

Antimicrobial Efficacy of *Penicillium amestolkiae* elv609 Extract Treated Cotton Fabric for Diabetic Wound Care

Nur Amiera Syuhada Binti Rozman¹, Nurhanis Syafiqah Binti Mohd Nor Hamin¹, Leong Chean Ring¹, Tan Wen Nee², Mahfuzah Binti Mustapha¹ and Tong Woei Yenn^{1,*}

¹Universiti Kuala Lumpur, Malaysian Institute of Chemical and Bioengineering Technology, 78000 Alor Gajah, Melaka, Malaysia

²School of Distance Education, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia

Abstract Diabetes mellitus is a chronic disorder which affects millions of population worldwide. Global estimates published in 2010 reported the world diabetic prevalence as 6.4%, affecting 285 million adults. Foot ulceration and wound infection are major forms of disabilities arising from diabetic diseases. This study was aimed to develop a natural antimicrobial finishing on medical grade textile that meets American Association of Textiles Chemists and Colorists (AATCC) standard. The textile samples were finished with the ethanolic extract of *Penicillium amestolkiae* elv609, an endophytic fungus isolated from *Orthosiphon stamineus* Benth (common name: cat's whiskers). Endophyte is defined as microorganism that reside in the living plant tissue, without causing apparent disease symptom to the host. The antimicrobial efficacy of the ethanolic extract of *P. minioluteum* was tested on clinical pathogens isolated from diabetic wound. The extract exhibited significant inhibitory activity against 4 bacteria and 1 yeast with the minimal inhibitory concentration ranged from 6.25 to 12.5 mg/mL. The results indicate different susceptibility levels of the test microorganism to the ethanolic extract. However, the killing activity of the extract was concentration-dependent. The finished medical textile showed excellent antimicrobial efficacy on AATCC test assays. All the microbial cultures treated with the textile sample displayed a growth reduction of 99.9% on Hoheinstein Challenge Test. The wash durability of the finished textile was found good even after 50 washes with commercial detergent. Besides, the gas chromatography mass spectrometry analysis showed that 6-octadecenoic acid and diethyl phthalate were the main bioactive constituents of the extract. In conclusion, the developed medical textile showed good antimicrobial efficacy on laboratory tests. This work can be extended to *in vivo* trials for developing healthcare textile products for antimicrobial applications.

Keywords Antimicrobial textiles, Diabetic ulcer, *Penicillium amestolkiae*

The National Health and Morbidity Survey revealed that 17.5% of Malaysian adult citizens are estimated to be affected by diabetes in 2015 [1]. About 24% of diabetes

patients were amputated due to microbial infection [2]. The issue identified with diabetic ulcer is the expensive medical treatment cost. Diabetic foot complications resulted in increased hospital bed occupancy and further increase the healthcare cost and resources. Besides, these sorts of wound need more in-depth research to comprehend the right and proficient approach to understand the right and efficient way to increase the chances for the wound to heal. Foot ulceration and wound infection are major forms of disabilities arising from diabetic diseases. The most common pathogens isolated from infected wound are *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter* spp. [3]. The severity of the wound infection can be influenced by the type and density of microorganisms, the host immune response, and the type of tissue involved [4]. The large number of pathogenic species in the diabetic wound fluid indicate the slow healing process.

Antimicrobial textiles are widely used as wound dressing materials. They provide a physical barrier to cover and protect

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***Corresponding author**

E-mail: wytong@unikl.edu.my

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the wound from microorganisms that can cause wound infection. According to Alavi *et al.* [5], silver is the most commonly used antimicrobial dressings in antimicrobial textile. However, silver dressing causes tissue toxicity and inhibits fibroblast growth. In addition, synthetic based antimicrobial textiles also cause skin rashes, eco-toxicity and induction of drug resistance [6]. Hence, new antimicrobial finishing is necessary due to the limitations of the current options available. However, only limited studies were conducted by using fungal extracts as the antimicrobial finishing.

