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Evaluation of Antimicrobial Activity and Identification of Yellow Pigmented Marine Sponge-Associated Fungi from Teluk Awur, Jepara, Central Java

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Abstract

Marine sponge associated fungi are known as potential source of metabolites with various biological activities. Natural pigment is one of metabolite which produced by microorganisms. Several researches reported the antimicrobial activity from natural pigment. Unfortunately there are lack of information about marine fungi natural pigment and its producer. The aims of this research were to identify yellow pigmented Indonesian marine sponge-associated fungi, to extract the pigment, and to study the antimicrobial activity of the pigment against clinical MDR bacteria and clinical pathogenic fungi. Sponge associated-fungus isolate MT23 was successfully identified as *Trichoderma parareesei*. The fungal pigment could be extracted only in methanol with yield 6.22±0.29%. The pigment could inhibited *Salmonella typhi* and *Escherichia coli* MDR strains. The biggest antibacterial activity was shown by concentration 1000µg/mL against *S. typhi* with inhibition zone was 4.03±0.06 mm.

Keywords: Associated fungi, pigment, *Trichoderma parareesei*

Introduction

Pigments are chemical compounds which give colours because of its ability to absorb light in the wavelength range at visible region (Delgado-Vargas *et al.*, 2000). The utilization of pigments is increasing every year due to the increase of demands in food, beverages, pharmaceutical and cosmeceutical industries (Rymbai *et al.*, 2011; Venil *et al.*, 2013). Natural pigments are known to have biological activities as its advantages over the synthetic pigments (Rostami *et al.*, 2016; Norman *et al.*, 2016; Yolmeh *et al.*, 2016).

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Antimicrobial activity is one of biological activities showed by natural pigments. Salem *et al.* (2014) successfully extracted natural pigment from *Carthamus tinctorius*. The pigments displayed antimicrobial activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* ATCC 25218 and *Candida albicans*. Another research reported that carotenoids pigment were able to inhibit *Salmonella enteritidis* (PTCC 1709) and *E. coli* (PTCC1260) (Rostami *et al.*, 2016). In addition, crude extract of pigment from *Streptomyces* sp. D25 was reported to have antibacterial activity against biofilm forming bacteria such as *Pseudomonas* sp. P1., *Bacillus* sp. P13 and *Alcaligens* sp. M28 (Radhakrishnan *et al.*, 2016).

Marine microorganisms are known as potential producer of natural pigment with great antibacterial activity. There are several microorganisms are known life associated

with marine organisms especially sponges such as bacteria, actinomycetes and fungi (Idraningrat *et al.*, 2016; Wu *et al.*, 2016). Ibrahim *et al.* (2014) studied antimicrobial activity of prodigiosin (red pigment) from sponge-associated bacteria *Serratia marcescens* IBRL USM 84. This pigment greatly inhibited MRSA and had good activity inhibited *Bacillus cereus, Bacillus licheniformis* and *Agrobacterium tumefaciens*. In 2015, Suresh *et al.* (2015) observed antibacterial activity of red pigment produced by *Halolactibacillus alkaliphilus* MSRD1 which isolated from seaweed against *S. aureus* and *Salmonella typhi*. In addition, Srilekha *et al.* (2016) successfully isolated marine pigmented bacteria and extracted the pigment then studied its antimicrobial activity. Result showed the pigment had great antimicrobial activity against bacteria such as *S. aureus*, *Proteus vulgaris*, *K. pneumoniae*, *E. coli*, and fungi such as *Fusarium* sp., *C. albicans*, *Mucor* sp., and *A. flavus* with inhibition zone range 18–28 mm. These reports show great potential of natural pigments from marine bacteria as antibacterial agent.

However, there are still a few researches about the potential of natural pigments from marine fungi and its antibacterial activity against clinical multidrug resistant bacteria (MDR) and clinical pathogenic fungi. MDR bacteria are bacteria which already resistant to several antibiotics that should be effective to inhibit the growth of the bacteria (Magiorakos *et al.*, 2012). This is a big issue for public health. The aims of this research were to identify Indonesian yellow pigmented sponge-associated fungi, to extract the pigment, and to study the antimicrobial activity of the pigment against clinical MDR bacteria and clinical pathogenic fungi.

