



Antimicrobial producing bacteria isolated from tropical peat swamp soil

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ABSTRACT

Aims: The aims of this study were to isolate and characterize antimicrobial producing bacteria from tropical peat swamp forest soils.

Methodology and results: Bacteria isolated from peat soil were screened for antimicrobial properties via agar overlay assay. Broth microdilution was performed using crude-cell free supernatant (CCFS) to determine the minimum inhibitory concentration (MIC). One isolate was selected due to its broad spectrum activity and identified as *Burkholderia* spp. with a maximum identity of 99% via 16s rRNA gene PCR. This isolate was able to produce antimicrobials that were active against several Gram positive bacteria, Gram negative bacteria and also yeast. The antimicrobial activity of the CCFS was stable at a pH range of 1 to 11, temperatures of -20 °C to 80 °C, and after treatment with several proteolytic enzymes: α -chymotrypsin, proteinase K and trypsin, indicating that the antimicrobial produced might not be proteinaceous in nature. It is possible that the isolate can produce polyketides, a type of antimicrobial compound produced by *Burkholderia* known to be resistant to proteolytic enzymes. However, further work needs to be done to confirm this.

Conclusion, significance and impact of study: The presence of antimicrobial producing bacteria signified that tropical peat swamps are indeed a potential source for antimicrobials to combat infections.

Keywords: Antimicrobial compounds, tropical peat swamp forest, antimicrobial resistant bacteria, *Burkholderia*

INTRODUCTION

Due to the extensive usage of antimicrobials to treat infections, there has been a global increase of antimicrobial resistant bacteria (ARB) (Oelsschlaeger, 2010). This limits treatment options and increases the healthcare costs due to the use of more expensive treatments. Moreover, mortality rates from infection by ARB will also increase as antimicrobial efficiency decreases (Davies and Davies, 2010). An example of ARB is methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA strains are resistant to all currently available beta-lactam antimicrobial agents, and hence must be treated by other non-beta lactam antimicrobial agents such as vancomycin, daptomycin and linezolid (Gorwitz *et al.*, 2008; Rivera and Boucher, 2011). However, *Staphylococcus aureus* strains resistant to these antibiotics are emerging and hence new antimicrobials are required to treat them (Garcia *et al.*, 2010; Locke *et al.*, 2010).

It is hypothesized that bacteria produce antimicrobials in the natural environment to gain an advantage in competing for resources and colonization of new habitats by killing or inhibiting the growth of competitors (Hibbing *et al.*, 2010). Therefore, it is worthwhile to look at unique natural environments such as tropical peat swamp forests for antimicrobials. Tropical peat swamp forests are unique

wetland ecosystems, growing on a substrate formed by the accumulation of layers of peat-partially decomposed organic matter up to 25 m deep. They are characterized by low pH (pH 2.9-4.0), low nutrient levels and low dissolved oxygen with a temperature range of 24-32 °C (Yule and Gomez, 2009; Yule, 2010). Hence, the antimicrobial compounds that were produced are likely to withstand those harsh conditions. Besides that, tropical peat swamp forests are underexplored and inhospitable environments and thus have great potential as a bioprospecting site.

Previous studies suggest that there is a huge diversity of microbes in tropical peat swamp forests. However, most research on tropical peat swamp forests mainly focus on bacteria related to nutrient recycling such as methanotrophic and acidobacteria, but none on antimicrobial producing bacteria (Jauhiainen *et al.*, 2005; Voglmayr and Yule, 2006; Yule, 2010). Therefore, tropical peat swamps are potential locations for the discovery of novel microorganisms, which leads to the objective of this study which was to isolate antimicrobial producing bacteria from tropical peat swamp soil

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MATERIALS AND METHODS

Test microorganism strains and culture conditions

Test microorganism strains that were used in this project includes *Bacillus cereus* (ATCC 14579), *Enterococcus faecalis* (ATCC 29212), vancomycin-resistant *Enterococcus* (ATCC 700802), MRSA (ATCC 700802), MRSA (ATCC 43300), *Staphylococcus aureus* (ATCC 6538P), *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 10031), *Salmonella enterica* (ATCC 14028), *Shigella flexneri* (ATCC 12022) and *Candida albicans* (obtained from the Institute of Medical Research). Strains were cultured on Brain-Heart Infusion (BHI) medium at 37 °C and maintained at -80 °C in BHI with 25 % (w/v) glycerol.

