ISOLATION AND ANTIBACTERIAL ACTIVITY OF SOIL-DERIVED FUNGI FROM TAMAN BOTANI NEGARA, SHAH ALAM, MALAYSIA

Marlia Singgih Wibowo*, Elin Julianti, Muhammad Daniaal Radzali

Author Information

Department of Pharmacochemistry, School of Pharmacy, Institut Teknologi Bandung
Jl. Ganesha 10 Bandung 40132-Indonesia

***Corresponding Author**
Marlia Singgih Wibowo

E-mail:
marlia@fa.itb.ac.id

ABSTRACT

Fungi are eukaryotic organisms that consist of unicellular organisms, namely molds and yeasts, and multicellular organism known as mushrooms. In the medical field, fungi have a significant contribution as they are widely used as sources for discovering a lot of novel antibiotics. As the preliminary study, this paper presents the isolation of soil-derived fungi from Malaysian forest as resources for finding the new antibiotics and their test of antibacterial activity against *Bacillus subtilis* and *Escherichia coli*. The fungi their selves were isolated by using Sabouraud dextrose agar (SDA) medium. The pure fungi isolates were screened for their antibacterial activity by using disk diffusion method. The active fungi were fermented in Sabouraoud dextrose broth (SDB) medium for 21 days. Culture media and mycelium were separated by filtration method. The culture broth was extracted by liquid-liquid extraction and mycelium was extracted by maceration method using ethyl acetate. The antibacterial activity of the dried extracts was determined by using the microdilution method. The isolation step resulted in five fungal strains coded S1-S5. The antibacterial assay showed that the extract of fungal broth medium of S3 had the highest antibacterial activity against *Bacillus subtilis* with MIC value of 64 µg/mL and S1 against *Escherichia coli* with MIC value of 32 µg/mL. Based on these MIC values, these can be classified as significant antibacterial activities as well. Thus, these extracts could be potentially useful for the development a new therapeutic agent bacterial infections.

Keywords: antibacterial activity, *Bacillus subtilis, Escherichia coli*, fungi, isolation.

ISOLASI DAN AKTIVITAS ANTIBAKTERI DARI JAMUR ASAL TANAH TAMAN BOTANI NEGARA, SHAH ALAM, MALAYSIA

ABSTRAK

Jamur merupakan organisme eukariotik yang terdiri dari organisme uniseluler yaitu kapang dan ragi, dan organisme multiseluler yang dikenal sebagai jamur. Jamur memiliki kontribusi yang besar terhadap bidang kesehatan karena merupakan sumber yang banyak digunakan dalam pencarian kandidat antibiotik baru. Tujuan dari penelitian ini adalah mengisolasi jamur yang berasal dari tanah hutan Malaysia sebagai sumber pencarian antibiotik baru dan menentukan aktivitas antibakterinya terhadap *Bacillus subtilis* dan *Escherichia coli*. Isolasi jamur dari sampel menggunakan media agar Sabouraud dextrose (SD). Skrining aktivitas antibakteri dilakukan terhadap isolat jamur murni dengan metode difusi agar. Isolat jamur yang aktif selanjutnya di fermentasi dengan medium cair SD selama 21 hari. Kultur media dan miselia dipisahkan dengan menggunakan metode filtrasi. Bagian kultur media di ekstraksi dengan ekstraksi cair-cair (ECC) sedangkan bagian miselium diekstraksi dengan cara maserasi menggunakan pelarut etil asetat. Ekstrak kering diuji aktivitas antimikrobanya terhadap bakteri uji dengan metode mikrodilusi. Hasil dari penelitian ini diperoleh lima strain jamur yang diberi kode S1-S5. Hasil uji aktivitas antibakteri menunjukkan bahwa ekstrak kultur media jamur S3 mempunyai aktivitas antibakteri yang paling tinggi terhadap *Bacillus subtilis* dengan konsentrasi hambat minimum (KHM) sebesar 64 µg/mL dan jamur S1 terhadap *Escherichia coli* dengan KHM sebesar of 32 µg/mL. Berdasarkan nilai KHMnya kedua jamur tersebut diklasifikan mempunyai aktivitas antibakteri yang signifikan. Ekstrak jamur tersebut berpotensi berguna untuk pengembangan senyawa terapetik yang baru untuk melawan infeksi bakteri.

