Antibacterial Activity of Semi Purified Extract of Marine-Derived *Trichoderma reesei* PDSP 5.7 using Bioguided Fractionation Method

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Abstract

Bioguided fractionation method is commonly used to obtained targeted fraction with certain biological activity. In this study, bioguided fractionation method using HPLC was applied to obtained antibacterial fractions from marine-derived fungus *Trichoderma reesei* PDSP 5.7. The result shows that fraction from mycelium and medium extract had antibacterial activity against ESBL *E. coli* and *S. enterica ser.* Typhi with a range of inhibition zone was 1.35±0.15 to 8.82±0.22 mm^2. From 32 fractions of each extract, the mycelial extract had 7 active fractions, 3 active fractions from broth medium which extracted using 1-butanol and 5 active fractions from ethyl acetate extract. This study indicated that crude extract of fungus *T. reesei* PDSP 5.7 was more potential as the source of antibacterial agents rather than the crude extract that obtained from its broth medium.

Keywords : Antibacterial, fractions, HPLC, MDR, Trichoderma

INTRODUCTION

Trichoderma is reported as an outstanding genus regarding to its ability to adapt in various ecological condition. This fungus commonly isolated from terrestrial and marine environment (Korkmaz *et al.,* 2017; Awad *et al.,* 2018; Greco *et al.,* 2018; Sibero *et al.,* 2018). Several of them were reported from extreme environment. Sing *et al.* (2018) isolated *Trichoderma velutinum* from cold habitat soil within temperature range of -5 to 2 ^oC while three species of *Trichoderma* were isolated from heavy metal polluted soil (Tansengco *et al.,*2018). With this wide niche, *Trichoderma* has been widely studied as source of natural products. A total of 578 compounds have been reported from *Trichoderma* with various biological activities (DNP, 2020). Several important chemical substances such as cyclopentenone derivatives, diterpenes, polyketides, and polypeptide were produced by this genus (Pang *et al.,* 2017; Yamada *et al.,* 2017; Zhou *et al.,* 2017; Singh *et al.,* 2018).Among all type of bioactive compounds that have been reported, *Trichoderma* is well known as a potential producer of polypeptide such as peptaibols.Peptaibols is defined as a group of bioactive peptides containing 5 to 20 unusual amino acids, which synthesized by a non-ribosomal biosynthesis pathway (Szekeres*et al.,*2005; Daniel and Filho, 2007). Plenty studies reported antibacterial properties of these peptides. Three new peptaibols were isolated from *T. arundinaceum* MSX70741 by Rivera-Chávez*et al.* (2017). In early 2018, Singh *et al.* (2018) published four novel *lipovelutibols (A-D)*from *T. velutinum* containing six amino acids with leucinol at the C-terminus and a fatty acyl moiety (*n-octanoyl*) at its N-terminus. Several compounds from *Trichoderma* were performed antibacterial activity against pathogenic microorganisms. Khamthong *et al.* (2012) isolated *coniothranthraquinone 1* and *emodin* from *T. aureoviride* PSU-F95. These compounds were reported as a potential antibacterial agent to inhibit methicillin-resistant *S. aureus* (MRSA) with MIC values of 8 and 4 µg/mL. Moreover, five new fungal polyketide and two known analogs were isolated from *T. koningiopsis* QA-3 by Shi *et al.* (2017). They mentioned that among seven compounds, *1,6-di-epi-koninginin A* and *trichoketide A* showed antibacterial activity against human pathogen *E. coli*. Adelin *et al.* (2017) isolated a new antibiotic compound named EA-2081 was isolated from *T. atroviridae* with MIC values 32 to 128 µg/mL against human pathogens strain ATCC.

Production of antimicrobial compounds by *Trichoderma* has a high possibility of

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beingapplied in the antibiotic industry. However, the human pathogen has evolved to resistant to several groups of antibiotics, known as multidrug-resistant organisms (MDRO). Every year, the infections caused by MDR bacteria areincreasing significantly due to the overuse of antimicrobial agents (Munita and Arias 2016). However, most of the antibiotic properties of bioactive compounds from *Trichoderma* were tested against ATCC bacteria. Therefore, it is interesting tostudy antibacterial compounds to inhibit MDR bacterial of *Trichoderma*.

