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

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ABSTRACT

The antibiotic resistance of phatogenic 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-poitive (*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 (derivated of leptosins), were extracted from the endophytic fungus Leptosphaeria sp. isolated from the brown alga *Sargasssum 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, yicathin 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 (Fujii *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. lagenarium* (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) J.Agardh 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 *Bostrychiara dicans* (Erbert *et al*. 2012); brevianamide M, 6,8-di-Omethylaverufin, and 6-O-methylaverufin secondary metabolites from an algicolous *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. Srikandace and Andayani (2015), have reported sicx 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 bacterias.

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 PDA, 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 ^{1}H NMR spectra were recorded in $CDCl_3$ on Agilent 500MHz with DD2 console system, while ^{13}C 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 30ºC for seven days in the dark. *A. elegant* SweF9 was cultivation in three different media: potato dextrose broth (PDB), potato malt peptone (PMP) and Czapek-Dox broth (CDB) incubated for ten days in room temperature at static condition. The culture media and mycelium were separated by filtration. The mycelium (B) and filtrate (F) was extracted with EtOAc. Each of organic extract was dried over anhydrous Na_2SO4, 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. elegan* 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.

Table I. Antibacterial activity from three different culture broths of *A. elegan* SweF9

No	Extract*	Inhibition zone (mm)			Activity index (%)		
		E. coli	*S. aureu*	*B. subtilis*	*E. coli*	*S. aureus*	*B. subtilis*
1	PDB (B)	11	14	16	48	70	59
2	PDB (F)	15	14	19	65	70	70
3	PMP (B)	14	15	16	61	75	59
4	PMP (F)	17	16	21	74	80	78
5	CDB (B)	7	-	7	30	-	26
6.	CDB (F)	8	-	8	35	-	30
7.	Streptomycin Sulfate	23	20	27			

Annotation: B = Biomasa; F = filtrate. *Sample concentration: 1%

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 ecxtracted 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 difusion 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.

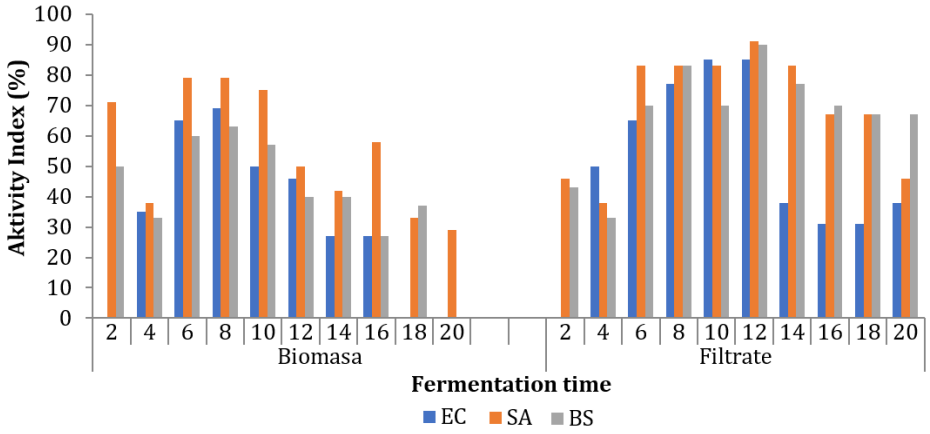

Figure 1. The index of antibacterial activity against the influence of fermentation time

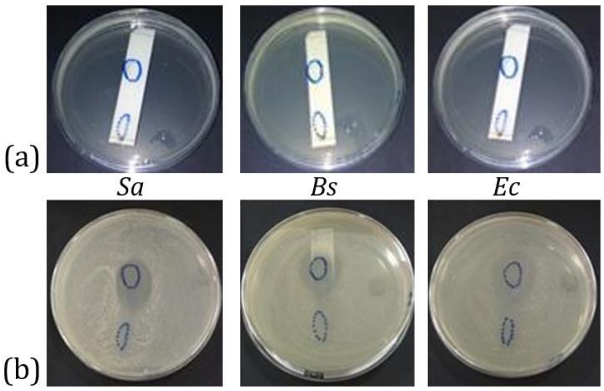

Figure 2. TLC-bioautographic of F extract of *A. elegan* SweF9 against *S. aureus*, *B. subtilis*, and *E. coli*. (a) before incubation; (b) after incubation

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 anti-microbial activity from several organic extracts, mainly plant extract (Jesionek *et al*., 2015).

Table II. Antibacterial activity of fraction from EtOAc extract of *A. elegans* SweF9

Fraction*	Yield (mg)	Inhibition Zone (mm)			Activity index (%)		
		E. coli	*S. aureus*	*B. subtilis*	*E. coli*	*S. aureus*	*B. subtilis*
Extract F	5500	30	27	29	100	100	96.6
F1	34.6	-	-	-	-	-	-
F2	115	22	20	22	73.3	76.9	73.3
F3	1399.5	23	22	20	76.6	84.6	66.6
F4	87.6	20	25	25	66.6	96.1	83.3
F5	233.8	10	-	10	33.3	-	33.3
F6	166.6	-	-	-	-	-	-
F7	637.7	-	-	-	-	-	-
F8	369.1	-	-	7	-	-	23.3
F9	67.2	-	-	-	-	-	-
F10	300.4	-	-	-	-	-	-
F11	70.2	-	-	-	-	-	-
F12	1580.3	9	8	-	30	30.7	-
Streptomycin Sulfate		30	26	30			

*Sample concentration: 1%

Table III. Antibacterial activity of compound **1**

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%

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 F4 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 1678cm^{-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 $C_7H_8O_3$ 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 δH 6.45 (brs, 1H, H-3) shows the proton shift bound to double bonded carbon (HC=C), δH 4.65 (brs, 1H, H-4), 3.78 (m, 1H, H-5), and 3.50 (m, 1H, H-6),

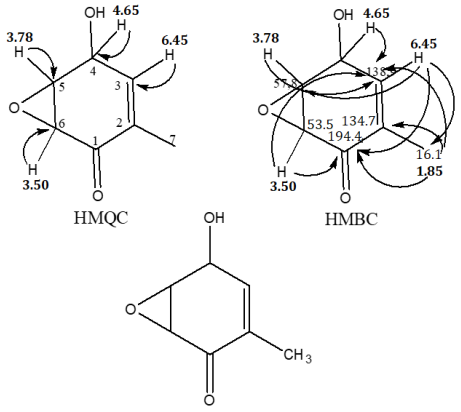

Figure 3. HMQC and HMBC ccorrelation of compound **1** ((+)-*epi*-Epoformin)

Table IV. NMR Data (400 MHz. Chloroform- $CDCl_3$) 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	-	136.6	-
3	6.45 (1H, m)	134.7	1,5,7	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

indicating the presence of 3 protons metin-oxy (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 ^{13}C-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 ^{1}H- and ^{13}C-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 ^{1}H- and ^{13}C-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

Uromyces which are pathogenic in legumes (Barilii *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.

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