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Antimicrobial activity and GC-MS analysis of bioactive constituents of *Aspergillus fumigatus* 269 isolated from Sungai Pinang Hot Spring, Riau, Indonesia

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Abstract. *Octarya Z, Novianty R, Suraya N, Saryono. 2021. Antimicrobial activity and GC-MS analysis of bioactive constituents of* Aspergillus fumigatus *269 isolated from Sungai Pinang Hot Spring, Riau, Indonesia. Biodiversitas 22: 1839-1845.* A total of 16 isolates of thermophilic fungi originating from hot springs in Riau and West Sumatra have been tested for their antimicrobial ability against pathogenic microbes *Candida albicans*, *Staphylococcus aureus*, and *Escherichia coli*. The antimicrobial test was carried out by using the disk diffusion method. Molecular identification of the most potential isolate (LBKURCC269) was carried out by amplifying the ITS (Internal transcribed spacer) sequence on rDNA using universal primer ITS-4 and ITS-5. ITS sequence results showed that LBKURCC269 has a 99% similarity to *Aspergillus fumigatus*. Ethyl acetate extract of LBKURCC269 (*Aspergillus fumigatus 269*) showed good antimicrobial activities against three pathogenic microbes tested with the inhibition of 17 mm, 13 mm, and 13 mm against *Staphylococcus aureus*, *Candida albicans*, and *Escherichia coli*, respectively. There were 24 identified chemical compounds in ethyl acetate extract. The major compounds were eicosane, eicosane 2-methyl, phenol, 2,6-bis(1,1-dimethyl ethyl)-4-methyl, hexadecane 2, and 11-octadecenoic acid, methyl ester. These findings suggest that thermophilic fungi isolated from hot springs could serve as reservoirs for new bioactive compounds of industrial and medical importance.

Keywords: Antibiotic, *Aspergillus fumigatus*, bioactivity, GC-MS, thermophilic fungi

INTRODUCTION

In many ways, fungi are very important and have a positive value for nature and humans. One of the most important parts is that fungi are used in biotechnology to produce various metabolites with specific purposes, such as alcohol, steroids, alkaloids, enzymes, organic acids, amino acids, nucleic acids, and antibiotics (Adrio and Demain 2003)(Chambergo and Valencia 2016). Over the years, antibiotics effectively treat diseases caused by microbial infections, but recently, pathogenic microorganisms can adapt to antibiotics to become more resistant. Bacteria continuously develop resistance to the antibiotic by different internal methods (such as mutations) and external methods (such as exchanging the resistant genetic sequence between distinct types of bacteria (Abushaheen et al. 2020). One of the most dangerous pathogens is methicillin-resistant Staphylococcus aureus (MRSA). Cases of MRSA and other resistant pathogens continue to increase, such as vancomycin-resistant Staphylococcus aureus. It causes an increased need for new antibiotics every day (Scheffler et al. 2013). Various efforts to find new antibiotics to overcome microbial resistance need to be done. Classical methodologies for discovering natural microbial products mostly relied on isolates in the laboratory, followed by a Bioactivity-guided fractionation and identification of the purified compounds. The arrival of the genomic era meant a radical change in discovering new natural products (Chávez et al. 2015).

The emerging demand for antibacterial, antifungal, and anticancer bioactive compounds requires further research exploring rare microbes from unique and unexplored ecosystems. Hot springs have not been fully explored for thermophilic microbes that produce secondary metabolites. The hot springs contain thermophilic fungi that can produce various secondary metabolites that might have antimicrobial activity. About 515 fungal genomes exist in published databases, of which 16 come from fungi isolated from extreme environments. The successful discovery of bioactive compounds from fungi that live in extreme environments made significant contribution (Chávez et al. 2015). A thermophilic fungi *Aspergillus fumigatus* isolated from the soil produces antimicrobial compounds (Abdelkareem et al. 2017). Thermophilic fungus *Penicillium* isolated from hot springs in Saudi Arabia produce two new antimicrobial compounds in a medium containing rice, i.e., 3-(furan 12-carboxylic acid)-6-(methoxycarbonyl)-4-methoxy-4-methyl-4 and 5-dihydro-2H-pyran 3α-methyl-7-hydroxy-5-carboxylic acid methyl ester-1-indanone in a medium containing rice (Orfali and Perveen 2019).