Materials and Methods

Media and chemicals

This research used Malt Extract Agar (MEA) M137 base with mycological peptone and MacConkey M081B agar from HiMedia Laboratories Pvt. Ltd. (Swastik Disha Business Park, Via Vadhani, Ind. Est., LBS Marg, Mumbai-400086, India), Nutrient Agar CM0003 from Oxoid Ltd. (Basingstoke, Hampsphire, England), Mueller Hinton Agar (MHA) ACC, to CLSI 1.05435.0500 from Merck KGaA (Darmstadt, Germany) while agarose was bought from Promega. Solvents such as methanol, ethyl acetate and n-hexane were bought from Merck. DNA extraction used Chelex®100 (Sigma-Aldrich), PCR master mix GoTaq Green Master Mix from Promega Corporation (2800 Woods Hollow Road·Madison, WI 53711-5399 U.S.A) while for primer (ITS1 and ITS4) from Macrogen (1330 Piccard Drive Rockville, MD, 20850 United States).

Fungal isolate

Fungus MT23 was culture collection of Marine Fungi Division of Tropical Marine Biotechnology Laboratory, Integrated Laboratory of Diponegoro University, Semarang, Central Java, Indonesia. This fungus was isolated from unidentified marine sponge from Teluk Awur Bay, Jepara, Central Java.

MDR bacteria and clinical pathogenic fungi isolates

MDR bacteria and clinical pathogenic fungi were obtained from Dr. Kariadi General Hospital Medical Center, Semarang, Central Java, Indonesia and Diponegoro National Hospital, Semarang, Central Java, Indonesia. Bacteria *E. coli*, *S. typhi* and fungi *Malassezia furfur* and *Trichophyton rubrum* were used in this study.

Fungal culture

Fungus was recultured according to Sibero *et al.* (2016) with particular modifications. Approximately 1 cm^2 fungus was taken from the stock culture then transferred to fresh Malt Extract Agar (MEA) and incubated in room temperature (23^oC) for 7 days with addition of chloramphenicol 5% (%w/w). After that, the morphology of the recultured fungus was compared to the culture stock. The same morphology indicate the fungus was not contaminated. Then fungus MT23 was reculture to new fresh MEA without chloramphenicol, pH 5.4, illumination using LED lamp (Philip LED 20 watt) and incubated in room temperature (23^oC) for 14 days.

MDR bacteria and clinical pathogenic fungi

Gram-negative bacteria *E. coli* and *S. typhi* were revified on MacConkey Agar while *M. furfur* and *T. rubrum* were revified on Potato Dextrose Agar (PDA). The bacteria were incubated for 24 h in 32°C while the fungi were incubated in at 27°C for 24 h.

Fungal identification

Morphological approach

Fungus MT23 was recultured on MEA for 5 days with slide cultured method (Qiu *et al.* 2005). Sterilized MEA was cut with size 2 cm^2 then transferred and put onto object glass then covered by cover glass. After that, fungal mycelia were inoculated on each side of the MEA then incubated at room temperature (set at 23°C). After 5 days of incubation, object and cover glasses were covered by fungal mycelia. MEA was separated from the glasses. Lactophenol cotton blue was dropped on the object and cover glasses then covered by its pairs. After that, the morphology of the fungi was observed under microscope.

Molecular approach

DNA of fungus MT23 was extracted using Chelex method from Turan *et al.* (2015) with particular modifications. Seven days old of mycelium were immersed in 100 µL ddH2O and 1000 µL of 0.5% saponin for 24 h in chilling temperature (4°C). Then it was separated by centrifugator with 12000 rpm for 10 min. The supernatant was discarded while the template was added 100 µL of ddH_2O followed by addition of 50 µL of 20% Chelex 100. Then it was heated in water bath at 80°C for 5 min. After that, the mixture was homogenized using vortex for 10 s and reheated for 5 min. After reheated, the DNA in mixture were separated using centrifugation at 12000 rpm for 10 min. Then the supernatant were transferred to new microtube and stored at -20°C for 24h.

DNA of fungus MT23 was amplified based on Internal Transcribed Spacer (ITS) region. The amplification using primer 1 µL of ITS 1 (5'-TCC GTA GGT GAA CCT GCG G-3') and 1 µL of ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). The primers were mixed with 12.5 µL of GoTaq Green Master Mix Promega, 0.5 µL of DNA extract and 10 µL of ddH_2O. The PCR profile consisted of 3 min of preheat at 93°C for 3 min, 30 cycles for denaturation at 95°C (1 min), annealing at 56.1°C (1 min) and extension at 72°C (1 min). Final extension was done at 72°C for 7 min. The quality of PCR product was checked using gel electrophoresis in agarose 1%. Then PCR product was sent to 1st Base Laboratories Sdn Bhd, Malaysia for DNA sequencing. DNA sequence was analyzed to its homology using Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov). Phylogenetic tree was reconstructed using MEGA 7.0 software package (Tamura *et al.* 2011). Neighbor-joining was applied as the statistical method with 1000 bootstrap replications.