By definition, endophytes are microorganisms that reside in living plant tissues for all or part of their life cycle [7]. They are known for the production of bioactive metabolites, particularly antimicrobials, antioxidants, and anti-inflammatory compounds [8, 9]. However, thus far, no study was conducted to evaluate the application potential of these compounds in medical textile to combat microbial infection. Thus, in this study, we aim to develop a natural antimicrobial finishing on medical grade textile with extract obtained from *Penicillium amestolkiae* elv609, an endophytic fungus residing in medicinal herb, *Orthosiphon stamineus* Benth. *P. amestolkiae* are known for enzyme production, especially cellulases, xylosidase and hemicellulases [10, 11]. They also known as the producer of monascus-like red pigments. Up to date, no report is available on antimicrobial activity of *P. amestolkiae*.

MATERIALS AND METHODS

Isolation of endophytic fungi. The endophytic fungus *P. amestolkiae* elv609 was previously isolated by Tong *et al.* [12]. The fungus culture was deposited at Upstream Bioprocess Laboratory, Universiti Kuala Lumpur.

Cultivation and extraction. Yeast extract sucrose broth (yeast extract, 20 g/L; sucrose, 40 g/L; magnesium sulfate 0.5 g/L) with pH 5.8 ± 0.2 was used to cultivate the fungal isolate [12]. Two agar plugs with 2 cm in diameter were inoculated into the medium. The cultures were cultivated at 30°C with 120 rpm rotational speed in a shake flask system. After 2 wk of cultivation, the fermented broth and fungal biomass were separated by using Whatman No.1 filter paper. Then, the granular fungal biomass was soaked in ethanol for overnight at ratio of 1 : 20 (w/v). The extract was concentrated under reduced pressure by using rotator evaporator at 60°C to obtain the crude extract paste. The extract was kept at 4°C in dark until further use.

Test microorganisms. The test bacteria used in this study include 4 gram-positive bacteria (*Bacillus cereus*, *Bacillus coagulans*, *Streptococcus* sp., and *Staphylococcus aureus*), 4 gram-negative bacteria (*Escherichia coli*, *Proteus mirabilis*, *Yersinia* sp., and *Pseudomonas aeruginosa*), and 2 yeasts (*Candida albicans* and *Candida utilis*). All the test microorganisms were isolated from wound of diabetic patient in Hospital Seberang Jaya, Penang. The test microorganisms were sub-cultured on nutrient agar prior to use for every

2 wk in order to maintain its viability. The microbial inoculum was prepared by transferring a loopful of microbial colonies into a universal bottle of sterile distilled water. The turbidity of the suspension was adjusted so that it matches with 0.5 Mc Farland standards which contain approximately 10^8 colony-forming unit (CFU)/mL.

Disc diffusion assay. The antimicrobial efficacy of the extract was evaluated according to standards created by the Clinical Laboratory Standards Institute (CLSI) with modifications [12]. The assay was performed by transferring the inoculums to the surface of Mueller Hinton agar (Merck, Darmstadt, Germany) using cotton swap. Sterile paper disc impregnated with 20 μ L of 50 mg/mL extract was placed on the inoculated medium. Then, 20 μ L of 100 μ g/mL chloramphenicol was used as positive control and 20 μ L of 20% Tween 80 was used as negative control. The experiment was done in triplicate in separate occasions. All plates were incubated at 37°C for 24 hr. After the incubation period, the diameters of inhibition zone surrounding discs were measured [13].

Broth microdilution assay. The minimal inhibitory concentration (MIC) was determined by using broth microdilution assay in sterile 96-well microtiter plate. A serial two-fold dilution of the extract was carried out with double strength Mueller-Hinton broth. One hundred microliters of inoculums and extract were then added into each wells for a final volume of 200 μ L. The final concentration of the extract was ranged from 0.78 to 100 mg/mL. Chloramphenicol was used as reference antibiotic. Then, the plate was incubated at 37°C for 24 hr. After incubation period, 40 μ L of 0.2 mg/mL p-iodonitrotetrazolium violet salt (Sigma, St. Louis, MO, USA) dissolved in 99.5% ethanol and was added into each well. The plate was incubated for 30 min at 37°C. The color changed from yellow to purple indicates the microbial growth in the well. The lowest extract concentration that produced a significant growth inhibition effect on test microorganism was identified as the MIC [14]. To check the viability of the test microorganisms, the mixture in every well was streaked on nutrient agar. The plates were incubated at 37°C for 24 hr. The minimal lethality concentration (MLC) was recorded as the lowest extract concentration to kill the test microorganisms.

