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Antimicrobial activities of endophytic fungi residing in *Aloe vera* against diabetic wound pathogens

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ABSTRACT

Aims: Endophytic fungi are organisms that colonize living plant tissues without causing significant symptoms of diseases. They are known as the repository of bioactive compounds. This study was aimed to isolate endophytic fungi associated with *Aloe vera* and to assess their antimicrobial activity against the diabetic wound pathogens that result to lower limb amputations.
Methodology and results: In this study, 84 endophytic fungi were isolated from *A. vera* by using 5% sodium hypochlorite and 70% ethanol as surface sterilants. On preliminary antimicrobial screening by agar plug assay, 12 (14.3%) isolates showed significant antagonistic activity on the test microorganisms. Two of the endophytes were identified as *Aspergillus flavus* and *Colletotrichum gloeosporioides* through morphological and molecular techniques. The fungal cultures fermented in shake flask were extracted subsequently with hexane, dichloromethane, ethyl acetate, and *n*-butanol. Ethyl acetate and dichloromethane extracts of *A. flavus* and *C. gloeosporioides* inhibited all test bacteria. The compounds in the extracts were seemed to be semi-polar in nature. On broth microdilution assay, minimal inhibitory and minimal lethality concentration of ethyl acetate extracts of the fungi were found in the range of 0.63–2.50 and 1.25–5.00 mg/mL, respectively.
Conclusion, significance and impact of study: This study reveals that *A. vera* harbours a wide diversity of endophytic fungi. *A. flavus* and *C. gloeosporioides* showing broad spectrum antimicrobial activity on wound pathogens may be potential sources of bioactive compounds. Further investigations should be conducted to isolate and identify the antimicrobial compound produced by these fungal isolates.

Keywords: Aloe vera, endophytic fungi, antimicrobial activity, *Aspergillus flavus*, *Colletotrichum gloeosporioides*

INTRODUCTION

Patients with chronic diabetic conditions are commonly suffered from serious foot ulcers. The ulcer is the leading cause of amputations of the lower extremities if not treated immediately (Won *et al*., 2014). Approximately 15% of diabetic patients suffered from foot ulcers (Haslam, 2010). Besides, they experienced 15 to 20-folds increased risk of lower limb amputations compared to non-diabetic patients, which primarily caused by microbial infections (Pscherer *et al*., 2012). Many studies on diabetic foot infections (DFI) reported that *Staphylococcus aureus* is the main causative agent along with *Streptococcus* spp., *Pseudomonas aeruginosa* and some members of *Enterobacteriaceae* (Citron *et al*., 2007; Ozer *et al*., 2010). The treatment of DFI is challenging to the clinical practitioners when multidrug-resistant bacteria like methicillin resistant *S. aureus* (MRSA) or extended-spectrum *β*-lactamase isolates colonize the wounds (Mendes *et al*., 2012; Akhi *et al*., 2017). Therapeutic alternatives with better efficiency are required to combat these pathogens.

Chronic wounds are easily infected if they are exposed to the colonization of microorganisms. Wound colonization refers to the multiplying of microbial species on wound, which eventually lead to apparent microbial growth (Deshmukh *et al*., 2019). The microbial species on chronic diabetic wound are diverse. They rarely exist in single species of planktonic cells, but they usually survived in a complex polymicrobial biofilm populations (Debrah *et al*., 2020). Biofilm is developed when the microbial cells embed to each other by forming a matrix of extracellular polysaccharides (Debrah *et al*., 2020). These substances help the bacteria to adhere to a host cell and

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protect them from environmental stress (Deshmukh *et al*., 2019). Thus, bacterial biofilm usually consists of multidrug resistant pathogens that causes nosocomial infection (Surendran *et al*., 2019). Bacterial biofilms significantly delay the diabetic wound healing, and eventually increase the treatment cost (Deshmukh *et al*., 2019). To tackle the infection of chronic wounds, a wide range of antimicrobial compounds were incorporated into wound dressing materials in order to improve the recovery of the damaged skin. Unfortunately, these antimicrobial compounds come with their fair share of drawbacks. Silver ions were widely used due to their excellent antimicrobial activities (Deshmukh *et al*., 2019). However, they have been reported to cause argyria due to extensive use of silver-containing wound dressing materials (Kim *et al*., 2019). Besides, silver ions are rapidly inactivated in wound environment (Kim *et al*., 2019). Medical grade honey is approved in many countries for the treatment of diabetic wound. However, it is ineffective against biofilm formed by the pathogenic microorganisms on wound (Oliveira *et al*., 2018). Antibiotics such as penicillin, chloramphenicol, bacitracin, tetracycline, gramicidin, polymyxin and aminoglycoside are used topically for the treatment of diabetic infections, however, they also cause hypersensitivity reactions, favour super-infections and select for resistant pathogens (Memon *et al.*, 2020). An alternative compound is needed for the treatment of diabetic wound infections.