Pigment extraction

Yellow pigment from fungus MT23 was extracted using solid-liquid extraction (Manikkam *et al.* 2015). The mycelia were separated from media, then the media were cut into smaller size and dried using silica gel in desiccator for 48 h. After that, solvent optimization for pigment extraction was done by immersing the dried agar in methanol, ethyl acetate and n-hexane with agitation using a shaker (118 rpm) for 24 h then filtered using filter paper (Macherey-Nagel 640d Ø 1125 mm). The filtrate was evaporated using rotary evaporator (Eyela SB-1100) set at 38°C. The pigment yield percentage was calculated using following equation:

$$\%\ Pigment\ yield = \frac{Pigment\ yield\ (gr)}{Agar\ weight\ (gr)} \times 100\%$$

Determination of inhibition zone

Antibacterial activity

Antibacterial activity of the pigment was evaluated using agar-disc diffusion method from Balouiri *et al.* (2016) with several modifications. Pigment extract was dissolved in methanol and diluted to five concentrations (50, 125, 250, 500 and 100 µg/mL). *S. typhi* and *E. coli* strain MDR were grown on MacConkey Agar for 24 h (32°C) then diluted in physiological saline solution into 0.5

McFarland. Then the bacterial solution was inoculated to MHA using sterile cotton swab. Each extract (10 µl) was injected onto sterilized paper disc then placed on the MHA and incubated at 32°C for 24 h. Amoxicillin + Clavulanic acid (30 µg/disc) was used as positive control. The presence of clear zone indicated the antibacterial activity of the pigment extract.

Antifungal activity

This assay was done according to Bhalodia and Shukla (2011) and Balouiri *et al.* (2016) with modifications. Pigment extract was dissolved in methanol and diluted to five concentrations (50, 125, 250, 500 and 100 µg/mL). *M. furfur* and *T. rubrum* were grown on PDA for 24 h at 27°C. Then the fungi were inoculated using pour plate method. Each extract (10 µL) was injected onto sterilized paper disc then placed on the PDA and incubated at 27°C for 24 h. Nystatin was used as positive control in this study. The presence of clear zone on PDA indicated the antifungal activity of the pigment extract.

Data analysis

The impact of solvents to pigment yields was calculated with One Way Analysis of Variance (ANOVA) with $P<0.05$ while antibacterial and antifungal activities were calculated using Two Way ANOVA with $P<0.05$. The data was analyzed using SPSS 5.0.

Results and Discussion

Fungal identification

Fungus MT23 is a marine sponge associated fungi which is isolated from sponge from Teluk Awur, Jepara, Central Java. Macroscopic characteristics of fungus MT23 were white colour mycelium with green radial growth ring, hyphae concentrated and produced yellow colour in the media. This fungus grown well in room temperature (23°C) and changed the colour of MEA from slightly orange to yellow colour. Colour change was started at fourth day of cultivation and successfully changed the colour of whole media at day 14 (Figure 1). Physical conditions of environment including pH and temperature were reported to give impact to the fungal pigment production. A fungus *Penicillium purpurogenum* GH2 produced highest yield of red pigment after 240 h (10 days) of cultivation with pH 5 at 24°C (Mendéz *et al.* 2011). On the other hand, Velmurungan *et al.* (2010) induced fungal pigment production using colour of lights. They figured out that the darker lights like red (780–622 nm) and blue (492–455) gave higher pigment productions compared to green (577–492 nm), yellow (597–577 nm) and white lights in 5 different fungi. This phenomenon could be explained by the postulating of the existance of photoreceptor in fungi. However, there was no clear explanation about the mechanisms of the light to induce pigment productions in fungi. Furthermore, Blumenstein *et al.* (2005) suggested that a phytochrome type of system may be operative in particular fungi, in his case was *A. nidulans*. The phytochrome suggested able to sense the red and far-red light through photointerconversion between the two stable conformations.

Figure 2 shows the morphology of fungus MT23 under microscope. This fungus had hyaline and thin mycelium with septum. The conidiophore were branching with lageniform phialide with green conidia, smooth, eguttulate, and ellipsoidal to cylindrical form. According to its characteristics, fungus MT23 was judged as member of genus *Trichoderma* (Atanasova *et al.*, 2010; Rahman *et al.*, 2011; Qin and Zhuang, 2016). To confirm the accuracy of morphological identification, we did identification through molecular approach.