Kata kunci: aktivitas antibakteri**,** *Bacillus subtilis, Escherichia coli,* isolasi, jamur

Introduction

Majority of fungi produces secondary metabolites which may be beneficial towards pharmaceutical chemist as these metabolites are widely used in medicine such as the development of antibiotics, anti-fungal, and anticholesterol (Zeilinger *et al*. 2015). For instance, the first well-known antibiotic produced, penicillin, was obtained from the fungi of *Penicillium notatum*. This discovery was made by Sir Alexander Fleming in 1928. There are hundreds of thousand species of fungi that have been identified. Also, more than millions may exist (Madigan *et al*. 2012).

One of the prolific sources of fungi is soil. Majority of forest soil community is dominated by fungi. On account of organic compounds in forest soil, fungi species can extend their filaments known as hyphae. A network of hyphae, known as mycelium, is used to absorb nutrients from the ground. Plenteous soil-derived fungi contain hyphae for growth.

This paper focuses on isolation of the soil-derived fungi from Malaysian forest as resources for new antibiotics. Moreover, the investigation of their antibacterial activity against *Bacillus subtilis* and *Escherichia coli* through determination the minimum inhibitory concentration (MIC) is presented in this paper.

Materials and Methods

Sample Collection and Preparation

The forest soil was collected in an opened area in Taman Botani Negara, Shah Alam, the state capital of Negeri Selangor Darul Ehsan, Malaysia. The coordinates, latitude, and longitude, of soil collection, are 3° 5'46.20"N and 101°30'42.73"E respectively. The collected soil was placed in a plastic container and kept in a freezer until the start of experimentation.

Isolation and Purification of Soil Derived Fungi

An amount of 1 gram soil sample was weighed and transferred into a centrifuge tube containing 10 mL of sterile water. The mixture was mixed for 1 minute. Then, 1 mL of the mixture was taken and transferred into a test tube containing 9 mL of sterile water. The mixture was gently shaken until become homogenous. From this mixture, 1 mL was taken and transferred into a new test tube containing 9 mL of water. The mixture was stirred gently until homogenous. Then, 1 mL of this mixture was transferred into a sterile petri dish. Lastly, 25 mL of sterile SDA was added into the petri dish and was shaken gently to ensure homogeneity. Once the medium solidified, the petri dish was labeled and incubated at 20^0C. Fungi grew after 3 to 4 days. The growing fungal was carefully transferred to a new media and repeated until getting a pure culture. Each pure fungal strain was observed under a microscope.

Screening of Antibacterial activity of Pure Fungal Isolates against Test Bacteria

Each pure fungal strains were cut to a size of 1 cm × 1 cm. These fungal strains were then placed on a surface of MHA containing 100 µL bacterial suspensions and incubated at 37 ^{0}C for 24 hours. Each fungal strain was observed its capability to inhibit the bacterium growth by measuring the zone of inhibition produced.

Fermentation of Fungal Strains

Each pure fungal isolates were cut to a size of 1 cm × 1 cm and were transferred to a 250 mL Erlenmeyer flask. Sterile SDB with the volume of 100 mL was added into each Erlenmeyer flask to undergo a fermentation process. The mouth of Erlenmeyer flask was covered with cotton which was wrapped with a sterile gauze pad. Fermentation was carried out at room temperature and was placed on a shaker for 21 days.

Extraction

After 21 days of fermentation, the medium was filtered under vacuum using Buchner funnel to separate the mycelium residue and the media culture. The mycelium residue was macerate for 48 hours in 100 mL of EA. On the other hand, the medium culture was extracted using liquid-liquid extraction (LLE) for 3 times using 300 mL of ethyl acetate. The solvent was removed under vacuum (150 mbar) by using a rotary evaporator until an

oily residue was formed. The residue was transferred into an empty vial that was weighed. The extract was then dried at room temperature for 24 hours. After a day, the weight of the dried extract formed was measured.