Hot springs are a habitat for thermophilic microorganisms. Hot springs contain organic and inorganic chemicals that can support the growth of thermophilic microorganisms

such as fungi and bacteria. There is no information on the potential of fungi collected from hot springs in Riau and West Sumatra that produce antimicrobial bioactive compounds. Hot springs in Riau and West Sumatra have their geographic uniqueness so that it is possible to find microorganisms that produce antimicrobial bioactive compounds. West Sumatra is mountainous and hilly, and the geographical of Riau is lowland and swamp. These two areas have different characteristics but have many hot springs. Each thermophilic microorganism habitat has biotic and abiotic factors, such as extreme salt concentrations, pH, and temperature. Therefore, these microorganisms have different adaptability and produce different bioactive compounds (de Oliveira et al. 2015). Thermophilic microbes can produce antimicrobial bioactive compounds. Thermophilic bacteria from hot springs in Saudi Arabia produced cyclohexyl acrylate, imiloxan, tabtoxinine β-lactam, and filberton. A study by Alrumman et al. (2019) showed that 50 fungi isolates have forty bioactive compounds capable of fighting pathogenic microbes (Alrumman et al. 2019). In this study, we identified selected *Aspergillus fumigatus* 269 capable of producing antimicrobial bioactive compounds and identify the bioactive components using gas chromatography-mass spectrometry (GC-MS) analysis. All fungus isolates were obtained from Biochemistry Laboratory, Riau University, Indonesia.

MATERIALS AND METHODS

Antimicrobial screening of thermophilic fungi

Sixteen thermophilic fungi used in this study are the Biochemistry Laboratory collection, Riau University, with the code of LBKURCC. All fungi were isolated from various hot springs in Riau and West Sumatra. One gram of sediment sample was taken and suspended into 10 mL sterile distilled water. An amount of 1 mL soil suspension was taken and poured into sterilized potato dextrose agar (PDA). All inoculated plates were incubated at 45°C for 8 days (Abdelkareem et al. 2017). All of the fungi have not been identified yet (Table 1).

Antimicrobial assays were carried out by primary and secondary screening tests against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. The microbial isolates used in this study were the Biochemistry Laboratory Culture, University of Riau. Microbia 1 isolates were grown aerobically at 37°C for 24 hours. Sterilized PDA (20 mL) was allowed to solidify and then inoculated with 100 µl of *Candida albicans* and allowed to stand for 30-60 minutes. Sterilized Nutrient Agar (NA) 20 mL was allowed to solidify and then inoculated with 100 µl of *E coli* and *Staphylococcus aureus* and allowed to stand for 30-60 minutes. In primary screening, the inhibitory potential of fungal was screened and determined by the cross-streak method. Secondary screening of cultivated broth was tested against microbe by paper disk (6 mm), which was given Cell-Free Extract (CFE) of the fungus and crude ethyl acetate extracts. CFE was obtained by centrifugation of liquid fungal cultures at 10.000 rpm for 20 min.

Table 1. Thermophilic fungal isolate codes and their regions of origin

Origin	Number of isolates	LBKURCC code
Sungai Pinang	8	265, 266, 267, 268, 269, 270, 271, 272
Bukik Kili	2	282, 283
Padang Gantiang	2	273, 274
Bukik Gadang	4	278, 279, 280,281

The inhibitory zone diameter around the discs was measured after 24 hours incubation at 37°C (Balouiri, Sadiki, and Ibnsouda 2016). Ampicillin (100µg/mL) was used as a positive control for antibacterial activity, and nystatin (100µg/mL) was a positive control for antifungal activity. The negative control was the growth medium. The assay was carried out in triplicate.