Fungal DNA was extracted using Chelex 100 method (Turan *et al.*, 2015). This method is commonly used to extract microorganisms' DNA (Liu *et al.*, 2015; Susilowati *et al.*, 2015). Chelex 100 able to prevent DNA damage by inhibiting DNase activity (Walsh *et al.*, 1997). Fungal mycelium was immersed in saponins and ddH2O for overnight. Saponins is detergent agent to lyse the cells (Dong *et al.*, 1997).To amplify fungal DNA, ITS1 and ITS4 were used as primer. These primers will amplify DNA from end of 18S rRNA gene region, internal transcribed spacer 1, 5.8S rRNA gene

region, internal transcribed spacer 2 region and the beginning of 28S rRNA gene region (White *et al.*, 1990; Larena *et al.*, 1999; Anderson *et al.*, 2003; Bellemain *et al.*, 2010; Schoch *et al.*, 2012). Figure 3 shows the location which amplified by ITS primer.

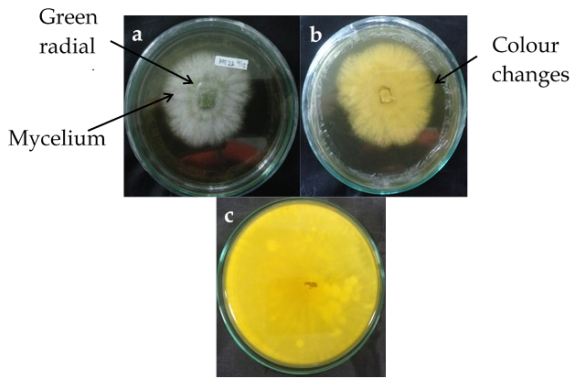

Figure 1. a) 4 days old of mycelium, b) 4 days old of reverse side, c) 14 days old of reverse side.

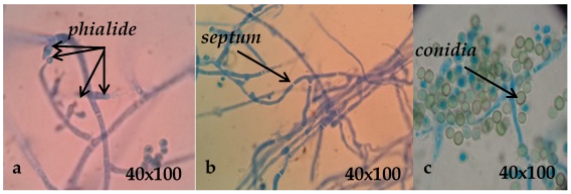

Figure 2. a) conidiophore, b) mycelium, c) conidia.

Fungal DNA amplification was done by using polymerase chain reaction (PCR) the quality of PCR product was checked using gel electrophoresis. The result of visualization of fungal DNA quality is shown by Figure 4.

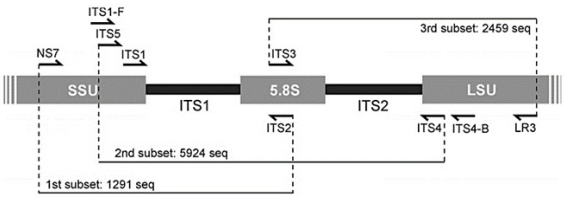

Figure 3. Diagram of the rDNA region of fungi and ITS primer (Source: Bellemain et al., 2010).

Figure 4 shows that the fungal DNA was successfully amplified in region between 500 to 1000 bp. This result is supported by the statement of Nilsson *et al.* (2009) who stated that the fungal DNA is normally obtainable in a single round of Sanger DNA sequencing in 650 bp region. PCR product was sequenced to get the sequence of fungal DNA, and then the sequence was compared to that of the database in gene bank using BLAS homology. According to fungal DNA comparison, fungus MT23 was 99% similar to *Trichoderma parareesei* ATCC MYA-4777 (Houseknecht *et al.*, 2011). Phylogenetic tree of fungus MT23 is shown by Figure 5.

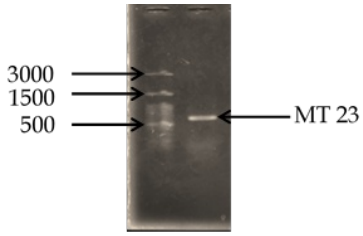

Figure 4. Visualization of DNA band of fungus MT23.