Endophytic fungi are the organisms that colonize the living tissues of various plants, which eventually develop a mutual relationship with the host, without causing any significant symptom of apparent illness (Yan *et al*., 2019). Usually, plants are the main reservoirs of endophytic microorganisms which are considered as the storehouses of various novel compounds that enabling them to be adapted to the environmental or ecological changes as well as diverse physiology of their hosts (Segaran and Sathiavelu, 2019). They have been reported to produce various bioactive molecules including alkaloids, flavonoids, phenolics, terpenoids, steroids, quinines and peptides, which act as antimicrobial, antiviral, anti-insecticidal, anticancer and antidiabetic agents (Strobel *et al*., 2004; Gouda *et al*., 2016). Hence, they have great potential in the applied fields including pharmaceutical, agriculture, biotechnology, food, and textile (Strobel, 2018).

Aloe vera is a succulent plant belongs to the member of the Liliaceae family. This plant is widely used for traditional medicines in many countries including Indian subcontinents, China, Japan, and West Indies (Maharjan and Nampoothiri, 2015). This simple perennial xerophyte consists of outer green rind and the inner parenchymal pulp having transparent mucilaginous gel. The gel in the pulp of *A. vera* is composed of approximately 99.3% water and the remaining solids contain polysaccharides, glucose, mannose, enzymes, phenolic compounds, vitamins, minerals, and organic acids (Hamman, 2008). The biological activities of *A. vera* were widely reported, including antimicrobial, wound healing (Tarameshloo *et al*., 2012), anti-aging, anti-inflammatory and immune-stimulatory (Budai *et al*., 2013), and anti-diabetic activity (Huseini *et al*., 2012). However, the studies on the endophytic fungi residing in *A. vera* are scarce. Hence, this study was conducted to isolate the endophytic fungi from this herbal medicinal plant. Besides, the antimicrobial activity of the fungal isolates was screened on pathogenic microorganisms isolated from diabetic wounds.

MATERIALS AND METHODS

Collection of plant sample

A total of 20 healthy and matured leaf samples of *A. vera* were collected from Taman Desa Bayu, Alor Gajah, Melaka, Malaysia (2°22′49.44″N, 102°12′32.04″E). The plant sample was deposited in the Herbarium, Universiti Kuala Lumpur with the specimen number (HERB/20/1741/34). The plant leaves were carefully cut off using clean and ethanol (70%) disinfected sharp knife. The samples were brought to the laboratory in an ice-box, in a sterile glass container. The samples were immediately kept at 4 °C and isolation of endophytic fungi was done within 12 h of sample collection.

Sample processing and surface sterilization

The leaves were washed in running tap water for 5 min and then washed again with sterile distilled water. The skin of the leaves was sliced carefully to avoid the attaching of gel. The peeled skins were subsequently cut into small pieces of 0.5 cm^2. Surface sterilization was carried out following the method stated by Burgdorf *et al*. (2014). The sliced leaves were separately treated with 70% ethanol for 2 min, 5% sodium hypochlorite solution for 2 min, then 70% ethanol for 1 min. Finally, the samples were double rinsed in sterile distilled water to remove the surface chemicals.

Validation of surface sterilization

To test the effectiveness of surface sterilization, the surface-sterilized leaf samples were imprinted onto potato dextrose agar (PDA) (Merck, Germany) according to Anjum and Chandra (2015). Besides, 100 µL aliquot of the final rinse water was spread on PDA. The plates were incubated at 30 °C for 5 days to check the appearance of microbial growth. The microbial growth indicates the growth of epiphytes.

Isolation of endophytic fungi

Surface imprinted samples were placed onto PDA plate supplemented with 50 mg/L of chloramphenicol (Sigma-Aldrich, USA) to restrict bacterial growth. The plates were incubated at 30 °C for 15 days to facilitate the growth of endophytic fungi. Fungal mycelia protruding out of leaf were inoculated onto the fresh PDA medium to obtain a pure culture. Replicated fungal isolates were discarded and only strains with differences in morphology were

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selected for subsequent antimicrobial screening tests. To ensure the viability, fungal isolates were periodically transferred onto a new PDA medium on every two months. The pure cultures of the fungal isolates were preserved at 4 °C.