Microorganisms produce secondary metabolites as a response to environmental stresses and threats. Ecologically, sponge-associated microorganisms produce bioactive compounds to protect themselves and its host from pathogens or predators (Taylor *et al.,* 2007; Brinkmann *et al.,* 2017). Moreover, as a filter feeder animal and it is continuously exposed to an array of microorganisms from the sea water, sponge obtains both of threat and opportunity for surviving. Sea water could bring harmful microorganisms such as biofilm forming bacteria and pathogenic bacteria. On the other hand, it also contains other microorganisms which may contribute to establishing symbiotic relationship to overcome the threat from harmful microbes. Selvin (2009) proved this theory by reporting the role of *Streptomyces* MSI051 in the protection of host sponge *Dendrilla nigra* against fouling processes. *Streptomyces* MSI051 showed strong inhibition against biofilm-forming bacteria *Pseudoalteromonas* sp. and *Thalassomonas* sp. The ability of sponge-associated microorganisms to produce antibacterial compounds to protect themselves and the host has been applied to produce antimicrobial compounds to inhibit human pathogen (Sibero*et al.,* 2016). As a potential source of antimicrobial compounds, sponge-associated microorganisms such as fungi is a very important to be explored to find new candidate of antibiotic against MDR bacteria (Sibero *et al.,* 2019).

Indonesia has broadmarine environment as an origin of potential sponge associated fungi. Due its potential, sponge-associated *Trichoderma* has been explored as an antibiotic compounds producer. As a result, several species of marine-derived *Trichoderma* have been reported from Indonesian marine sponges (Sibero *et al.,*, 2016; Sibero *et al.,* 2017; Trianto *et al.,* 2018). In addition, the previous study showed the ability of crude extract from sponge-associated fungus *T. reesei* PDSP 5.7 inhibited clinical pathogenic multidrug resistant extended spectrum β-lactamase (ESBL) *E. coli*, MRSA, *S. enterica* ser. Typhi, and *S. haemoliticus*.The crude extract had widest antibacterial against *S. enterica* ser. Typhi then followed by ESBL *E. coli* after cultivated for 15 days. The crude extract had wider inhibition zone than *Amoxicillin+Clavulanic acid* against *S. enterica* ser. Typhi (Sibero *et al.,* 2018). It shows that marine derived *Trichoderma* is a potential source of new antibiotic agent against MDR bacteria. Our current study was aimed to obtain and characterize the fractions with antibacterial activity against clinical pathogenic bacteria ESBL *E. coli* and *S. enterica* ser. Typhi strain MDR using bioguided fractionation method.

MATERIAL AND METHODS

This research utilized marine derived *Trichoderma reesei* PDSP 5.7 with accession number MG547722.1. This fungus was isolated from sponge *Cinachyrella* sp. PPD.SP.05 which collected from Pandang Island, North Sumatra (Sibero *et al.,* 2018). Fungus was revived on *Malt Extract Agar* (MEA) *HiMedia* for 7 days and incubated at 25^{o}C.

Clinical pathogenic ESBL *Escherichia coli*, and *Salmonella enterica* ser. Typhi strain multidrug-resistant (MDR) wasobtained from General Hospital Dr. Kariadi, Semarang, Central Java. These bacteria were isolated from the patient and confirmed as MDR.

Fungus *T. reesei* PDSP 5.7 was recultivated on *Malt Extract Broth* (MEB) *Difco*^{TM} for 15 days in static condition (stand culture) at room temperature (25^{o}C). After cultivation, fungal mycelium and broth was separated using filter paper (*Advantec* Ø 125 mm). Mycelium wasdried at 37^{o}C then extracted using 1-butanol. Fungal metabolites in broth were extracted using a single extraction method with 1-butanol (*Wako,* Japan)and ethyl acetate (*Wako,* Japan). Solvent was added into fungal broth (2:1) then agitated using a shaker (200 rpm) for 1 h after that it was separated by orbital centrifugation (6000G, 15 min, 4^{o}C). Solvent was evaporated at 35^{o}C.