Cultivation and extraction

Fungal isolates were grown in a500 mL conical flask containing 200 mL Potatoes Dextrose Broth on an orbital shaker (150 rpm) at 45°C temperature. The culture filtrate was taken after 8 days of incubation. The bioactive compounds of the promising fungal isolates were extracted with ethyl acetate at the same volume (v/v) of the solvent and culture filtrate. The extraction was carried out in the separating funnel. The mixture of the filtrate with organic solvents appears in two layers, an organic layer containing secondary metabolites and an aqueous layer. Ethyl acetate extract was concentrated with a rotary evaporator (IKA Rotary Evaporator model) at 60°C, and the concentrated extract was stored at 4°C for antimicrobial testing (Al-Dhabi et al. 2016).

Molecular identification of LBKURCC269 thermophilic fungal isolate

The thermophilic fungal mycelia (24 hours old) was scraped off as much as 0.3 g and put into an Eppendorf tube. Fungal DNA was isolated by using a modified Promega Wizard Genomic DNA Purification Kits. The PCR (Polymerase Chains Reaction) reaction was carried out by mixing 1 µL of isolated DNA and 3 µL $MgCl_2$, 10 µL ITS 4 primer(TCC TCC GCT TAT TGA TAT GC) and ITS 5 (GGA AGT AAA AGT CGT ACA AGG) (Raja et al. 2017), 0,125 µL Taq polymerase, 10 µL buffer, dan 5 µL dNTPs. PCR reactions were carried out in 35 cycles with a reaction program of 1.5 minutes denaturation at 94°C, 1 minute of annealing at 47°C, 3 minutes of extension at 72°C, and followed by a final extension of 3 minutes at 72°C. 6 µL of PCR samples were analyzed using 0.32 g of agarose dissolved in 40 mL of 1 X TAE buffer, then added 4 µL of red Gel. Gel electrophoresis was run at 110 volts for 1 hour. The electropherogram was viewed and photographed under UV light in the Gel documentation system. Bands of the isolate were compared to the 1 kb DNA standard to verify PCR results. PCR products are purified and then sequenced using 2 primers

(ITS 4 and ITS 5). Sequencing data analysis was carried out using the BioEdit software program. Sequence alignment analysis was carried out by comparing the sequences obtained from sequencing with those already in GeneBank using BLAST (Basic Local Alignment Search Tool) on the site http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were determined using the MEGA6 program.

GC-MS (Gas Chromatography-Mass Spectroscopy)

Ethyl acetate fraction was subjected to GC-MS analysis, and the conditions used for the GC-MS analysis are presented in Table 2. The mass spectra of the chemical components were compared to the known chemical compounds in the National Institute Standard and Technology (NIST) library (Chaudhary and Tripathy 2015).

Table 2. GC-MS condition

GC program	
Column	Rtx 5 (fused silica), 30 m × 250 µm × 0,25 µm.
Equipment	GCMS-QP2010S SHIMADZU
Carrier gas	Helium gas 0,5 mL/min
Pressure	13.7 kPa
Detector	Mass detector
Sample injection	3 µl
Column Oven temperature	70°C
Injection temperature	300°C
Total GC run time	80 min
MS program	
Inlet line temperature	250°C
Source temperature	300°C
Electron energy	70 Ev
Mass scan m/z	28-600 amu