Test microorganisms

Antimicrobial activity of the fungal isolates was tested against the pathogenic microorganisms previously isolated from patients with chronic diabetic wounds (Rozman *et al*., 2018). The test microorganisms were included Gram-positive bacteria (*Bacillus subtilis*, *S. aureus*, Methicilin resistant *S. aureus* [MRSA] and *Streptococcus* species), Gram-negative bacteria (*Escherichia coli*, *Proteus mirabilis*, *Shigella boydii*, *Salmonella typhimurium,* and *Yersinia* sp.), and yeasts (*Candida albicans* and *Candida utilis*). The microbial cultures were maintained at Upstream Bioprocess Laboratory, Universiti Kuala Lumpur, Malaysia. To prepare the microbial inocula, a loopful of microbial colonies was suspended into 5 mL of sterile distilled water. Then, the inoculum was mixed vigorously. The turbidity of the suspensions was adjusted match to 0.5 McFarland standard. The final inocula size of bacteria and yeast were 1× 10^8 CFU/mL and 1× 10^6 CFU/mL, respectively.

Preliminary antimicrobial screening: agar plug assay

Agar plug technique was conducted following the procedure described by Elleuch *et al*. (2010) with some modifications. Fungal agar plug of 10 mm diameter was excised from 14 days old culture of endophytic fungi. The agar plugs were placed underlying the mycelium surface onto the nutrient agar (Merck) plate inoculated with the microbial inoculum using a sterile cotton swab. The experiment was done in triplicate. The plates were kept in a freezer at 4 °C for 48 h to facilitate diffusion of antimicrobial compounds from the mycelial plugs into the surrounding medium. Then, the plates were then incubated at 37 °C for 4 h. After the incubation period, the presence of clear zones of inhibition surrounding the agar plugs was observed.

Fungal identification

Isolate L21 and L42-2 were selected for further study based on their significant antimicrobial activity on agar plug assay. Cultural and morphological characteristics were observed for 14 days of incubation. Cell morphology of the isolates was observed under light microscopy (Olympus, Japan) following lactophenol cotton blue (LPCB) staining. The DNA extraction and polymerase chain reaction (PCR) were performed by NHK Bioscience Solutions Sendirian Berhad, Malaysia for molecular identification of the isolates. ITS-3 (Internal Transcribed Spacers) and ITS-4 were used as forward and reverse primers, respectively. Each DNA sequence was generated from the corresponding electropherogram data. The complementary DNA sequence of the nucleotide sequences by the reverse primer was retrieved using Chromas software (version 2.6.4). The forward and complementary DNA sequences were aligned with Molecular Evolutionary Genetics Analysis (MEGA) software (version 5.05). The aligned DNA sequence was searched for homology through the NCBI Gene Bank database using basic local alignment search tool called nucleotide BLAST.

Fungal fermentation

The isolates were cultivated in shake flask system in yeast extract sucrose (YES) broth [0.4% yeast extract powder (Merck), 2% sucrose, supplemented with 0.1% potassium dihydrogen phosphate (KH_2PO_4) and 0.05% magnesium sulfate ($MgSO_4$)]. The pH of the medium was adjusted to 5.5 before sterilization with an autoclave at 121 °C for 15 min. The flask was inoculated with fungal agar plugs of 6 mm in diameter excised from the periphery of 7 days old culture on PDA medium. The opening of the flasks was blocked with autoclaved cotton stoppers then covered with aluminum foil to prevent contamination. The flasks were incubated at 30 °C with a rotational speed of 120 rpm (revolutions per minute) in a rotatory shaker for 20 days.

Liquid-liquid extraction

The fermented culture was treated with ultra-sonicator (Sonics and Materials, USA) at a high-frequency sound with a wave density of 20 kHz/s along with 80% of amplitude for 20 min to break the fungal cells. The cell lysates were then separated into fungal biomass and clear lysate by centrifugation (Eppendorf, Germany) at 3200 *g* for 1h. The supernatant was extracted sequentially with an equal volume (1:1, v/v) of hexane (Hex), dichloromethane (DCM), ethyl acetate (EA) and butanol (BtOH). The solvents were mixed thoroughly and allowed to form two clear immiscible layers. The organic phase containing the desired compounds was evaporated to concentrate under reduced pressure at 50 °C by using a rotatory evaporator (Buchi, Germany). The concentrated extracts were dried at room temperature to remove the remaining solvents. Finally, the crude extracts were stored at 4 °C prior to using for antimicrobial assays.

Antimicrobial activity on disc diffusion assay

Disc diffusion assay was performed according to Leong *et al*. (2017). The microbial suspension was streaked on the surface of Muller-Hinton agar (Merck). Sterile filter paper discs of 6 mm in diameter were impregnated with 20 µL of crude extract dissolved in 25% methanol at a concentration of 50 mg/mL. The disc was placed on the surface of the medium. Chloramphenicol (30 µg/mL) and amphotericin B (10 µg/mL) were taken as positive controls for bacteria and yeast respectively and 25% methanol was used as the negative control. The experiment was done in triplicate. After elapsing the

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incubation period of 18 h at 37 °C, the diameter of the clear zones surrounding the disc was measured.