Result of morphology observation was supposed fungus MT23 was member of genus *Trichoderma* and supported by the molecular identification which successfully identified fungus MT23 as *T. parareesei*. Genus *Trichoderma* is well known as terrestrial fungus and give advantages for agricultural sector (Rinu *et al.*, 2013; Saravanakumar *et al.*, 2013; El Komy *et al.*, 2015; Hamed *et al.*, 2015). The finding of *T. parareesei* as marine sponge associated fungi showed that this fungus was a marine facultative (marine derived) fungi. Several species from *Trichoderma* such as *T. longibrachiatum, T. harzianum*, and *T. atroviride* were reported to tolerate to the increasing of salinity (Gal-Hemed *et al.* 2011). Genus of *Trichoderma* was also known to produced pigment and bioactive compounds (Rubeena *et al.*, 2013; Vacondio *et al.*, 2015; Benkada *et al.*, 2016).

Pigment extraction

Fungus MT23 changed the colour of MEA to yellow because it produced extracellular pigment. Extracellular pigments production is indicated by colour changing of the environment where the fungi live. Previous research successfully identified black extracellular pigment which produced by endophytic fungi as melanin having photoprotector activity (Sibero *et al.*, 2016). Fungus MT23 produced yellow pigment started at the fourth day of cultivation and

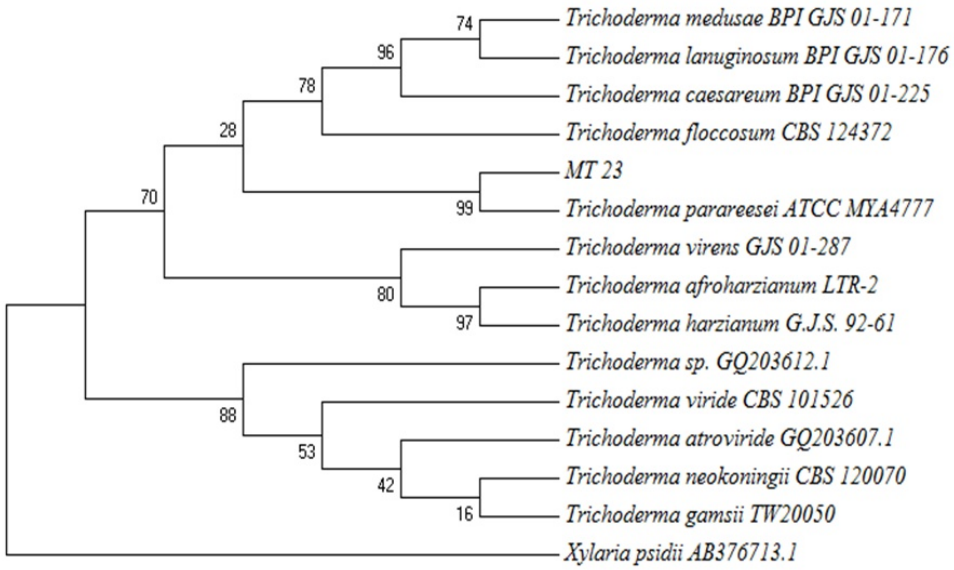

Figure 5. Phylogenetic tree of fungus MT23.

ready to be harvested on day fourteenth. Pradeep and Pradeep (2013) studied the optimum cultivation condition for pigment production of fungus *Fusarium moniliforme*. The fungus had highest pigment absorbance (AU 500nm) at temperature 28–30°C, pH 5.5, glucose as carbon source, peptone as nitrogen source and cultivated for 8 days. Yellow pigment from fungus MT23 were extracted using organic solvents. Environmental conditions have important role in fungi growth. Pradeep and Pradeep (2013) studied the optimum cultivation condition for growth of fungus *Fusarium moniliforme*. The fungus had highest biomass (g/L) at temperature 28°C, pH 5.5, glucose as carbon source, peptone as nitrogen source, KH_2PO_4 as source of salt and addition of methionine as additional amino acid. Stationary phase of this fungus was on eighth day of cultivation. In this study, we used Malt Extract Agar (MEA) from HiMedia M173 which contained malt extract and mycological peptone. Peptide and amino acid in peptone are easily metabolized by fungi which induced the production of fungal metabolites, including pigment (Calestino *et al.*, 2014). Da Costa Souza *et al.* (2016) used several media including malt extract to induced pigment production in filamentous fungi. As a result, pigment production in malt extract was higher than other medium. In addition, nutrient content in media can regulate the expression of genes to activate the metabolic path way for pigments production (Pradeep *et al.*, 2013).