RESULTS AND DISCUSSION

Antimicrobial test of thermophilic fungal isolates

In this study, the antimicrobial screening was carried out 16 thermophilic fungi isolates living at 45°C. The antimicrobial activities varied, ranging from none to good antimicrobial activity. Antimicrobial activity categorized based on the diameter of the inhibitory zone, - (none) no activity, +/- (very low activity) 7-9 mm, + (moderate activity) 9.1-12 mm, and ++ (good activity) 12.1-21 mm (Aka-Gbezo et al. 2018), (Rajashree and Borkar 2018). Previously known that antibacterial and antifungal activities of *Streptomyces Al-Dhabi-2* inhibited the growth of tested microbes in the streak method (Al-Dhabi et al. 2019). Al-Dhabi et al (2016) reported that thermophilic isolates were isolated from the Tharban hot spring of Saudi Arabia. The isolates were showed antimicrobial activity against pathogenic microbial. In primary screening, the strains LBKURCC269 and LBKURCC 272 exhibited good activity against pathogenic microbes (Table 3). It can be seen that there are fungal isolates from hot springs that have antimicrobial properties, so they have the potential to be used as a source of antibiotics. All thermophilic fungal inhibit the growth of *Candida albicans* and *Escherichia coli*, except LBKURCC272 inhibits *Candida albicans*. The strain LBKURCC269 has good antimicrobial activity against *E. coli* (Figure 1c), and LBKURCC272 is potential against *C. albicans*. Fungal isolates with moderate activity against *S. aureus* were LBKURCC269 and LBKURCC281. The LBKURCC282 and LBKURCC283 isolates also can inhibit it less than LBKURCC269 and LBKURCC281. Fungi are the primary producers of bioactive compounds, with around 42% and 60% from plants (Scheffler et al. 2013). The highest antimicrobial activity based on preliminary screening was the LBKURCC269 isolate originating from the Sungai Pinang's hot water. LBKURCC269 has good activity inhibition against pathogenic microbial and the most potential because it can inhibit more than one pathogen. Based on the result of preliminary screening, further studies have been conducted in the secondary screening.

Table 3. Antimicrobial activity of LBKURCC thermophilic fungi against *C. albican, S. aureus,* and *E. coli*

Isolate number	Inhibition		
	Candida albicans	***Staphylococcus aureus***	***Escherichia coli***
Sungai Pinang			
LBKURCC265	+/-	-	+
LBKURCC266	+/-	-	+
LBKURCC267	+/-	-	+
LBKURCC268	+/-	-	+
LBKURCC269	+	+	++
LBKURCC270	+/-	-	+
LBKURCC271	+/-	-	+
LBKURCC272	++	-	-
Bukik Kili			
LBKURCC282	+	+/-	+
LBKURCC283	+	+/-	+
Padang Gantian			
LBKURCC273	+	-	+
LBKURCC274	+	-	+
Bukik Gadang			
LBKURCC278	+	-	+
LBKURCC279	+	-	+
LBKURCC280	+	-	+
LBKURCC281	+	+	+

Note: -: none, +/-: very low activity, +: moderate activity, ++: good activity

LBKURCC269 is a fungal isolate that changes colour when grown on PDA media depend on the length of incubation time. The initial colour was white, turn to turquoise, dark green, and finally blackish-gray, as seen in Figures 1 (a) and (b). This fungus has more dense hyphae in the centre. Changes in color and morphological characteristics of the LBKURCC269 fungus are similar to Aspergillus. Ethyl acetate extract was assayed for antimicrobial activity. Ethyl acetate is a semi-polar solvent so that it can attract polar compounds, has low toxicity and is quickly evaporated so that it can be used for the