Broth microdilution assay

This assay was performed in a sterile 96-well plate (Nest) to determine the minimal inhibitory concentration (MIC) and minimal lethality concentration (MLC) of ethyl acetate (EA) extract of selected isolates. The assay was performed as described by Tong *et al*. (2014) on test microorganisms that showed significant sensitivity on disc diffusion assay. The assay was done in triplicate. The microbial culture was first diluted 1:10 (v/v) with double strength sterile Muller Hinton broth (Merck). The test concentrations were ranged from 0.02–5.00 mg/mL. An aliquot of 100 µL sample was dispensed in each well of microtiter plate followed by 100 µL of microbial inoculum. A growth control was maintained by replacing the extract with 100 µL of 25% methanol and MHB was considered as the sterility control. The plate was incubated at 37 °C for 24h. After sampling for MLC, 40 µL of *p*-iodonitrotetrazolium (INT) violet salt at the concentration of 0.2 mg/mL prepared by dissolving in 95% ethanol was added into each well as a microbial growth detector. Change of broth color was observed after 30 min of incubation. The MIC against the test organisms was marked as the lowest concentration of EA extract that prevents any apparent growth of test microorganisms. Prior to MLC determination, a loopful of the sample from each well was streaked on nutrient agar medium. Then the plates were incubated at 37 °C for 24 h and the viability of the test microorganisms was checked. The MLC was recorded as the lowest concentration of the extract to kill the test microorganisms completely. The experiment was done in triplicate in separate occasions.

RESULTS AND DISCUSSION

Isolation of endophytic fungi

To ensure endophytic fungi, only healthy and matured *A. vera* plants with no visible symptoms of the disease were selected to avoid the potentially pathogenic fungi. The plant was cultivated without fungicide and pesticide that would otherwise inhibit the growth of the endophytic fungi. As several factors like the type of host plant, age, and type of tissue affect the symbiosis of the endophytes with the host plant, thus the plant must be carefully selected (Cao *et al*., 2004). In order to isolate the endophytes, unwanted microbial contaminants of epiphytes should be removed from samples by treating with some chemical disinfectants. Hence, prior to inoculating onto the growth media, the desired samples were surface sterilized with ethanol, sodium hypochlorite, and ethanol to ensure the removal of epiphytes. The agar plates for surface imprinting and culturing of the last rinsing water were found no visible growth. These validate that the combined surface sterilization treatments were successful in terms of the elimination of the epiphytic microorganisms present on the plant surface. The result is agreed with Coombs and Franco (2003) and Cao *et al*. (2004). The surface sterilization technique was effective, thus the fungal isolates growing from the leaf samples were, in fact, endophytic in nature.

The hyphal tips of different fungal isolates mostly arose within 4 days of inoculation. In this study, a total of 84 morphologically diverse fungi were obtained from *A. vera.* The assemblage of significantly large numbers of fungi with varied morphology may be the result of the selection of different healthy plants during sampling. It also indicates that the isolates may be non-phytopathogenic or less pathogenic, or the host's immunity was enough to hold their pathogenicity at the dormant phase (Sadrati *et al*., 2013). The occurrence of huge numbers of fungi implies that *A. vera* facilitates fungal biodiversity with unique ecological niches and the fungi have a good affinity to be colonized through symbiosis with the plant. The tendency may also be a resemblance to the fungal ability of adaptation and utilization of the host's specific nutrients (Huang *et al*., 2008). These interactions between the host and endophyte reflection their mutual agreements of providing nutrients and protection to each other through chemical species (Tetard-Jones and Edwards, 2016).

Preliminary antimicrobial screening

In this screening test, 25 isolates showed significant antagonistic activity with the presence of clear inhibition zones surrounding the fungal agar plugs against the pathogenic microorganisms. The restriction of the pathogens without direct contact of fungal mycelia implies that the isolates secreted potential bioactive compounds that restricted the growth of the microbes (Nourozian *et al*., 2006). A total of 12 (14.3%) isolates were found to be significantly inhibited 3 or more test pathogens, including *S.aureus* and *C. utilis*. However, 61 out of 84 fungi showed no antimicrobial activity. The probable reasons might be either unfavorable physicochemical conditions in the media, generation of insufficient bioactive metabolites, or the isolates may not produce any potent compound enough to suppress microbial growth (Ritchie *et al*., 2009). It is noteworthy that 7 of the isolates (Isolate L1, L9, L12-1, L21, L42-2, and L67) inhibited the growth of all microorganisms tested (Table 1). In addition, *C*. *utilis*, an opportunistic pathogen found in wound exhibited the highest susceptibility, followed by *B*. *subtilis* and *S*. *aureus* as presented in Figure 1. The increased sensibility of yeast might be the result of reduced expression of trans-membrane drug efflux (Lamping *et al*., 2010). Having established successful colonization, the endophytes can prevent further infections of their host from the terrestrial pathogenic species through the antagonistic actions (Powthong *et al*., 2013).

