seven peaks in the range δC 19-194ppm. The shift at δC 194.4 ppm is the typical shift for ketone carbonyl (C=O), shift at δC 134.7ppm, and 138.9ppm is the shift of carbon double bond (C=C), 63.5ppm, 57.8 ppm, 53.5ppm is metin C oxy which CH binds to O and 16.1 ppm is C methyl (-CH3). The complete structural elucidation of 1 as well as the unambiguous assignment of all 1H- and 13C-NMR signals was based on 2D NMR experiments (HMQC and HMBC) (Figure 3). An experiment with the HMQC spectrum shows protons at δH 6.45 (H-3) correlate with carbon at C 138.9. The proton at 4.65 (H-4) correlates with carbon at δC 63.5. Proton δH 3.78(H-5) correlates with carbon at δC 57.8, proton δH 3.50 (H-6) correlates with δC 53.5, and the presumption of the presence of one methyl group is supported by proton correlation δH 1.84 with carbon at δC 16.1. The proton at δH 6.45 has a long-distance correlation with three atoms at δC 194.4; 57.8; and 16.1 ppm. The proton at 4.65 (H-4) correlates with carbon at C at 138c 138.9, proton 3.78 (H-5) correlates with C-3 (δC 138.9). Proton δH 3.50 (H-6) correlates remotely with two carbon atoms at δC 194.4 (C-1) and δC 134.7 (C-2). While the proton 84H 1.84 (H-7) shift ppm correlates with three carbon atoms in the area δc194.4 (C-1), δC 134.7 (C-2), and δC 138.9 (C-3). The 1H- and 13C-NMR of compound 1 (Table IV).

According to the spectroscopic data and literature as comparisons (Cala et al., 2018; Nagasawa et al., 1978), compound 1 was determined as 5-hydroxy-3-methyl-7-oxabicyclo[4.1.0] hept-3-en-2-one, trivial name is epi-epoformin compounds. (+) - Epi-Epoformin was isolated firstly from unidentified fungus, which was separated from the diseased leaf of crapemyrtle (Lagerstroemia indica L.) and showed the marked inhibition against the germination of lettuce seeds, Lactuca sativa L. (Nagasawa et al, 1978). Recently, this compound was also used as a fungicide to inhibit the growth of Puccinia pathogenic fungi and

Table IV. NMR Data (400 MHz. Chloroform- CDCl3) of Epi-epoformin (Cala et al., 2018)

| Compound 1 | Epi-epoformin |
|------------|---------------|
| No | δ (J in Hz) | δC mult | HMBC (H to C) | No | δ (J in Hz) | δC mult | HMBC (H to C) |
| 1 | - | 194 | - | 1 | - | 193.5 | - |
| 2 | - | 138.9 | - | 2 | - | 134.9 | - |
| 3 | 6.45 (1H, m) | 134.7 | 15.7 | 3 | 6.46 (1H, brs) | 134.3 | 1,5,7 |
| 4 | 4.65 (1H, br) | 63.5 | 3 | 4 | 4.67 (1H, brs) | 64.5 | 3 |
| 5 | 3.78 (1H, m) | 57.8 | 3 | 5 | 3.78 (1H, m) | 55.0 | 3 |
| 6 | 3.50 (1H, m) | 53.5 | 1, 2 | 6 | 3.53 (1H, m) | 52.9 | 1, 2 |
| 7 | 1.84 (3H, s) | 16.1 | 1,2,3 | 7 | 1.86 (3H, s) | 16.05 | 1,2,3 |
Uromyces which are pathogenic in legumes (Barilli et al., 2016). The biological activity of epiformin is better known as an antifungal or fungicide, while antibacterial activity has not been reported before. Furthermore, this compound has not been reported to be isolated from A. elegans isolated from marine biota.

CONCLUSION
In summary, endophytic fungi A. elegans SweF9 isolated from Euchema sp, possess antibacterial activity against tested bacteria, and revealed (+)-Epi-Epoformin as the active compound. Our results suggest that the fungal endophytes of A. elegans SweF9 could be an appropriate source of bioactive secondary metabolites.

ACKNOWLEDGEMENT
This work was supported by from Indonesian Institute of Sciences (LIPI). The author also thanks to Mrs. Andini Sundowo, M.Si for the mass spectra measurement, Dr. Nina Artanti and Dr. Sofia Fajriyah for valuable discussion.