Sample collection

Peat soil samples were collected from the Southeast Pahang peat swamp forest (N 3° 01' 19.56"; E 103° 39' 29.67"). Fifty grams of peat soil were obtained from the peat surface using a sterile hand shovel and at a depth of 50 cm using a core borer from three different locations that were 20 m apart to increase the chances of isolating different type of bacteria. The collected peat soil samples were placed in sterile containers and transported at ambient temperature within 18 h for laboratory analysis.

Isolation and screening for antimicrobial activity

One gram of peat soil was immersed in 0.85% (w/v) saline and was serially diluted up to 10⁻⁸. The samples were then spread plated onto 1/10 tryptone soy agar (TSA) and incubated at 30 °C for 5 days. Harrison's disc method (Harrigan and McCance, 1976) was used to select 20 representative colonies based on the prevalent microbes that were developed on each dilution in a random statistical manner. These isolates were then restreaked onto 1/10 TSA to obtain pure cultures. Agar overlay assay was performed by adding the test microorganism strains into 0.8% (w/v) brain heart infusion (BHI) soft agar, overlaid on top of the patched plate and incubated at 37 °C for 18 h.

DNA extraction and 16S rRNA gene PCR analysis

All isolates (in total of 7 isolates) that showed antimicrobial activity against all test microorganisms via the presence of zone of inhibition using agar overlay assay were subjected to 16S rRNA gene PCR for bacterial identification. DNA template was obtained by boiling the colonies at 100 °C for 3 min and the suspension was centrifuged at 13000 × g for one minute. The lysate containing the bacterial DNA was then used as DNA template for the following PCR reaction. PCR was performed in a 50 µL of reaction mixture containing 5 × MyTaq Red Reduction Buffer, 1.25 U MyTaq DNA polymerase, 0.5 µM of 63f forward primer

(5'-CAG GCC TAA CAC ATG CAA GTC-3'), 0.5 µM of 1387 reverse primer (5'-GGG CGG WGT GTA CAA GGC-3') and 5 µL of DNA template (Marchesi *et al.*, 1998). The expected product size was 1300 bp. PCR amplification was performed using MJ Research PTC 200 thermocycler with initial denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 15 sec, annealing at 60 °C for 45 sec and elongation at 72 °C for 45 sec. PCR product was purified using Wizard SV Gel and PCR Clean-up and sent to 1st Base, Malaysia for sequencing. Sequencing analysis was done using Basic Local Alignment Search Tool (BLAST) from National Centre for Biotechnology Information (NCBI) to identify the isolates.

Preparation of crude cell free supernatant (CCFS)

Isolates which showed positive antimicrobial activity were grown in 1/10 TSB broth at 30 °C, 150 rpm for 8 days in a Smith SI-100 shaking incubator. The culture was centrifuged at 4000 × g for 30 min. The crude cell free supernatant (CCFS) was obtained and freeze dried.

Determination of Minimum Inhibitory Concentration (MIC)

MIC was performed to determine the lowest concentration of the antimicrobial compound to inhibit visible growth of a microorganism after overnight incubation. The freeze-dried CCFS was reconstituted in sterile water to 50 mg/mL, followed by filter sterilization and then subjected to MIC determination using broth microdilution, as according to CLSI (2010). One hundred microliters of bacterial suspension was added to each well containing 100 µL of different concentrations of reconstituted CCFS. Sterile water was used as a negative control, while 1 mg/mL of chloramphenicol, a broad spectrum antibiotic was used as the positive control. The antimicrobial activity of PS(14) CCFS was determined from the visible turbidity in each well after 24 h of incubation at 37 °C.

Partial characterization of antimicrobial compounds

In order to determine the pH stability of the antimicrobial compounds in a range from 1 to 13, CCFS was reconstituted in the following buffers: HCl-KCl buffer (pH 1), citrate buffer (pH 3 and 5), phosphate buffer (pH 7, pH 9 and pH 11), NaOH-KCl buffer (pH 13), and incubated at room temperature for 15 min (Lisboa *et al.*, 2006). The temperature stability of CCFS was determined by incubating it at -20 °C, 4 °C, 37 °C, 60 °C, 80 °C, 100 °C and 121 °C for 15 min. The CCFS was also treated with trypsin, α-chymotrypsin and proteinase K with a final concentration of 1 mg/mL for an hour to determine its susceptibility to proteolytic enzyme. The antimicrobial activity was then assayed using broth microdilution. The antimicrobial activity was regarded as stable if the MIC value was either one fold lower, one fold higher or similar to the controls used for each assay (CLSI, 2010).