Identification of the selected fungi

Having significant antimicrobial efficiency, Isolate L21 and L42-2 were selected for morphological and molecular identification. Figure 2 shows the colony morphology and

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Table 1: Antimicrobial activity of endophytic fungi on agar plugs assay against the clinical pathogens.

Test microbes	Diameter of inhibition zone (mm)											
	L1	L9	L12-1	L21	L22-1	L27	L40	L42-2	L58-1	L67	L75-1	L75-2
B. subtilis	+++	++++	++++	+++	+++	+++	++++	++++	+++	++++	+++	+++
S. aureus	++++	++++	++++	+++	++++	++	++++	++++	++++	++++	+++	++
Streptococcus sp.	++++	++++	++++	++++	++++	++	++++	++++	++++	++++	+++	+++
E. coli	++++	++++	++++	+++	++++	++	++++	++++	++++	+++	++	+++
P. mirabilis	+	++	++	++	-	-	-	+	-	+++	-	-
S. typhimurium	++++	++++	++++	+++	+++	+	++	++	++	++	++	+
C. utilis	+++	++++	++++	++++	++++	++	++++	++++	++++	+++	++	++

Zone inhibition spectrum: - (10 mm, no inhibition), + (11–15 mm), ++ (16–20 mm), +++ (21–25 mm), ++++ (≥26 mm). The fungi with inhibitory activity to at least six microbes have been included here.

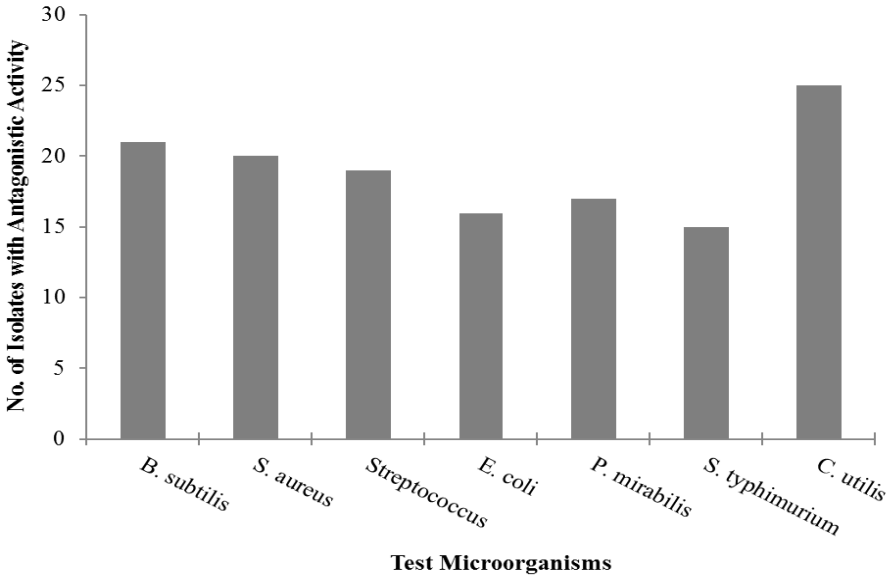

Figure 1: Number of endophytic fungi isolates with antagonistic activity against the clinical pathogens on agar plugs assay.

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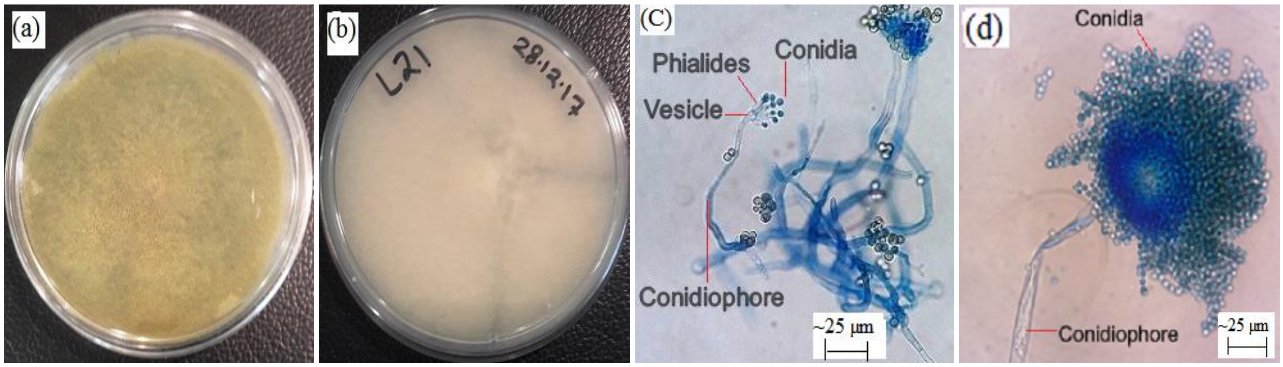