Minimum Inhibitory Concentration (MIC) Assay

The Minimum Inhibitory Concentration was determined using microdilution method (CLSI 2010). The dried extract was dissolve using methanol to achieve a stock concentration of 2048 µg/mL. Each well of the microplates was filled with 100 µL MHB. Extract with the volume of 100 µL and concentration of 2048 µg/mL was added in well number 12. A series of dilution was done by pipetting 100 µL of the mixture from well number 12 to well number 3. From well number 3, 100 µL of mixtures was discarded. Then, the bacterial suspension was added from well number 2 to well number 12. From this series of dilution, well number 1 and 2 shows negative and positive control respectively. Also, the highest concentration of extract was 1024 µg/mL, located on well number 12, while the lowest concentration of extract was 2 µg/mL, found on well number 3. The microplate was incubated at 37 ^{0}C for 24 hours. The minimum inhibitory concentration was measured by observing the turbidity of the mixture after incubation.

Results and Discussion

The morphological characteristic of each pure fungal strain was observed and identified. Some features such as the color, surface, form, elevation, and margin were observed and recorded. The result of morphological characterization was shown in Table 1. Microscopic analysis was done to further analyze the morphological characteristics of each fungal colony, such as the types of spores, hyphae, and mycelium. The results of fungi hyphae observation showed that pure fungal colonies of SA 4 and SA 5 contained septate hyphae, whereas SA 2 contained aseptate hyphae. Different results on the fungal colonies of SA 1 and SA 3, it could not be observed any hyphae on them. The results of spore observation showed that the spores of pure fungal colonies SA 1 and SA 3 were blastospore, whereas pure colonies of SA 2, SA 4, and SA 5 were conidia as illustrated in Figure 2. Therefore, based on the morphological characteristic, the suggested genus of SA 1, SA2, and SA 5 were *Aspergillus*. On the other hand, the proposed genus of SA 3 and SA 4 were *Penicillium* and *Rhizopus* respectively as tabulated in Table 2. In this case, the molecular biological identification is necessary to confirm their species.

Table 1. Morphological Characteristic of Pure Fungal Strain (Leung and Liu 2005).

Fungal Isolate	Colour	Surface	Form	Elevation	Margin
SA 1	Yellowish green with white edges	Cotton-like	Irregular	Convex	Filiform
SA 2	Light green	Cotton-like	Irregular	Raised	Filiform
SA 3	Greenish blue with white edges	Sand-like	Circular	Convex	Entire
SA 4	White	Smooth	Circular	Raised	Entire
SA 5	Black	Rough	Irregular	Raised	Undulate

Each fungal isolates was tested its capability to inhibit the growth of bacterium surrounding the fungus. Two types of bacteria, gram positive and gram negative, used in this analysis were *B. subtilis* and *E. coli*, respectively. The fungi that exhibited a diameter of zone inhibition, namely S1, S3, and S4, (as shown in Figure 3, Table 3) proceeded towards fermentation process to produce their secondary metabolites.