Approximately 2 mg of fungal extract was dissolved in 1 mL dimethyl sulfoxide (DMSO) then sonicated for several secs then filtered using cosmonice filter pore size 0.45 µm (Nacalai Tesque, Inc) and injector (Terumo, 1 mL). Sample was injected into HPLC (Agilent 1100 Series), reverse phase column (Microsorb C_{18}, particle size 3 µm, pore size 50×4.6 mm) with buffer 0.1% formic acid (HCO_2H) and acetonitrile (CH_3CN) (*Wako,* Japan). Crude extract was fractionated

with acetonitrile presentation 15% for min 0-3, 85% for min 25-29 and 15% at 32 min, speed 1.0 mL/min. Detection was performed with diode array detector (DAD) at wave length 210 nm, 230 nm, 254 nm and 400 nm. Each fraction was collected using fraction collector (Gilson FC 203B) then dried using cooling vacuum centrifugation (Savant Speed Vac® Plus SC210A and Thermo RVT400 Vapor Trap) then kept at 2^oC.

For the antibacterial assay, each fraction was dissolved in 50 µL DMSO then sonicated for 1 min. MDR clinical pathogenic ESBL *Escherichia coli* and *Salmonella enterica* ser. Typhi were refreshed on *MacConkey HiMedia* for 24 h before assay. For the assay, pathogens were diluted to be 0.5 McFarland in physiological saline solution, then mixed in *Meuller Hinton Agar* (MHA) *Difco*^{TM} and poured into a square disc. Approximately 10 µL of each fraction was injected into a paper disc (*Advantec Ø* 6 mm), then placed onto MHA and incubated for 24 h at 32^oC. The presence of inhibition zone indicated the antibacterial activity.

Data of antibacterial activity were analyzed statistically using PASW Statistic software package for Windows with confidence interval 95% ($P<0.05$).

RESULT AND DISCUSSION

The microbial secondary metabolite could be secreted to the environment (extracellular metabolites) or kept inside the cell (intracellular metabolites). Most of the extracellular metabolites were produced to protect the producer (Arumugam *et al.*, 2014). This study extracted fungal intracellular metabolites from mycelium and extracellular metabolites from the broth medium.Fungus *Trichoderma reesei* PDSP 5.7 has been studied to has antibacterial activity against several multidrug-resistant bacteria. Crude extract from this fungus performed the strongest antibacterial activity against *S. enterica* ser. Typhi with inhibition zone value was 14.72±0.07 mm^2,while *Amoxicillin + Clavulanic acid* had 12.20±0.28 mm^2 (Sibero *et al.*, 2018). In this study, fungal secondary metabolites were extracted from broth medium using 1-butanol and ethyl acetate, while mycelial bioactive was extracted using 1-butanol. We tried to figure out the profile of fungal extract from mycelium and broth in different solvents. However, the application of crude extract for drug development is not suggested, so the isolation of single antibacterial compounds is highly recommended. To obtain pure compound with antibacterial property, bioguided fractionation and isolation method is commonly applied (Lo *et al.*, 2004; Pieters dan Vlietinck 2005). This research performed bioguided fractionation using HPLC. The crude extract was fractionated to 32 fractions based on the retention times. Each fraction was tested against ESBL *E. coli* and *S. enterica* ser. Typhi strain MDR. The result of the antibacterial assay is presented in Table 1.

In Table 1, fractions from mycelial extract showed antibacterial activity against the tested pathogenic bacteria. ESBL *E. coli* was inhibited by fr. 20, 21, and 22 with widest inhibition zone was exhibited by fr. 21 (8.00±0.00 mm) mm while *S. enterica* ser. Typhi was inhibited by fr. 18, 19, 20, 21, 22, 23 and 25 with the widest inhibition zone performed also by fr. 21 (8.82±0.22 mm). Fungal extract from broth medium which extracted using 1-butanol had active fractions at retention time 20, 21 and 22 against both of tested bacteria. The strongest antibacterial activity was performed by fr. 21 with inhibition zone 6.05±0.05 mm against ESBL *E. coli* and 8.95±0.05 mm against *S. enterica* ser.