Figure 2: Colony morphology with front view (a), reverse view (b) and LPCB staining (c) and 7 days old culture (d) of *Aspergillus flavus* (Isolate L21) under light microscope at 40× magnification.

microscopic observation of 7 days old culture of Isolate L21. On PDA medium, initially, it showed white and tiny mycelia that turned into olive to dark green spores after 3 days of incubation. A white border of growing mycelia encircled colony changed into the green to brownish color on day 5. On day 7, the texture of the colony was plain and flat at the edge but the center was comparatively raised with powdery granules of spores without exudates. Green to brownish color dominated the entire colony and the reverse side appeared to be slightly pale as the spores acquired the maturity. Light microscopy of LPCB staining unambiguously showed smooth and hyaline conidiophore, clear and subglobose vesicles, flask-shaped uniseriate phialides, and thin-walled globose conidia. The cultural and microscopic characteristics clearly indicate that the isolate L21 belongs to the genus of *Aspergillus* and it might be the species of *Aspergillus flavus* (Thathana *et al*., 2017). BLASTN using NCBI database found that more than 97% of the query sequence of L21 is 100% identical to the internal transcribed spacers and ribosomal RNA genes of *A. flavus*. This fungus has been well documented with extensive studies for the production of aflatoxins. Many researchers found it as an endophyte in various hosts and described it to be involved in the production of different bioactive metabolites (Chowdhury *et al*., 2018; Liu *et al*., 2019).

Isolate L42-2 initially showed fast-growing white and fluffy mycelia on the PDA medium. The fungus occupied the entire surface of the Petri dish within 10-12 days of inoculation. The aerial mycelia changed into grayish-white and pale brown color with concentric rings of conidial masses that darken with age (Figure 3). The reverse side of the colony was slightly whitish creamy or grayish orange with a circular halo of mycelium. LPCB staining of actively growing cells showed clear, septate and branched mycelia with hyaline, long, single-celled, cylindrical or slightly curved conidia. The cultural and morphological features imply that the isolate L42-2 could be a member of the genus of *Colletotrichum* (Photita *et al*., 2005). BLASTN of NCBI database showed isolate L42-2 is more than 99% identical with the internal transcribed spacers and ribosomal RNA genes of *Colletotrichum gloeosporioides*. *C. gloeosporioides* is commonly associated with various plant diseases (Sharma and Kulshrestha, 2015). Many studies have been reported it to be generated various compounds including as colletotric acid and colletoic acid (Zou *et al*., 2000), phillyrin (Zhang *et al*., 2012), Piperine (Chithra *et al*., 2014), acorane sesquiterpenes (André *et al*., 2017).

Fermentation of selected fungi

Prior to fermentation, YES medium was supplemented with KH_2PO_4 as a buffering agent since pH is changed due to the production of acidic and basic compounds over the period of fermentation (Peňa *et al*., 2015). In addition, $MgSO_4$ was provided to maintain cellular homeostasis of the essential ion playing vital roles in cell division, cellular integrity, enzyme activation, and stress suppression during fermentation (Udeh and Kgatla, 2013). Initially, a low pH of 5.5 was adjusted as acidic media provide physical stress which maximizes the production of secondary metabolites (Mathan *et al*., 2013). *A. flavus* and *C. gloeosporioides* produced significantly heavy growth in the fermented medium. This reflects that the YES medium as well as the physical parameters comprising pH, incubation temperature, incubation period, and rotational speed of shaker favored the production of sufficient fungal growth (Bhattacharyya and Jha, 2011).

Antimicrobial activity on disc diffusion assay

Results of the antimicrobial activity of 8 crude extracts collected from *A. flavus* and *C. gloeosporioides* are presented in Table 2. Generally, the finding supports the conformity of the results of antagonistic activity on agar plug assay. All of the extracts exhibited inhibitory activity to 3 or more test microorganisms in this study. In addition, *B. subtilis* and *S. boydii* were susceptible to all the crude extracts. On disc diffusion assay, Gram-positive bacteria were more sensitive than the Gram-negative bacteria. A possible explanation might be the anomalies of their cell wall chemistry. In addition to the underlying single

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Table 2: Antimicrobial activities of different crude extracts of *A. flavus* and *C. gloeosporioides* on disc diffusion assay.