Table 1: The annular radius of inhibition via agar overlay assay and the CCFS minimum inhibitory concentration (MIC) of isolate PS(14) against 12 test microorganisms.

	Test microorganisms	Annular radius (mm)	MIC (mg/mL)
Gram positive	<i>Bacillus cereus</i> (ATCC 14579)	4.3 ± 0.6	0.625
	<i>Enterococcus faecalis</i> (ATCC 29212)	13.0 ± 1.0	0.156
	VRE (ATCC 700802)	13.3 ± 1.5	0.313
	MRSA (ATCC 700699)	7.0 ± 1.0	1.250
	MRSA (ATCC 43300)	1.7 ± 0.6	0.625
	<i>Staphylococcus aureus</i> (ATCC 6538P)	2.0 ± 1.0	2.500
	<i>Staphylococcus aureus</i> (ATCC 29213)	1.7 ± 0.6	1.250
Gram negative	<i>Escherichia coli</i> (ATCC 25922)	2.7 ± 0.6	1.250
	<i>Klebsiella pneumoniae</i> (ATCC 10031)	6.3 ± 0.6	2.500
	<i>Salmonella enterica</i> (ATCC 14028)	3.0 ± 1.0	2.500
	<i>Shigella flexneri</i> (ATCC 12022)	4.3 ± 0.6	1.250
Yeast	<i>Candida albicans</i> (IMR)	9.7 ± 0.6	2.500

Scanning electron microscopy (SEM)

SEM was performed to determine the effect of PS(14) CCFS on the cellular morphology of the bacteria. MRSA (ATCC 700699) were grown in BHI broth at 37 °C for 18 h and the turbidity was adjusted to 0.5 McFarland standard. The MIC of CCFS was added to the adjusted bacterial culture. An untreated control was used as a negative control, while treatment with 1 mg/mL chloramphenicol was used as the positive control. All samples were incubated at 37 °C for 4 h. Fifty microliters of the mixture was placed on a glass slide and air dried for an hour. After incubation, culture was fixed with 2.5% (v/v) glutaraldehyde in phosphate buffer saline (PBS) at room temperature for 30 min. It was then washed with PBS three times to remove excess glutaraldehyde. The fixed glass slide was serially dehydrated with ethanol in PBS and left inside a desiccator for 12 h. The glass slide was then coated with platinum using Q150R Rotary-Pumped Sputter Coater before viewing using S-3400N VP SEM.

Determination of leakage of intracellular components

This assay was conducted to determine the effect of PS(14) CCFS on cell permeability thereby leading to leakage of intracellular components. Ten milliliters of MRSA (ATCC 700699) cultures were prepared in BHI broth. Cells were harvested by centrifugation (4000 × g, 10 min) at room temperature, washed twice and resuspended in sterile Tris/HCl buffer (pH 7.2). The MRSA suspension was adjusted to 1.5 OD₄₇₀ of 1.5. At time t=0 min, MIC of the CCFS was added to the cell suspension. Untreated cultures of MRSA were used as a negative control. Aliquots of both samples were removed at regular intervals of 30 min, centrifuged (12000 × g, 2 min) and the absorbance at 260 nm and 280 nm of the sample supernatants were measured. Absorbance at 260 nm was used to detect leakage of nucleic acid from the bacterial cell, while absorbance at 280 nm was used to detect the presence of protein.

Statistical analysis

Statistical analysis to determine significance of results were performed using t-test and one way analysis of variance (ANOVA) at the significance level of α = 0.05. Statistical analysis was performed using IBM SPSS Statistics 20.

RESULTS AND DISCUSSION

Isolation and identification of antimicrobial producing bacteria

Out of 500 isolates screened, 7 isolates demonstrated antimicrobial activity against all test microorganisms using agar overlay assay and were all identified as *Burkholderia* spp., with a maximum identity of 99% by comparing the 16S rRNA gene consensus sequence with NCBI database. However, only one isolate PS(14) was further studied as agar overlay assay showed similar zone of inhibition against all test microorganisms for all 7 *Burkholderia* sp. isolates.