Antimicrobial finishing of cotton textile. Plain, 100% cotton woven textile, made up of Ne60 combed yarn with 110 ends/cm and 80 picks per inch was chosen for antimicrobial finishing [15]. Firstly, the textile was mordanted with acetic acid at a liquor ratio of 1 : 40 (w/v) and boiled for 3 hr at 100°C. Then, followed by finishing of extract at 55°C for 45 min with continuous stirring. Lastly, textile was soaked in 10% citric acid at 50°C for 5 min with continuous stirring in order to fix antimicrobial finishing. The coated textile was dried at 50°C for 24 hr. A solvent control was prepared by replacing the extract with methanol.

Hoheinstein Challenge Test (AATCC-100). The textile samples were cut to the size of 2 × 2 cm, 50 µL microbial inoculum was inoculated into 100 mL of nutrient broth followed by the transfer of textile sample. The cultures were incubated at 37°C for 24 hr with a rotational speed of 120 rpm. After the incubation period, the culture was suitably diluted and plated on nutrient agar plate. The colony counts were obtained after 24 hr of incubation at 37°C. The antimicrobial efficiency of the sample was determined by comparing the percentage reduction of bacteria relative to the solvent control.

Wash durability test (AATCC-147). The wash durability of the developed textile was evaluated after different wash cycles according to Sarkar *et al.* [16]. The samples were washed using 1% standard detergent. The antimicrobial activity was assessed after 30 and 50 washes according to protocol described in previous section (AATCC-100).

Gas chromatography mass spectroscopy analysis. The analysis was performed by using gas chromatography instrument (Hewlett-Packard 6890N, Palo Alto, CA, USA) with mass spectrometer (Hewlett-Packard 5973 inert mass selective mass detector) with mass spectrometry (Hewlett-Packard 5973 inert mass selective detector). The column HP-MS (30.0 m × 0.25 mm; Agilent Technologies, Santa Clara, CA, USA) was used for this analysis. The instrument was calibrated using absolute methanol (blank sample). The oven temperature was fixed at 70 to 285°C at 30°C/min and a hold for 2 min. Helium was used as carrier gas with a flow rate of 1.2 mL/min. The injector temperature was 280°C, injection volume of 1 µL with a split ratio of 1 : 5. The mass spectra were taken at 70 eV with a mass scan range of 35–650 amu. The identification of compounds was based on the comparison of their mass spectra with National Institute of Standards and Technology (NIST 02) Library.

RESULTS AND DISCUSSION

Microbial infections of foot ulcers are the major cause of amputation among diabetic patients. In present study, we assess the antimicrobial potential of *P. amestolkiae* elv609 against microorganisms isolated from diabetic wound. In this study, we reported that *P. amestolkiae* elv609 exhibited significant antimicrobial activity against 5 out of 10 test microorganisms (Table 1). Even so, the size of inhibition zones varied among all microbes, indicating different susceptibility of the test microorganism to the extract. Among the microbial species, the largest clear zone was shown by *Streptococcus* sp. which is a gram-positive bacteria. The results obtained are in agreement with previous studies that most of the fungal extracts are generally exhibit lower antimicrobial activity against gram-negative bacteria compared to gram-positive bacteria [17]. It has been reported that gram-negative bacteria have an outer membrane containing lipopolysaccharide that can protect peptidoglycan cell wall which makes them more resistant to antimicrobial compounds [18, 19].

P. amestolkiae elv609 exhibited broad spectrum antimicrobial activity, it also exhibited significant inhibitory effect on *C. utilis*. Bacterial infections of diabetic ulcers are commonly polymicrobials, hence a broad spectrum antibacterial agent is effective to reduce the bacterial population present on the ulcer. No data is available for comparison as this is the first report on the antimicrobial activity of *P. amestolkiae*.