Test microbes	Diameter of inhibition zone (mm)									
	L21 Hex	L21 DCM	L21 EA	L21 BtOH	L42-2 Hex	L42-2 DCM	L42-2 EA	L42-2 BtOH	PC	NC
B. subtilis	+	+ +	+ + +	+ +	+	+ +	+ + +	+	+ + + +	-
S. aureus	+ +	+ + +	+ + +	-	+	+ + +	+ + +	+	+ + + +	-
MRSA	+	+ + +	+ + + +	+	-	+ +	+ + +	+ +	+ + +	-
Streptococus sp.	+ +	+ +	+ + + +	-	-	+ +	+ + + +	+	+ + +	-
E. coli	-	+ +	+ + +	-	-	+	+ + +	+	+ + +	-
S. typhimurium	-	+	+ + +	-	-	+ +	+ + + +	+ +	+ + + +	-
S. boydii	+	+ +	+ + +	+	+	+ + +	+ +	+	+ + + +	-
Yersinia sp.	+	+ + + +	+ + + +	+	+ + +	+ + + +	+ + + +	-	+ + + +	-
C. albicans	-	+ +	-	-	-	-	-	-	+ + +	-

Hex = Hexane, DCM = Dichloromethane, EA = Ethyl Acetate, BtOH = Butanol; Zone inhibition spectrum: - (6 mm, no inhibition), + (7–10 mm), ++ (11–15 mm), +++ (16–20 mm), ++++ (≥21 mm); PC = Positive control (30 µg Chloramphenicol and 10 µg amphotericin B) and NC = Negative control (25% methanol).

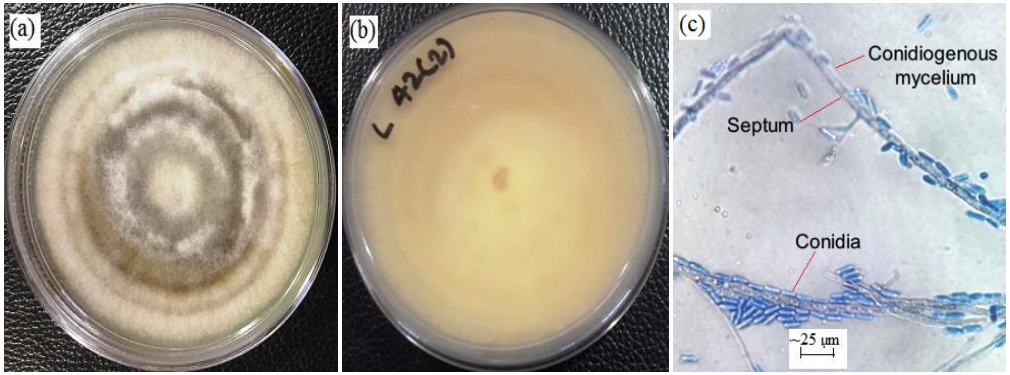

Figure 3: Colony morphology with (a) front view, (b) reverse view and (c) LPCB staining of 7 days old culture of *Colletotrichum gloeosporioides* (Isolate L42-2) under light microscope at 40× magnification.

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peptidoglycan layer, Gram-negative bacteria have an extra outer membrane which maintains selective permeability of toxic molecules including antibiotics (Delcour, 2009). Since the outer membrane is not fully hydrophobic hence it allows only some metabolites to enter into the cytoplasm through the porin channels (Gaysinsky *et al*., 2005). On the contrary, *C*. *albicans* was the least susceptible as only inhibited by the DCM extract of *A. flavus* (Isolate L21). The result was inversed with primary antimicrobial screening although the test was conducted with *C*. *utilis*, a different species. Hence, two endophytic fungi, namely *A. flavus* and *C. gloeosporioides* are able to produce bioactive metabolites with broad-spectrum antimicrobial activity.

The selection of extraction solvent is crucial to ensure that the antimicrobial compounds are successfully extracted from the culture broth. Besides, Ng and Rosman (2019) and Ng and See (2019) also reported the varying solubility of bioactive compounds through different mechanism based bioassays that measure the hydrophilicity of the compounds present. On disc diffusion assay, the extracts from non-polar solvent hexane (Hex) and polar solvent butanol (BtOH) were found to be significantly less bioactive than those with semi-polar solvents dichloromethane (DCM) and ethyl acetate (EA). In addition, EA extracts of *A. flavus* and *C. gloeosporioides* exhibited significantly better antimicrobial activity than the DCM extracts judging on the sizes of inhibition zones. These results agreed with Chowdhury *et al*. (2018) and Nurunnabi *et al*. (2018). However, it was found that both EA and DCM extracts of *A. flavus* and *C. gloeosporioides* inhibited all test bacteria, with the largest inhibition zone observed on *Yersinia* sp. The negative control (methanol) showed no antimicrobial activity which indicates that the antimicrobial activities were due to the presence of bioactive compounds in the extract.