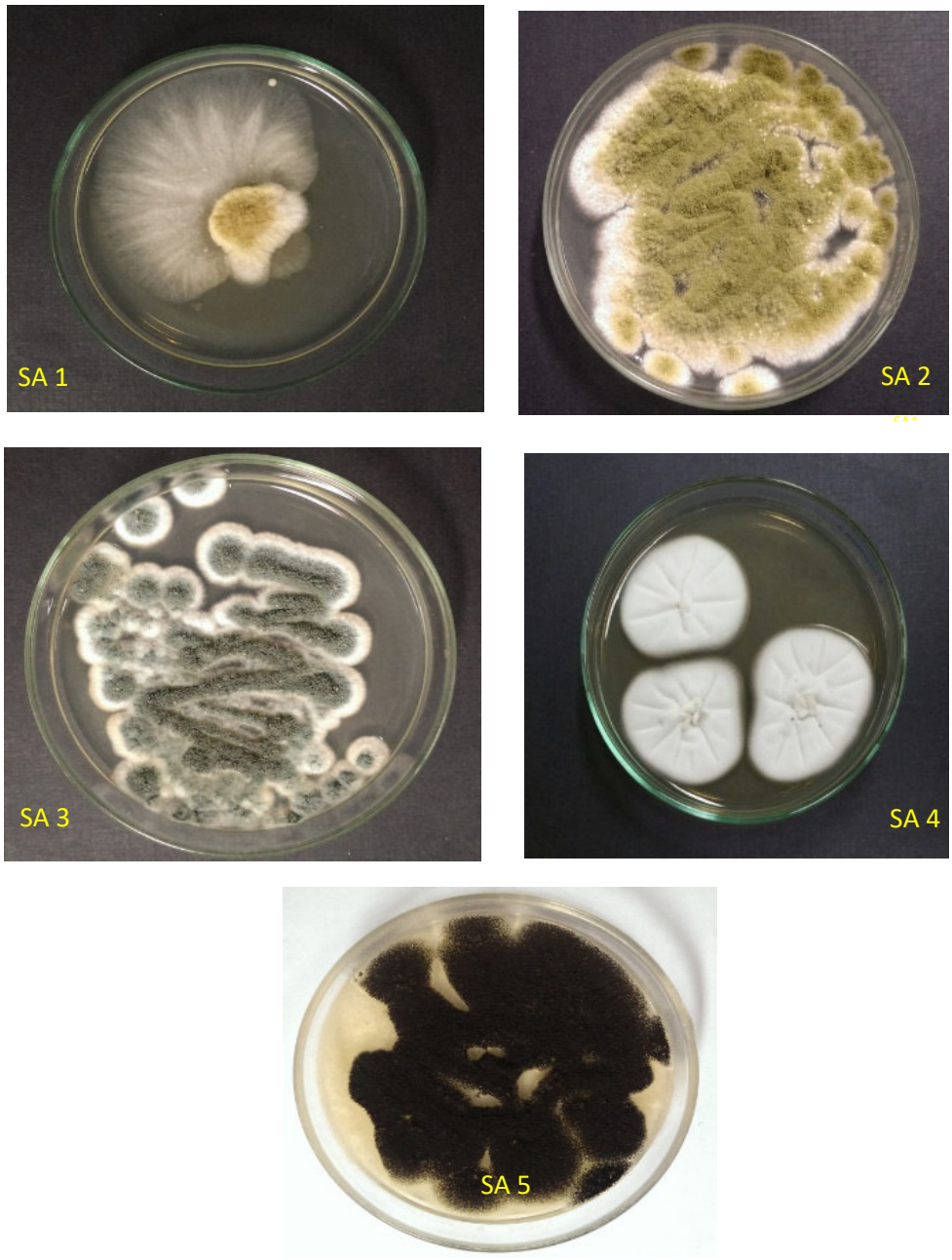

Figure 1. Pure Isolated Colonies of Fungi.