extraction of semi-polar fungal bioactive compounds. The incubation time was adjusted to the optimum time for antibacterial production from the fungus *Aspergillus fumigatus* isolated from the soil on synthetic broth media (Abdelkareem et al. 2017). The ethyl acetate extract was tested against pathogenic microbes using the disk diffusion method (Table 4). Inhibitory zone of cell-free extract and ethyl acetate extract of LBKURCC269 against *C. albicans* was 15 mm and 13 mm, respectively. The highest inhibition zone (17 mm) of LBKURCC269 ethyl acetate extract was against *S. aureus*. Previously reported that an inhibition zone significantly smaller than the control. The detected activity can be considered very promising since disk diffusion assay is carried out in an aqueous environment. The tested samples (crude ethyl acetate extracts) are poorly hydrosoluble (Takahashi et al. 2008). Ethyl acetate is also used to extract antimicrobial compounds from 2 species of Penicillium thermophilic fungal in Saudi Arabia (Orfali and Perveen 2019). Several solvents were used to extract antimicrobial compounds from the thermotolerant bacterium *Bacillus subtilis* KFSB5 isolated from compost soil, and the result showed that ethyl acetate extract had the highest activity against *C. albicans* (Kanse, Kadam and Dnyanoba 2014). The results showed that cell-free extract and ethyl acetate extract of LBKURCC269 were able to inhibit pathogenic microbes characterized by the presence of a clear zone (Figure 2).

Fungal identification and phylogenetic analysis

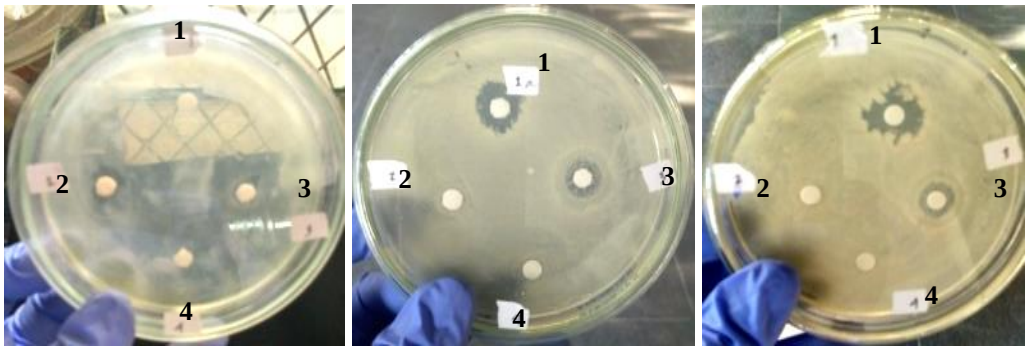
In this study, it was found that the PCR annealing reaction for LBKURCC269 isolate was 47°C. The ITS-1 and ITS-2 rDNA regional sequencing produced 613 bp (Figure 3). LBKURCC269 fungal isolate was identified using ITS (Internal Transcribed Spacer) sequences in the ribosomal DNA region which is one way to identify the current fungus. The internal transcribed spacer (ITS) region can be easily amplified with a universal primer that fits almost all fungal species. The ITS area was used as a molecular marker because it shows genetic changes in fungi and serves as one of the phylogenetic classification bases (de Oliveira et al. 2015). DNA isolates of LBKURCC269 were amplified according to the primer position map state by White et al, (1990), that the region ITS-1and ITS-2 use ITS-4 and ITS-5 as a pair primer.

Table 4. The diameter of the inhibitory zone of LBKURCC269 fungal ethyl acetate extract

Sample	Diameter of the inhibition (mm)		
	Candida albicans	***Staphylococcus aureus***	***Escherichia coli***
Cell-free extract	15,3	11,7	12,3
Ethyl acetate extract	13	17	13
Nystatin	17,3	-	-
Ampicillin	-	20	14,6
Negative control	-	-	-

A **B** **C**

Figure 1. A. LBKURCC269 fungus 24 hours, B. LBKURCC269 fungus 8 days, C. LBKURCC269 clear zone against *E. coli*



A **B** **C**

Figure 2. Antimicrobial test of LBKURCC269 ethyl acetate extract against pathogenic microbes: A. *Candida albicans*, B. *Staphylococcus aureus*, C. *Escherichia coli*. 1. Positive control, 2. Cell-free extract, 3. Ethyl acetate extract, 4. Negative control