The MIC of the extract was ranged from 6.25 to 25 mg/mL. The results indicate the different susceptibility levels of test microorganisms to the extract. The lowest MLC value obtained in this study was 12.5 mg/mL on *B. cereus*. Based on the results presented Table 2, the MLC was significantly higher than MIC, as a higher concentration of extract was needed to kill the test microorganisms, instead of inhibiting the growth. Therefore, the results showed that the ethanolic

Table 1. Antimicrobial activity of *Penicillium amestolkiae* elv609 extract on disc diffusion assay

Test microorganism	Diameter of inhibition zone (mm)		
	Ethanolic extract	Chloromphenicol	Negative control
Gram-positive bacteria			
<i>Streptococcus</i> sp.	17.0 ± 0.1	25.0 ± 0.1	-
<i>Bacillus cereus</i>	7.0 ± 0.2	12.3 ± 0.2	-
<i>B. coagulans</i>	-	17.0 ± 0.1	-
<i>Staphylococcus aureus</i>	-	7.0 ± 0.1	-
Gram-negative bacteria			
<i>Escherichia coli</i>	10.3 ± 0.2	12.0 ± 0.2	-
<i>Yersinia</i> sp.	-	15.0 ± 0.1	-
<i>Proteus mirabilis</i>	-	9.0 ± 0.1	-
<i>Pseudomonas aeruginosa</i>	14.0 ± 0.1	17.7 ± 0.2	-
Yeasts			
<i>Candida utilis</i>	10.0 ± 0.1	12.70 ± 0.1	-
<i>C. albicans</i>	-	15.0 ± 0.1	-

-, no inhibitory activity.

Table 2. Minimal inhibitory concentration and minimal lethality concentration of test microorganisms to *Penicillium amestolkiae* elv609 extract on broth microdilution assay

Test microorganism	Fungal extract		Chloramphenicol	
	Minimal inhibitory concentration (mg/mL)	Minimal lethality concentration (mg/mL)	Minimal inhibitory concentration ($\mu\text{g/mL}$)	Minimal lethality concentration ($\mu\text{g/mL}$)
<i>Streptococcus</i> sp.	12.5	50	8	16
<i>Bacillus cereus</i>	6.25	12.5	8	32
<i>Escherichia coli</i>	25	25	4	8
<i>Pseudomonas aeruginosa</i>	25	50	8	16
<i>Candida utilis</i>	25	50	8	32

Table 3. Growth reduction of microbial cultures treated with medical cotton finished with *Penicillium amestolkiae* elv609 ethanolic extract

Test microorganism	Microbial load (CFU/mL)		Reduction (%)
	Control	Treated textile	
<i>Streptococcus</i> sp.	5.20×10^6	-	100
<i>Bacillus cereus</i>	2.82×10^7	-	100
<i>Escherichia coli</i>	3.26×10^8	-	100
<i>Pseudomonas aeruginosa</i>	4.28×10^6	-	100
<i>Candida utilis</i>	1.64×10^8	1,050	100

CFU, colony-forming unit.

Table 4. The wash durability of medical cotton finished with *Penicillium amestolkiae* elv609 ethanolic extract

Test microorganism	Control (CFU/mL)	30 Washes (CFU/mL)	Reduction (%)	50 Washes (CFU/mL)	Reduction (%)
<i>Streptococcus</i> sp.	5.2×10^6	-	100	-	100
<i>Bacillus cereus</i>	2.82×10^7	320	99.99	3,020	99.98
<i>Escherichia coli</i>	3.26×10^8	1,670	99.99	7.5×10^5	99.78
<i>Pseudomonas aeruginosa</i>	4.28×10^6	-	100	-	100
<i>Candida utilis</i>	1.64×10^8	1,170	99.99	2,910	99.99

The wash durability of the textile was determined based on the percentage reduction in the growth of test microorganisms.

CFU, colony-forming unit.

extract was concentration-dependent. The extract exhibited microbialicidal effect to all test microorganisms, based on the MLC obtained [20]. The results showed that *P. amestolkiae* elv609 are potential source of antimicrobial compounds to combat microbial infection in diabetic wound.