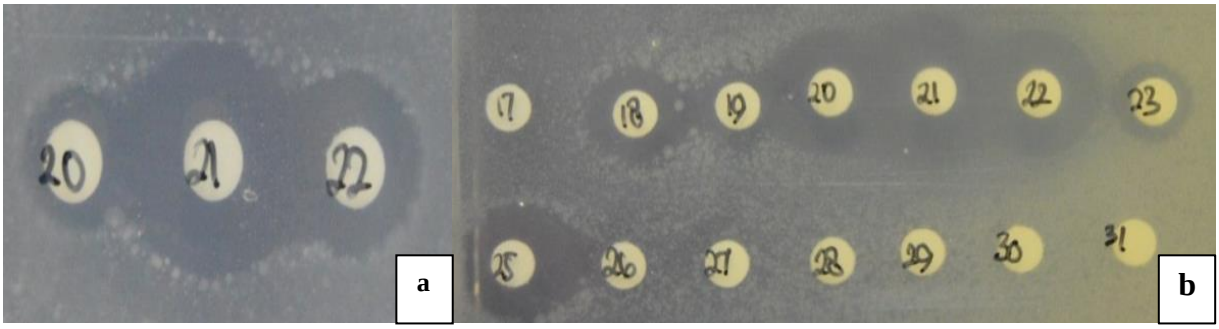

Figure 1. Inhibition zones of mycelial active fractions against clinical pathogenic ESBL (a) *E. coli* and (b) *S.enterica ser. Typhi*

Table 1. Antibacterial activity of fungal fractions against clinical pathogenic bacteria strain MDR

Fraction	Mycelium		Medium			
			1-butanol		Ethyl acetate	
	Inhibition Zone (mm^2)		Inhibition Zone (mm^2)		Inhibition Zone (mm^2)	
	ESBL *E.coli*	*S. enterica*	ESBL *E.coli*	*S. enterica*	ESBL *E.coli*	*S. enterica*
1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
2	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
4	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
5	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
6	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
8	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
9	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
11	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
12	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
13	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
14	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
15	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
16	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
17	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
18	0.00±0.00	$4.10±0.10^{d}$	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
19	0.00±0.00	$3.05±0.05^{e}$	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
20	$3.00±0.10^{c}$	$7.20±0.10^{b}$	$1.35±0.15^{c}$	$4.05±0.05^{c}$	$6.00±0.00^{a}$	$8.00±0.00^{b}$
21	$8.00±0.00^{a}$	$8.82±0.22^{a}$	$6.05±0.05^{a}$	$8.15±0.05^{a}$	$5.95±0.05^{a}$	$8.00±0.00^{b}$
22	$4.95±0.05^{b}$	$7.05±0.05^{c}$	$2.00±0.00^{b}$	$7.45±0.05^{b}$	$5.95±0.05^{a}$	$8.15±0.15^{a}$
23	0.00±0.00	3.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	$6.15±0.05^{c}$
24	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	$1.95±0.05^{d}$
25	0.00±0.00	$7.15±0.05^{b}$	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
26	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
27	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
28	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
29	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
30	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
31	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
32	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

(Data are mean±SD. Values with different shared letters in each MDR denote significant difference at $P<0.05$)

Typhi Furthermore, there were more fractions from ethyl acetate extract showed antibacterial activity. Fractions 20, 21 and 22 inhibited both of tested bacteria while fr. 23 and 24 only inhibited *S. enterica* ser. Typhi. Most extensive inhibition zone was performed by fr. 20 against ESBL *E. coli* with diameter 6.00±0.00 mm while for *S. enterica* ser. Typhi fr. 22 showed strongest antibacterial activity with diameter 8.15±0.15 mm. The inhibition zone and HPLC chromatographic profiles of active fraction are shown by Figure 1-2.