A phylogram of LBKURCC269 thermophilic fungi was obtained by the N-J Tree method with 10.000 bootstrap replicates. Based on the homology of nucleotide sequences in Genbank and phylogram with 15 strains, thermophilic fungi LBKURCC269 isolate had the closest relationship with *Aspergillus fumigatus strains IHEM19376* and *Aspergillus fumigatus ATCC1022* (Figure 4) with a branching rate of 10.000. *Aspergillus fumigatus* has also been isolated from hot water on the shores of Lake Bogoria in Kenya. These isolates can produce beneficial enzymes for biotechnological applications (Odilia et al. 2018). Moreover, *Aspergillus fumigatus* isolated from Sudanese Indigenous soil can produce antibacterial compounds using different growth parameters (Abdelkareem et al. 2017).

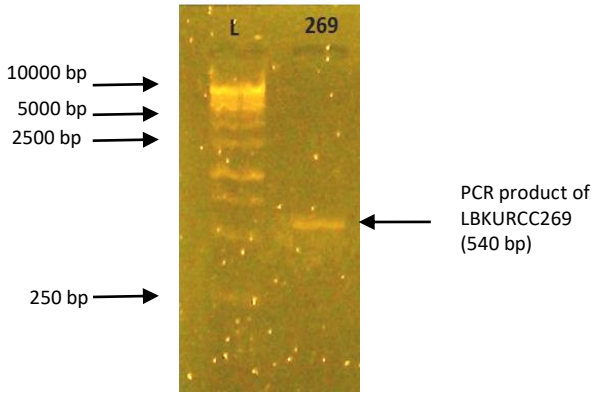


Figure 3. PCR amplification product of the LBKURCC269 isolate. L: leader as standard, 269: DNA band of LBKURCC269 isolate

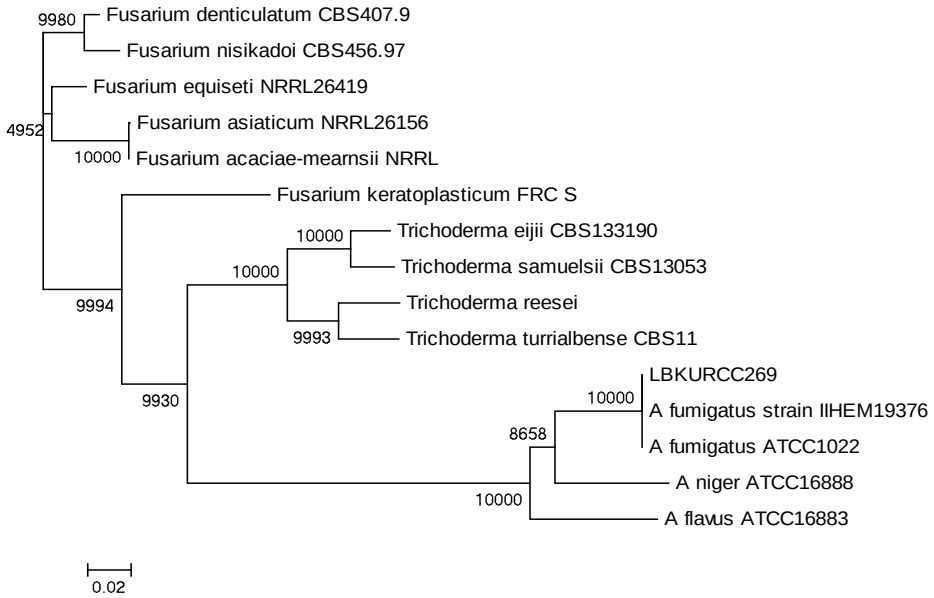


Figure 4. Phylogenetic tree of LBKURCC269 isolates and other closest species. The tree was by Neighbor-joining analysis based on 18S rDNA sequences