Broth microdilution assay

Table 3 shows the MIC and MLC of EA extracts of *A. flavus* and *C. gloeosporioides* on test microorganisms. The bacteriostatic and bactericidal activities of the extracts were shown to be varied with the concentration gradients. As a whole, the values of MIC and MLC of the fungi were found in the range of 0.63–2.50 and 1.25–5.00 mg/mL, respectively. A lower MIC and MLC were recorded against *B. subtilis*, *Yersinia* sp. and *C. utilis*. MLCs were significantly higher than MICs which indicates that a higher concentration of EA extracts was needed to kill the test microorganisms rather than inhibiting the microbial growth. However, in a study of methanolic extract of *C. gloeosporioides*, Arivudainambi *et al*. (2011) claimed even lower MIC with a value of 0.0315 mg/mL against multidrug-resistant *S. aureus*. In this study, the MIC of *A. flavus* and *C. gloeosporioides* was noticed to be the same toward most of the microbial species and the value was not more than 2.5 mg/mL. On the other hand, the values of MLC were just double than those of MICs that caused complete massacre *in-vitro* under the experimental conditions. Besides, the bioactive compounds are generally considered to be bactericidal when the MLC is not more than four bits of MIC (Leong *et al*., 2017). On broth microdilution assay, the lowest MIC with a value of 0.63 mg/mL was recorded against *C. albicans* that showed the least susceptibility to disc diffusion. The explanatory reasons may be the rate of diffusion, the hydrophilicity of active compounds, and surface water activity that affect the diameter of the inhibition zone on solid media. Thus, the broth microdilution assay is more effective; hence it confirms significant result on the justification of the bioactive compounds (Torrico *et al*., 2010).

Table 3: MIC and MLC of ethyl acetate extracts *A. flavus* and *C. gloeosporioides* on broth microdilution assay.

Microorganisms tested	*A. flavus*		*C. gloeosporioides*	
	MIC (mg/mL)	MLC (mg/mL)	MIC (mg/mL)	MLC (mg/mL)
B. subtilis	1.25	2.50	1.25	2.50
S. aureus	2.50	5.00	2.50	5.00
Streptococus sp.	2.50	5.00	2.50	5.00
E. coli	2.50	5.00	2.50	5.00
S. typhimurium	2.50	5.00	2.50	5.00
Yersinia sp.	2.50	2.50	1.25	2.50
C. albicans	2.50	5.00	0.63	5.00
C. utilis	1.25	1.25	1.25	2.50

Several natural products were isolated from *A. flavus*. Patil *et al*. (2015) reported the isolation of several antimicrobial compounds from *A. flavus*. Most of the antimicrobial compounds isolated were phenolic and flavonoid in nature. Flufuran and 5-acetoxymethylfuran-3-carboxylic acid, two furan derivatives that exhibited significant antimicrobial activity were also produced by *A. flavus*. *C. gloeosporioides* was frequently isolated as an endophytic, and it is known as a fungus that produces natural compounds with novel structures (Zou *et al*., 2000; Ganadevi and Muthumary, 2008; Chithra *et al.*, 2014). The antimicrobial activity of the fungus was previously reported by Arivudainambi *et al.* (2011) and Chithra *et al.* (2014). Colletotric acid, an aromatic compound was previously identified as the key antimicrobial compound produced by *C. gloeosporioides* (Zou *et al.*, 2000). Further investigations should be conducted to isolate and identify the antimicrobial compounds produced by these two endophytic fungi isolated from *A. vera*. Besides, it is also crucial to test the efficiency of the extracts and the antimicrobial compounds on more clinical pathogens, particularly *P. aeruginosa*.

CONCLUSION

The findings reveal that the *Aloe vera* plant magnificently facilitates the diversity of endophytic fungi. Initially, a total of 84 morphologically different isolates were obtained in this study. However, 12 of 84 isolates showed significant

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antagonistic activity against diabetic wound microorganisms on agar plug assay. Two endophytic fungi identified as *A. flavus* and *C. gloeosporioides* exhibited broad-spectrum antimicrobial activities against the wound pathogens. The bioactive compounds in the extracts were semi-polar in nature and their bioactivity was dependent on concentrations. Further investigations should be conducted to isolate and identify the antimicrobial compounds produced by these two fungal isolates.

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