Organic solvents such as n-hexane, ethyl acetate, chloroform, methanol and dichloromethane were usually used to extract natural pigments from microorganisms (Saravanan and Radhakrishnan, 2016; Weber *et al.*, 2016). In this research, n-hexane, ethyl acetate and methanol were used to extract fungal pigment. The purpose of this step was to know the best solvent to obtain highest fungal pigment yield. The result of pigment extraction is shown by Figure 6 and Table 1.

Table 1 shows that only methanol was able to extract fungal extracellular pigment from fungus MT23. Statistic analysis showed that solvents gave significant difference to the fungal pigment yield ($P<0.05$). The polarity of pigment will influence the suitability of

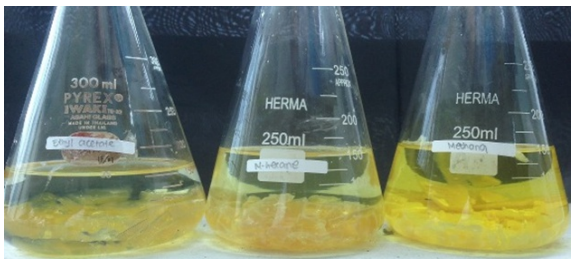

Figure 6. The result of maceration of agar media in organic solvents: a) in ethyl acetate, b) in n-hexane, c) in methanol.

Table 1. Fungal pigment yield with different solvents.

Solvent	Fungal pigment yield (%)
n-Hexane	0
Ethyl acetate	0
Methanol	6.26±0.29

optimum solvent to extract the pigment. Several pigments such as astaxanthin, chlorophyll a, chlorophyll b and xanthophyll were able to be extracted with methanol (Sasidharan *et al.*, 2013, Sumanta *et al.*, 2014; Bhat and Marar 2015).

Antibacterial and antifungal activities

Fungal pigments are known to produce antimicrobial activity. Geweely (2011) successfully investigated antimicrobial of several fungal pigments. The result showed that fungus *Aspergillus nidulans, Fusarium moniliforme, Penicillium purpurogenum*, and *Phoma herbarum* had weak to moderate antimicrobial activity against dermatophyte fungi, non dermatophyte fungi and several bacteria. Another research showed antibacterial activity of astaxanthin pigment from marine yeast (Ushakumari and Ramanujan, 2013). Fungal pigment from fungus MT23 was tested against several clinical pathogenic microorganisms. The result of antibacterial and antifungal is shown in Table 2.

According to Table 2, fungal pigment from MT23 showed weak antibacterial activity against clinical MDR bacteria *S. typhi* and *E. coli*. The biggest antibacterial activity showed at the concentration of 1000 µg/mL against *S. typhi*. The pigment didn't show any antifungal activity against clinical pathogenic fungi. The weak antimicrobial activity means that this fungal pigment is not potential as a source of antibiotic against clinical pathogenic and MDR microorganisms. Carotenoid is one of natural pigment which produced by fungi with yellow, orange to reddish colors. The production of this pigment is related to stress tolerance or with the synthesis of physiologically active by product (Avalos and Limȯn, 2015). Carotenoid pigment is mostly reported has antimicrobial and antioxidant potential (Ernawita *et al.*, 2016; Yoo *et al.*, 2016). The antimicrobial mechanisms of this pigment could lead to the accumulation of lysozyme enzyme that digest bacterial cell walls (Abu-Ghannam *et al.*, 2013).

Conclusions

Sponge associated-fungus isolate MT23 was successfully identified using morphological and molecular approaches as member of *Trichoderma* and had 99% similarity to *Trichoderma parareesei*. The fungal pigment could be extracted only in methanol with yield 6,22±0,29%. The pigment showed weak antibacterial activity against *S. typhi* and *E. coli* strain MDR, furthermore the pigment did not show any activity against clinical pathogenic fungi.

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Table 2. Antibacterial and antifungal activities of fungal pigment MT23.

Concentration (µg/mL)	Diameter of Inhibition Zone (mm)			
	Clinical MDR Bacteria		Clinical Pathogenic Fungi	
	S. typhi	*E. coli*	*M. furfur*	*T. rubrum*
50	0.00	0.00	0.00	0.00
125	0.00	0.00	0.00	0.00
250	0.00	0.00	0.00	0.00
500	1.13±1.00[a]	0.00	0.00	0.00
1000	4.03±0.06[b]	2.50±0.01[a]	0.00	0.00
Positive control	6.40±0.24[c]	11.58±0.00[b]	19.70±0.38	20.10±0.02
Negative control	0.00	0.00	0.00	0.00

Superscript shows statistically significant difference (P<0.05)

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