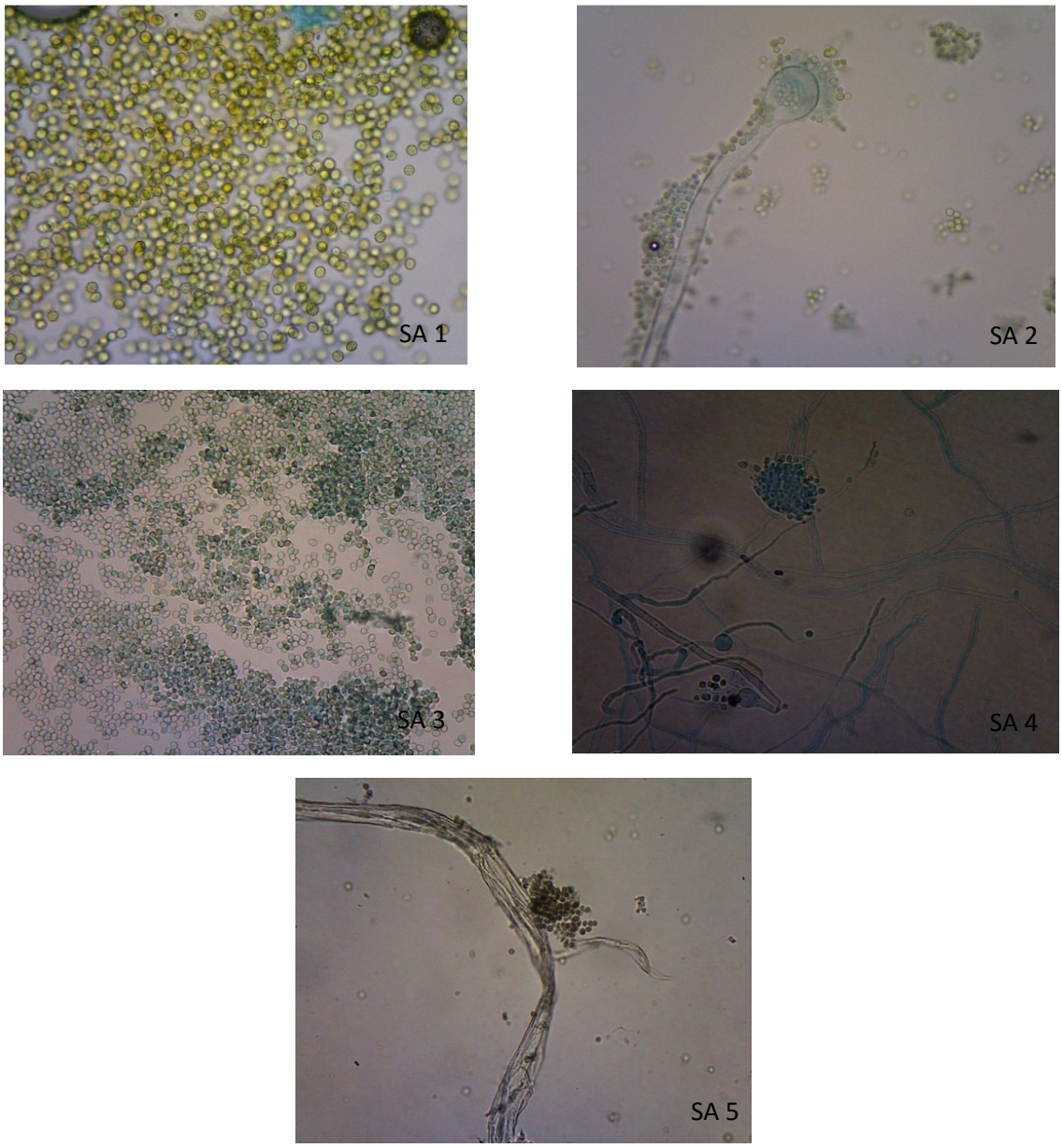

Figure 2. The Microscopic Observation of Pure Fungal Isolates, Magnification 100x.

Table 2. The Microscopic Characteristic of Pure Fungal Strain (Leung and Liu 2005).

Fungal Isolate	Spore	Hyphae and mycelium
SA 1	Blastospore	-
SA 2	Conidium	Aseptate
SA 3	Blastospore	-
SA 4	Conidium	Septum
SA 5	Conidium	Septum