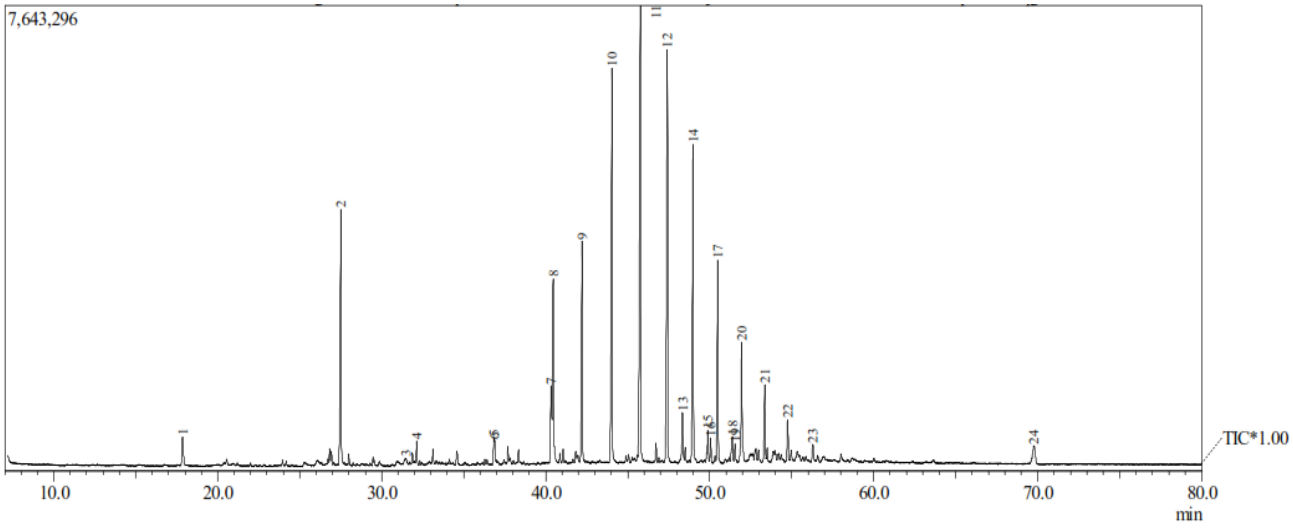


Figure 5. GC-MS chromatogram of the ethyl acetate extract of *Aspergillus fumigatus 269*

Table 5. Identified chemical compounds in the ethyl acetate extract of *Aspergillus fumigatus 269* by GC-MS analysis

Compounds	Chemical formula	Molecular weight	RT (min)	Area (%)	Similarity index (%)
Dodecane	$C_{12}H_{26}$	170	17.848	1.39	96
Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl	$C_{17}H_{27}NO_2$	277	27.504	8.06	78
9-octadecenal	$C_{18}H_{34}O$	266	31.480	0.81	86
Hexadecane 1	$C_{16}H_{34}$	226	32.128	0.75	93
Decane, 2,3,6-trimethyl	$C_{13}H_{28}$	184	36.835	0.65	89
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	36.880	0.69	85
Dodecane, 2,6,11-trimethyl	$C_{15}H_{32}$	212	40.329	2.70	90
11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	40.449	6.05	93
Hexadecane 2	$C_{20}H_{42}$	226	42.217	6.23	96
Eicosane 1	$C_{20}H_{42}$	282	44.037	12.33	97
Eicosane 2	$C_{20}H_{42}$	282	45.766	15.33	97
Eicosane 3	$C_{20}H_{42}$	282	47.409	13.14	96
Octadecane,2-methyl	$C_{19}H_{40}$	268	48.337	1.79	92
Eicosane,2-methyl	$C_{20}H_{42}$	296	48.983	9.82	96
Heptadecane,2-methyl	$C_{18}H_{38}$	254	49.887	1.17	93
Eicosane.3-methyl	$C_{21}H_{44}$	296	50.057	0.70	93
Nonadecane,2-methyl	$C_{20}H_{42}$	282	50.492	5.97	97
Tetradecane	$C_{14}H_{30}$	198	51.379	1.08	93
Pentadecane	$C_{15}H_{32}$	212	51.548	0.70	92
Nonadecane,2-methyl	$C_{20}H_{42}$	282	51.948	4.35	96
Pentacosane	$C_{25}H_{52}$	252	53.356	2.44	95
Triacontane	$C_{30}H_{62}$	422	54.752	1.61	94
Octadecane	$C_{18}H_{38}$	254	56.285	0.82	94
Propane,2-(1,1 dimethyl ethyl)sulfonil	$C_{18}H_{18}O_2S$	178	69.761	1.41	76