According to Figure 2, the mycelial extract of *T. reesei* PDSP 5.7 had 6 active fractions with 10 major peaks. In fractions 18, 19, 22, 23 and 25 we found one peak for each, while fraction 20 contained 3 peaks and fraction 21 had 2 peaks. Fractions 20 and 21 showed stronger antibacterial activity rather than other fractions. We highlighted it with two hypotheses whether it was caused by synergic effect of compounds in each fraction or there was a major compound with great antibacterial activity. Moreover, Figure 2 shows that peaks in fraction 21***b*** (Figure 2) had 1200 mAU which considered as main antibacterial compounds. In addition, it also needs to emphasize that a single peak does not indicate a single compound in the fraction. Figure 2 shows most of the peaks have a similar pattern. Although it had a similar UV pattern, it does not indicate the same compound in the fractions. Several compounds such as *chrysophanol*, *pachybasin*, *sorbicillin* and*ergosterol*were reported as mycelial intracellular metabolite from *Trichoderma* (Tarus

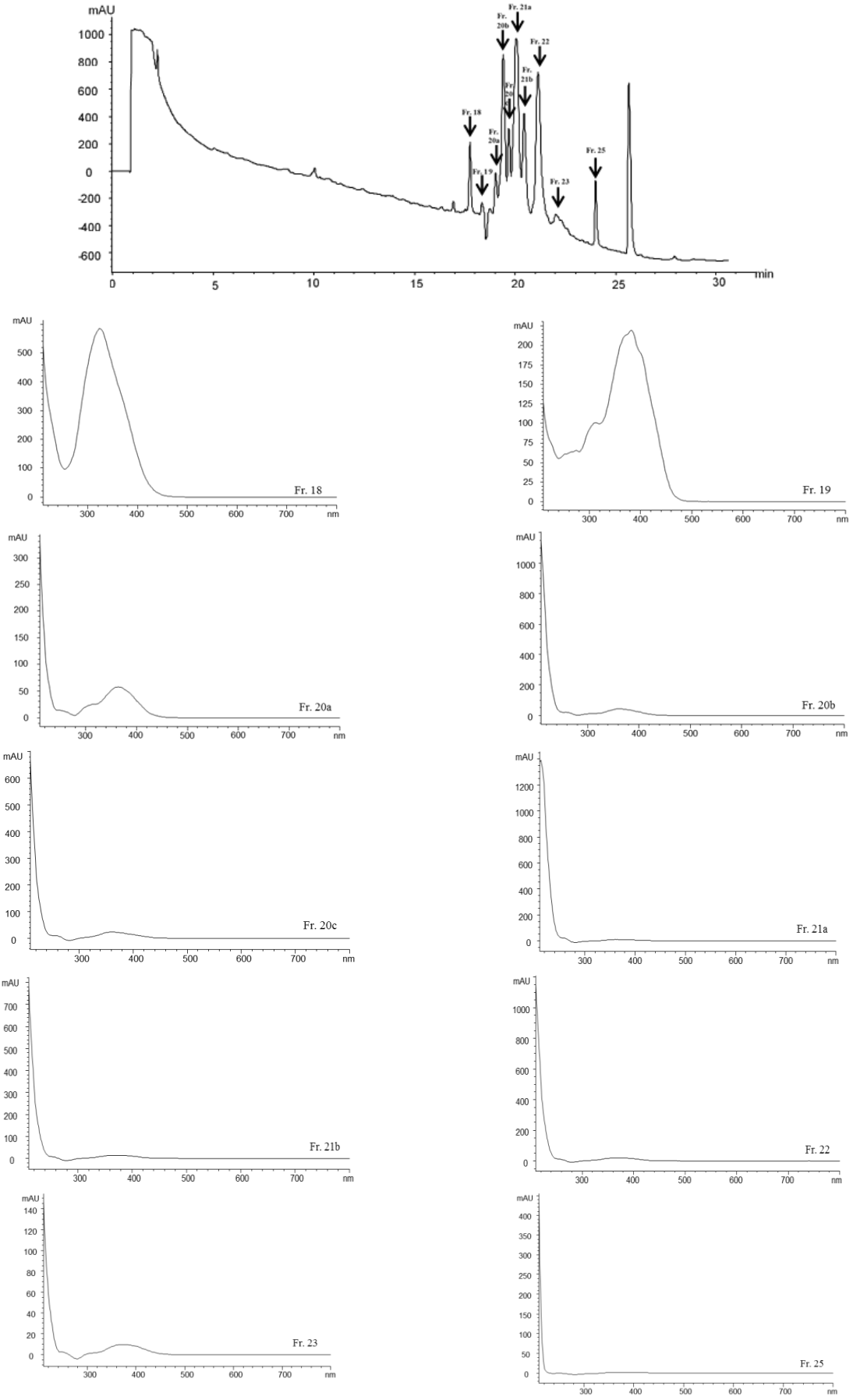

Figure 2. HPLC chromatogram profile and UV spectrum of active fractions from the mycelial extract