REFERENCES
Andrew J., Jessica C., Christopher A. and John A. 2015. Endophytes from marine macroalgae: promising sources of novel natural products. Current Science. Vol 1: 175-187.
Barilli E., Cimmino A., Masi M., Evidente M., Rubialesa D. and Evidente A. 2016. Inhibition of early development stages of rust fungi by the two fungal metabolites cyclopaldicacid and epi-epoformin. Pest Manag Sci.: 1-9.
Calà A., Masi M., Cimmino A., Molinillo JMG, Marcias FA, Evidente A. 2018. (+)-epi-epoformin, a phytotoxic fungal cyclohexeneoxepoxide: structure activity relationships. Molecules. 23: 1-12.
Calvo AM., Wilson RA., Bok JW. and Keller NP. 2002. Relationship between secondary metabolism and fungal development. Microbiol. Mol. Biol. Rev. 66, 447–459.
Choma IM. and Grzelak EM. 2015. TLC-Direct Bioautography as a High Throughput Method for Detection of Antimicrobials in Plants. Chromatography. 2: 225-238.
Elisha IL., Botha FS., McGaw LJ. and Elloff JN. 2017. The antibacterial activity of extracts of nine plant species with good activity against Escherichia coli against five other bacteria and cytotoxicity of extracts. BMC Complement Altern Med. 17 (133):1-10.
Elsebai MF., Kehraus S., Lindequeut U., Sasse F., Shaaban S., Gütscw M., et al. 2011. Antimicrobial phenalenone derivatives from the marine-derived fungus Coniothyrium cereal. Org. Biomol. Chem. 9: 802-808.
Erbert C., Lopes AA, Yokoya NS., Furtado NAJC., Conti R., Pupo MT., et al. 2012. Antibacterial compound from the endophytic fungus Phomopsis longicola isolated from the tropical red seaweed Bostrychiara dicans. Bot. Mar. 55: 435440.
Hasan S., Ansari MI., Ahmad A. and Mishral M. 2015. Major bioactive metabolites from marine fungi: A Review. Bioinformation 11(4): 176-181.
Jesione W., Alberti A., Ott GP., Kocsis B., Horváth G. and Choma IM. 2015. TLC-Direct Bioautography as a Bioassay Guided Method for Investigation of Antibacterial Compounds in Hypericum perforatum L. Journal of AOAC International. 98 (4): 1013-1020.
Kalyanasundaram I., Nagamuthu J. and Muthukumaraswamy S. 2015. Antimicrobial activity of endophtic fungi isolated and identified from salt marsh in Vellar Estuary. Academic Journals 7: 13-20.
Miao F., Li X., Liu X., Cichewicz RH. and Ji N. 2012. Secondary metabolites from an algicolous Aspergillus versicolor strain. Mar. Drugs 10: 131-139.
Nagasawa H., Suzuki A. and Tamura S. 1978. Isolation and structure of (+)-desoxyepoxydon and (+)-epoxydon, phytotoxic fungal metabolites. Agric. Biol. Chem. 42: 1303–1304.
de Felício R., Pavão GLB., de Oliveira A., Erbert C., Conti R., et al. 2015. Antibacterial, antifungal and cytotoxic activities exhibited by endophytic fungi from the Brazilian marine red alga Bostrychiaternella (Ceramiales). Revista Brasileira de Farmacognosia 25,641–650.
Santos IP., Silva LCN., Silva MV., Araújo JM., Cavalcanti MS. and Lima VLM. 2015. Antibacterial activity endophytic fungi from leaves of Indigofera suffruticosa Miller (Fabaceae). Front. Microbiol. 6:350.
Antibacterial Compound from Aspergillus elegans SweF9

Sarasan M., Puthumana J., Neema Job N., Han J., Lee JS. and Philip R. 2017. Marine Algicolous Endophytic Fungi - A Promising Drug Resource of the Era. J. Microbiol. Biotechnol. 27(6), 1039–1052.

Srikandance Y. and Andayani DSG. 2015. Preliminary of Antibacterial Activity from Indonesia Marine-Derived Endophytic Fungi. Adv Environ Biol. 9(9): 143-149.

Sun RR., Miao FP., Zhang J., Wang G., Yin XL. and Ji NY. 2013. Three new xanthone derivatives from analgicolous isolate of Aspergillus wentii. Magn. Reson. Chem. 51, 65–68.