Hohenstein challenge test was widely applied to evaluate the antimicrobial efficacy of antimicrobial textile. The results revealed that the reduction of bacterial colonies was significant on all test microorganisms (Table 3). The textile with *P. amestolkiae* elv609 extract finishing significantly reduced the microbial load of all test microorganisms. One hundred percent microbial load reductions were observed for all test microorganisms. The results showed excellent potential of the textile in inhibiting the growth of the diabetic wound microorganisms. Besides that, the textile finishing technique also affects the antimicrobial efficiency of antimicrobial textile. Jothi [21] also applied the same finishing technique on medical grade cotton with *Aloe vera* extract, they also reported a 99.9% growth reduction of *S. aureus*.

The developed textile also showed excellent wash durability, by using citric acid as mordant. The textile sample showed significant bacterial reduction, even after 50 washes with standard detergent. However, the microbial load significantly increased compared to 30 cycles ($p \leq 0.05$) (Table 4). The good wash durability of the developed textile can be due to the presence of the Van der Waals and hydrogen bonds that exists between fungal extract and textile cellulosic material, that allows the slow release of the extract from the textile [22]. According to Nithya *et al.* [23], there are a few factors that contribute to the good result for wash durability test which are cross linking of extract, antimicrobial finish by binding agent and due to hydrophilicity. In this study, citric acid was used as mordant to act as cross linking agent. Based on previous studies citric acid can improve the tensile strength and thermal stability [24]. So, this study suggested that treated textile can be use in the textile application.

A total of 12 compounds were detected in *P. amestolkiae* elv609 ethanolic extract via gas chromatography mass spectrometry analysis. The retention time, molecular weight,

Table 5. GC-MS analysis of *Penicillium amestolkiae* elv609 ethanolic extract

No.	Retention time	Compound	Molecular weight	Area (%)
1	3.182	1,3-Cyclopentanedione	98	3.9
2	3.443	Dihydro-2-methyl-3(2H)-furanone	100	9.3
3	4.626	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	144	0.9
4	6.497	2-Butyl-2-ethyl-oxazolidine	157	4.3
5	8.285	2-Propenoic acid, 3-(dimethylamino)-3-[(1-methylethyl)amino]-methyl ester	186	2.5
6	10.683	2,4-Bis(1,1-dimethylethyl)-phenol	206	4.0
7	12.28	Diethyl Phthalate	222	27.0
8	20.76	n-Hexadecanoic acid	256	6.6
9	25.227	9,12-Octadecadienoic acid	280	3.1
10	25.322	6-Octadecenoic acid	282	29.6
11	25.92	Octadecanoic acid	284	2.2
12	33.249	1,2-Benzenedicarboxylic acid, diisooctyl ester	390	6.6

GC-MS, gas chromatography mass spectrometry.

and peak area were depicted in Table 5. In general, 2 major compounds were predominant in the extract, they are diethyl phthalate and 6-octadecenoic acid, based on their percentage of peak area. 6-Octadecenoic or known as stearic acid was found to be present as major constituent with highest peak area of 29.6% and retention time 25.322. It has been reported that 6-octadecenoic acid exhibited significant antimicrobial and antioxidant activity, particularly on gram-positive bacteria [25, 26]. The results obtained are in agreement with the antimicrobial activity screening results, where the extract exhibited better inhibitory effect on gram-positive bacteria. Premjanu and Jayanthi [27] reported the antimicrobial and antioxidant activity of diethyl phthalate, which is one of the main constituent in *P. amestolkiae* elv609 ethanolic extract. The compound was widely used as cosmetic ingredients for topical application [27].

In conclusion, *P. amestolkiae* elv609 showed significant inhibitory effect on diabetic wound microorganisms. The GCMS of the bioactive extract also showed the presence of diethyl phthalate and 6-octadecenoic acid. We also successfully incorporated the extract into medical grade cotton, and the finished textile showed excellent antimicrobial activity on *in vitro* assays, with good wash durability.

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