The chemical compounds in ethyl acetate extract of *Aspergillus fumigatus 269* were identified by using a GC-MS analysis (Figure 5). The major compounds of the extract were phenol 2, 6-bis(1,1-dimethyl ethyl)-4-methyl (8.06%), 11-octadecenoic acid, methyl ester (6.05%), hexadecane (6.23%), eicosane 1 (12.33%), eicosane 2 (15.33%), eicosane 3 (13.14%), eicosane,2-methyl (9.82%), nonadecane, 2-methyl (5.97%) and other minor compounds were also presented in Table 5. The various chemical compounds may contributed to antimicrobial activity. Dodecane (1.39%) with retention at 17.848 min were reprted to have antibacterial activity (Togashi et al. 2007). Retention time at 44 to 47 min corresponds to the compounds eicosane 1, 2, and 3 with peak area 12.33%, 15.33%, and 13.14%. Alkane such as eicosane has also been reported to have antibacterial activities (Boussaada et al. 2008) and antifungal activities (Ahsan et al. 2017). Several other compounds such as dodecane, 2,6,11-trimethyl (2.70%), hexadecanoic acid, methyl ester (0.69%), and 11 octadecanoic acids, methyl ester (6.05%) were reported to have antimicrobial activity (Nahid Rahbar 2012). Hexadecanoic acid extracted with ethyl acetate can also inhibit Staphylococcus aureus. This compound was isolated from Penicillium crustosum which lives in the sea (Amer et al. 2019).

Previous studies reported that some of the antibiotic compounds had been identified from the fungus *A. Fumigatus*, i.e., fumagillin. Fumagilin is a monobasic acid containing 4 conjugated double bonds (Hicham et al. 2008); and helvaholic acid (Laureti et al. 2011). *A. fumigatus* also produce secondary metabolites of indole alkaloid, i.e., fumigaclavines and fumitremorgens which have mycotoxins activity (Keller et al. 2005). *A.fumigatus* is a filamentous fungus that live anywhere, especially in garbage piles that contain a lot of organic material. A study by Svahn et al. (2012) showed filamentous fungi isolated from rivers with high antibiotic content were dominated by *Aspergillus spp*. They have antimicrobial properties against pathogenic bacteria and fungi, and one of which is *Aspergillus fumigatus* which produce gliotoxin metabolites (Svahn et al. 2012). The discoveries of the antimicrobial compounds from *A. Fumigatus 269* will increase the finding of this type fungus in hot springs.

Sixteen thermophilic fungi have been isolated from hot springs in West Sumatra and Riau, Indonesia. LBKURCC269 is the most potential against 3 pathogenic microbes (*Candida albicans, Staphylococcus aureus, and Escherichia coli*). The results of the sequencing analysis showed that this isolate was 99% similar to *Aspergillus fumigatus*. The ethyl acetate extract which have antimicrobial properties contain phenol 2, 6-bis(1,1-dimethyl ethyl)-4-methyl (8.06%), 11-octadecenoic acid, methyl ester (6.05%), hexadecane (6.23%), eicosane 1 (12.33%), eicosane 2 (15.33%), eicosane 3 (13.14%), eicosane,2-methyl(9.82%), nonadecane, 2-methyl (5.97%). There is a need to isolate bioactive compounds' antimicrobials that might be used to overcome pathogenic microbial infections.

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