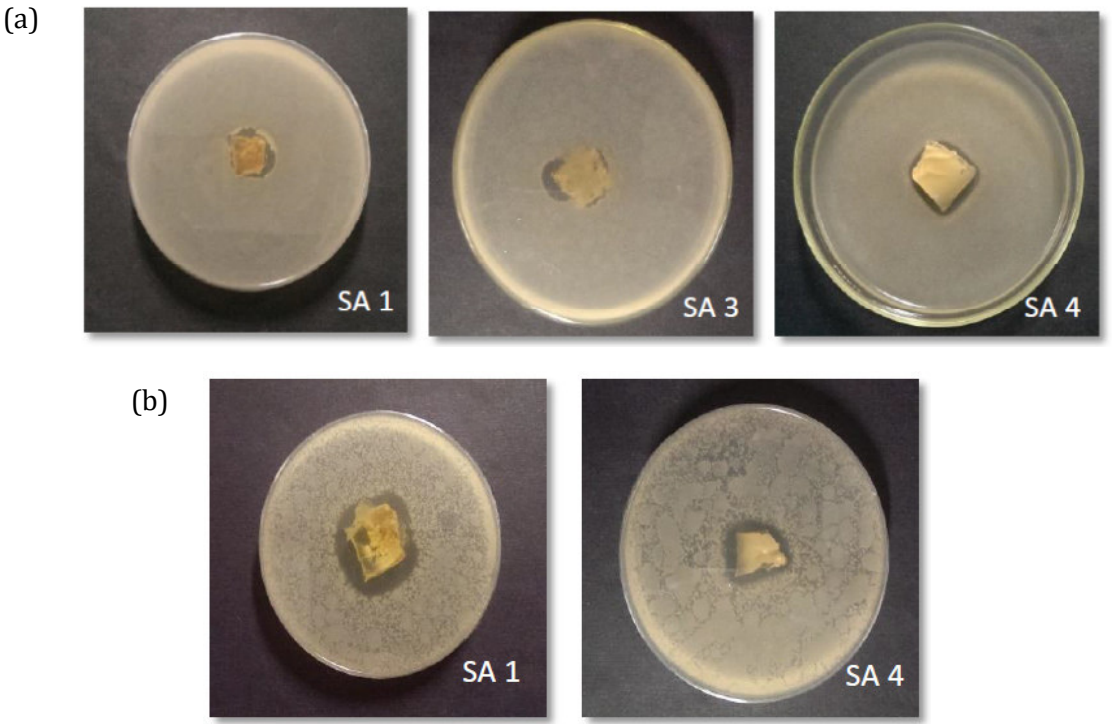

Figure 3. Zone of Inhibition Produced by Pure Fungal Colony Against *B. subtilis* (a), *E. coli* (b).

Table 3. Inhibition Zone Produced by The Pure Fungal Isolates Against Test Bacteria.

Test bacteria	Inhibition Zone (mm)				
	SA1	SA2	SA3	SA4	SA 5
Bacillus subtilis	15.7	-	6.6	14.9	-
Escherichia coli	23.1	-	-	18.7	-

Note : - , no inhibition zone

Extraction of fermentation product was done on both mycelium and liquid medium because fungal secondary metabolites can exist either in extracellular (liquid medium) or intracellular (mycelium residue). Then, the dried extracts of SA 1, SA 3, and SA 4 were used for determining their minimum inhibitory concentration (MIC).

The medium extract of SA 3 gave the lowest MIC value against *B. subtilis* at 64 µg/mL, followed by SA 1 with MIC 256 µg/mL. However, fungal medium extract of SA 4 showed growth until the concentration of extract was 2048 µg/mL against *B. subtilis* and *E. coli*. The fungal extract medium of SA 1 also gave the lowest MIC against *E. coli* at 32 µg/mL. However, the fungal medium extract of SA 3 and SA 4 did not show any inhibition against *E. coli*.

The extract mycelium of S3 and S4 have the same MIC value against *B. subtilis* at 256 µg/mL, while the SA 1 gave the MIC value of 512 µg/mL. The lowest MIC value against *E. coli* was shown by extract mycelium of SA 4 at 64 µg/mL, followed by SA 1 with the concentration of 256 µg/mL (Table 4). Meanwhile, fungal extract SA 3 did not give any MIC value against *Escherichia coli*. The classification of antimicrobial activity of extracts based on MIC value as follows: significant if MIC values are below 100 µg/ml, moderate when 100<MIC<625 µg/mL and weak if MIC>625 µg/mL (Kuete *et al*. 2011, Dzoyem *et al*. 2013). From the result shown that fungal

medium extract of S3 had the highest antibacterial activity against *B. subtilis* and S1 against *E. coli*. Based on their MIC values, they can be classified as the extracts with significant antibacterial activities.