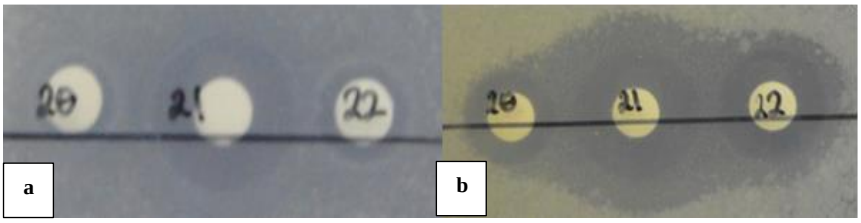

Figure 3. Inhibition zones of active fractions from medium extract in 1-butanol against clinical pathogenic ESBL (a) *E. coli* and (b) *S. enterica* ser. Typhi

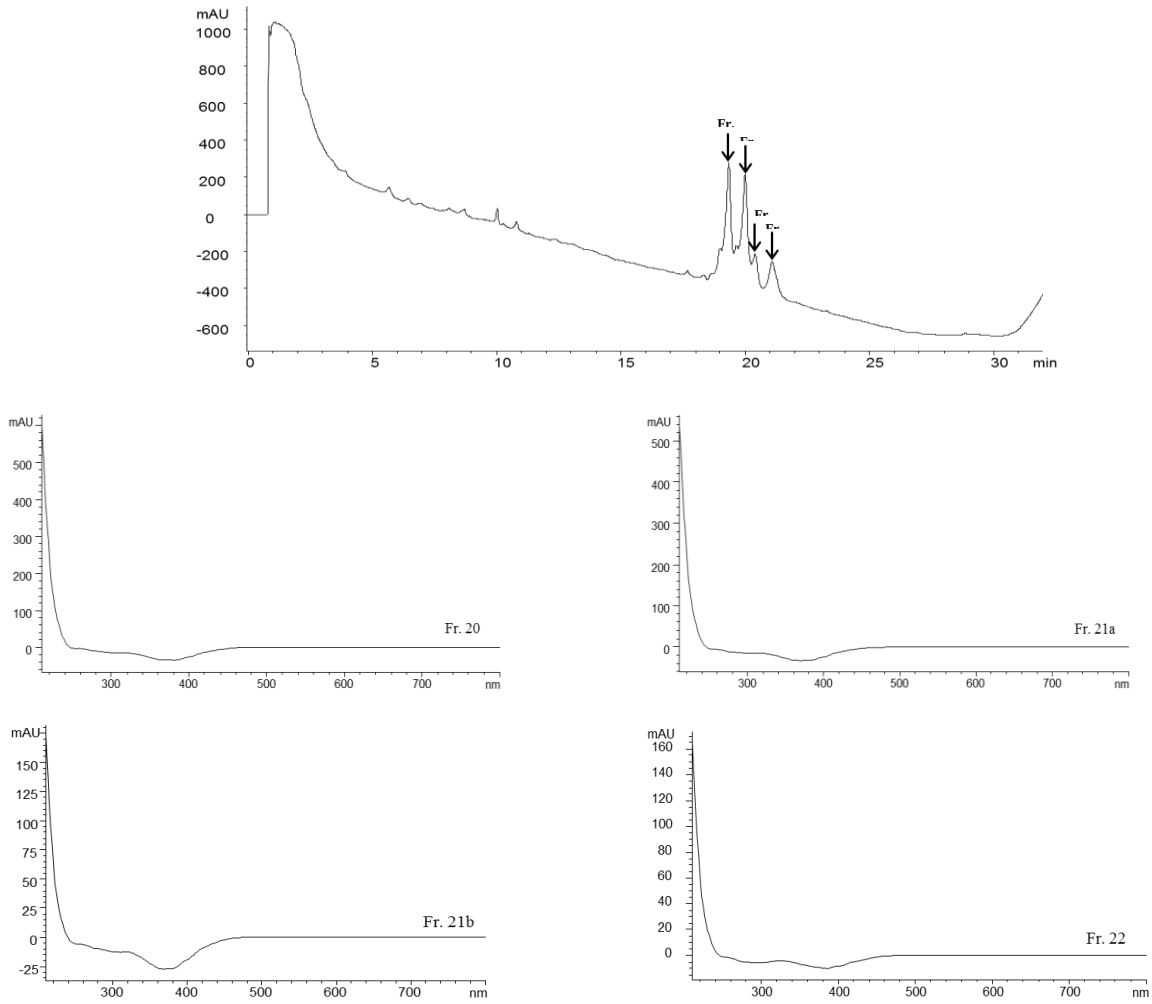

Figure 4. HPLC chromatogram profile and UV spectrum of active fractions from medium extract in 1-butanol

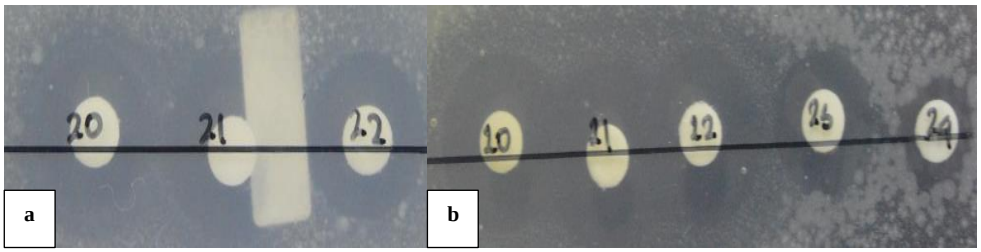

Figure 5. Inhibition zones of active fractions from medium extract in ethyl acetate against clinical pathogenic ESBL (a) *E. coli* and (b) *S. enterica* ser. Typhi