Table 4. The MIC Value of Fungal Media And Mycelium Extract.

Fungi	Concentration of extract (μg/mL)			
	Media Culture		Mycelium	
	B. subtilis	*E. coli*	*B. subtilis*	*E. coli*
SA 1	256	32	512	256
SA 3	64	-	256	-
SA 4	-	-	256	64

Note: -, MIC > 2048 μg/mL

Among the test bacteria, *Bacilus subtilis* is the most sensitive to the extracts. Meanwhile, *E. coli* was the most resistant. The different response towards antibacterial substances is due to their outer membrane. The bacterial envelope of *B. subtilis*, a Gram positive bacterium, consists of lipoteichoic acid, teichoic acid, peptidoglycan, protein, and phospholipid. The peptidoglycan layer is located on the outermost part or the surface of the bacterium. On the other hand, *E. coli*, a Gram negative bacterium, consist of lipopolysaccharide, lipoprotein, peptidoglycan, protein, and phospholipid. The structures of gram negative bacterium differ from a gram positive bacterium regarding the location of peptidoglycan. The peptidoglycan in gram negative bacterium is situated in between the outer membrane, which is the cell wall, and the cell membrane (Madigan *et al*. 2012). This outer layer prohibits certain molecules such as drugs, from penetrating the bacterium cell, consequently making the bacterium more resistant to drugs in contrast with a gram positive bacterium (Kaplan 2000).

Conclusion

A total of five types of soil-derived fungi (SA1-SA5) were isolated from Taman Botani Negara, Shah Alam, Malaysia. Based on the morphological characteristic, the suggested genus of SA 1, SA2, and SA 5 were *Aspergillus*, whereas SA 3 and SA 4 were *Penicillium* and *Rhizopus,* respectively. The antibacterial assay showed that fungal medium extract of S3 had the highest antibacterial activity against *B. subtilis* and the extract mycelium of S4 against *E. coli*. Also based on their MIC values, they were classified as extracts with significant antibacterial activities. These extracts could be potentially useful for the development of new therapeutic agents against bacterial infections.

Acknowledgment

The authors also thank School of Pharmacy ITB-Bandung Institute of Technology for providing the facilities and reagents to conduct this research.

References

Clinical and Laboratory Standard Institute (CLSI), 2010, Method for Dilution Antimicrobial Susceptibility Test for Bacteria that Grow Anaerobically, Approved Standard M07-A8 8th ed.; National Committee for Clinical Laboratory Standards: Wayne, Philadelphia, USA.

Dzoyem JP, Guru SK, Pieme CA, Kuete V, Sharma A, Khan IA, Saxena AK, Vishwakarma RA, 2013, Cytotoxic and antimicrobial activity of selected Cameroonian edible plants, BMC Complementary and Alternative Medicine, 13 (78): 1-6.

Kaplan M, 2000, Melissa Kaplan's Herp care collection: the problem with gram-negative bacteria, http://www.anapsid.org/gramnegative.html, accessed on 3rd May 2016.

Kuete V, Krusche B, Youns M, Voukeng I, Fankam AG, Tankeo S, Lacmata S, Efferth T, 2011, Cytotoxicity of some Cameroonian spices and selected medicinal plant extracts, J Ethnopharmacol 134:803–812.

Madigan M, Martinko J, Stahl D, Clark D, 2012, Brock biology of microorganisms, 13th edn, Pearson, San Fransisco.

Zeilinger S, Martin J, Estrada C, 2015, Biosynthesis and molecular genetics of fungal secondary Metabolites, 2nd edn, Springer, New York.