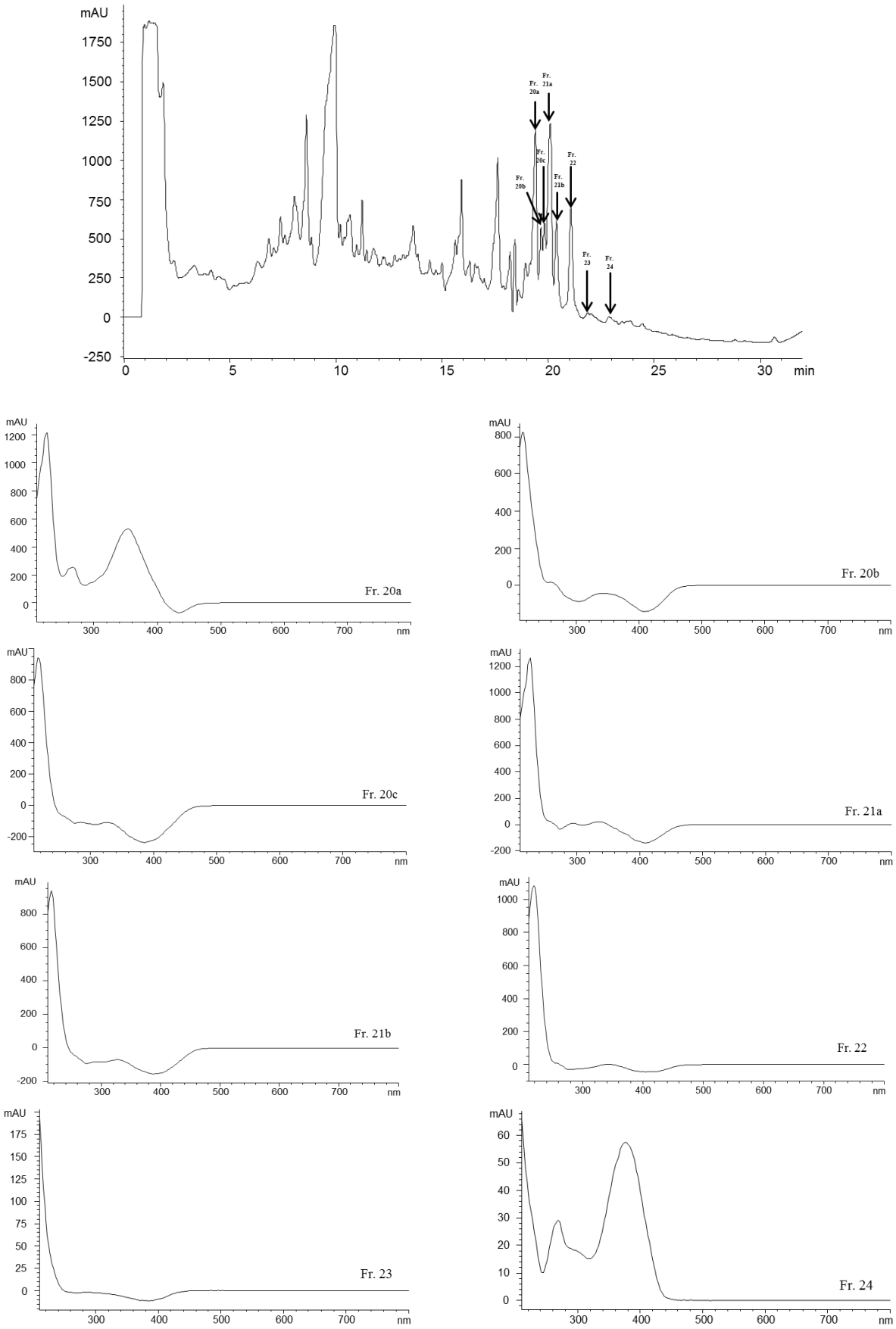

Figure 6. HPLC chromatogram profile and UV spectrum of active fractions from medium extract in ethyl acetate

et al., 2003; Reino *et al.,* 2008).Further, the inhibition zones, HPLC chromatogram and UV spectrum of active fractions from medium extract using 1-butanol are shown by Figure 3-4 while medium extract using ethyl acetate are shown by Figure 5-6.

The result showed that both of mycelia and medium extract contained antimicrobial compounds that inhibited ESBL *E. coli* and *S. enterica* ser. typhi strain MDR. This similar result also reported by Malhadas *et al.* (2017). They found that fungal extract from mycelium and broth medium could inhibit pathogenic bacteria in a certain concentration. Figure 3 and Figure 5 show the inhibition zones of fungal extract from broth medium in a different solvent. The extract obtained from 1-butanol performed antibacterial property against both of tested bacteria at fraction 20, 21 and 22 while the extract from ethyl acetated inhibited the bacteria at fraction 20, 21, 22, 22, 23 and 24. Both extracts showed the widest inhibition zone at fraction 20 and 21 (Table 1). Figures 4 and 6 show that 1-butanol extract had fewer peaks than ethyl acetate extract. This result was correlated to the profile of each extract. Fraction 20 and 21 from ethyl acetate extract consisted of 5 peaks, while fractions from 1-butanol extract consisted of 3 peaks.

Besides, all extract had active fractions in similar retention time. According to HPLC condition, we considered the fraction as semi-polar to non polar because it appeared when the concentration of solvent around 79-83%. Most of the active fractions had peaked with similar UV patterns. However, the similarity of the UV pattern is caused by the presence of similar functional group in the active compounds. In this study, we consider that ethyl acetate able to extract more compounds from broth medium rather than 1-butanol. Based on the inhibition zone from fungal fractions, we propose that fungus *T. reesei* PDSP 5.7 is a good candidate as an antibiotic producer to overcome infection of ESB *E. coli* and *S. enterica* ser. Typhi strain MDR.

CONCLUSION

Fraction from mycelium and medium extract of marine-derived fungus *T. reesei* PDSP 5.7 had antibacterial activity against ESBL *E. coli* and *S. enterica* ser. Typhi with range of inhibition zone was 1.35±0.15 to 8.82±0.22 mm. Mycelial extract had activity at fractions 18, 19, 20, 21, 22, 23 and 25, active fractions from broth medium which extracted using 1-butanol were 20, 21 and 22 while active fractions from ethyl acetate extract were 20, 21, 22, 23 and 24. Most of the active fractions had similar UV absorption patterns.

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