Partial characterization of the antimicrobial compounds

In order to determine the pH and temperature stability of the antimicrobial compounds, the CCFS was subjected to different pH levels and temperature for 15 min. To perform preliminary characterization of the CCFS, MRSA (ATCC 700699) was chosen as the test microorganism to represent all Gram positive bacteria including the other *S. aureus* strains tested previously. It was found that the antimicrobial activity of PS(14) CCFS against MRSA (ATCC 700699) was stable at a wide range of pH ranging from pH 1 to pH 11 (Figure 1) and it was temperature stable from -20 °C to 80 °C (Figure 2). It is possible that the antimicrobial compounds produced by isolate PS(14) can withstand low pH to ensure that other microorganisms can be inhibited despite the low pH condition in tropical peat swamp.

Proteolytic enzymes treatment was also performed to determine whether the antimicrobial compounds are

proteinaceous in nature. This is because some pH and temperature stable antimicrobial compounds such as bacteriocin can be digested by these proteolytic enzymes (Marshall *et al.*, 2010). The antimicrobial activity of PS(14) CCFS was retained even after being treated with three different proteolytic enzymes: α -chymotrypsin, proteinase K and trypsin (Figure 3). From these results it can be deduced that the antimicrobial produced by isolate PS(14) might not be a protein, but further studies are required to confirm this. This result is not unusual as it is known that certain *Burkholderia* species such as *Burkholderia ubonensis* can produce pH and heat stable bacteriocin-like inhibitory substances that were also stable in the presence of several proteolytic enzymes, for instance pepsin, trypsin and papain (Marshall *et al.*, 2010). Quan *et al.* (2006) also showed that the antimicrobial compound (CF661) produced by *Burkholderia cepacia* demonstrated high stability when treated at a pH range of pH 3-11, temperature range from 30-100 °C and with proteolytic enzymes (proteinase K, pepsin and papain).

Mode of action

The mode of action of the antimicrobial activity against MRSA (ATCC 700699) was investigated by performing scanning electron microscopy (SEM) and intracellular component leakage assay (Figures 4 and 5). There was no change in the cellular morphology of the MRSA (ATCC 700699) but there was a reduction in bacterial viable cells after treatment with PS(14) CCFS (Figure 4). Furthermore, there was significant nucleic acid and protein leakage from MRSA (ATCC 700699) after 90 minutes of treatment with PS(14) CCFS (Figure 5). However, there was no conclusive result to determine the exact mode of action of PS(14) and therefore further studies are required to determine the mechanism of action of PS(14) CCFS. For instance, other membrane damage assays such as potassium ion leakage assay and ATP leakage assay can be performed (Al-Adham *et al.*, 1998; O'Neill *et al.*, 2004).

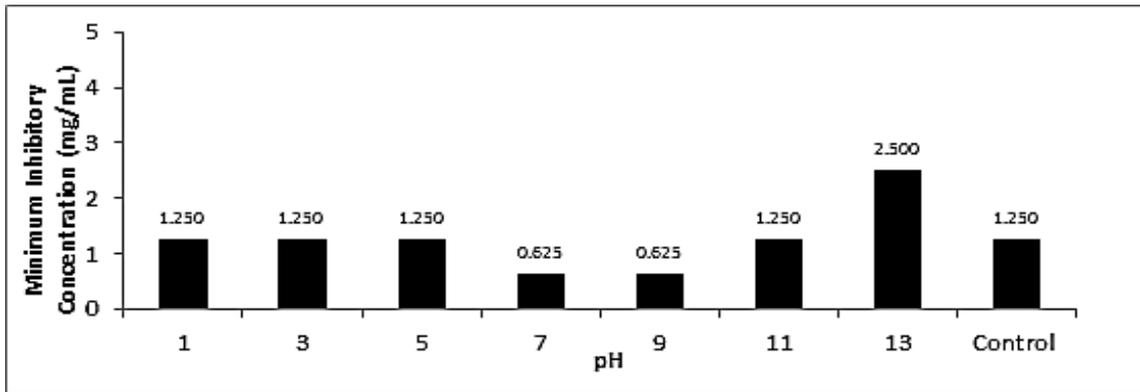


Figure 1: MIC of PS(14) CCFS against MRSA (ATCC 700699) after treatment at different pH for 15 min, CCFS reconstituted in sterile distilled water (pH 6.7) was used as control.

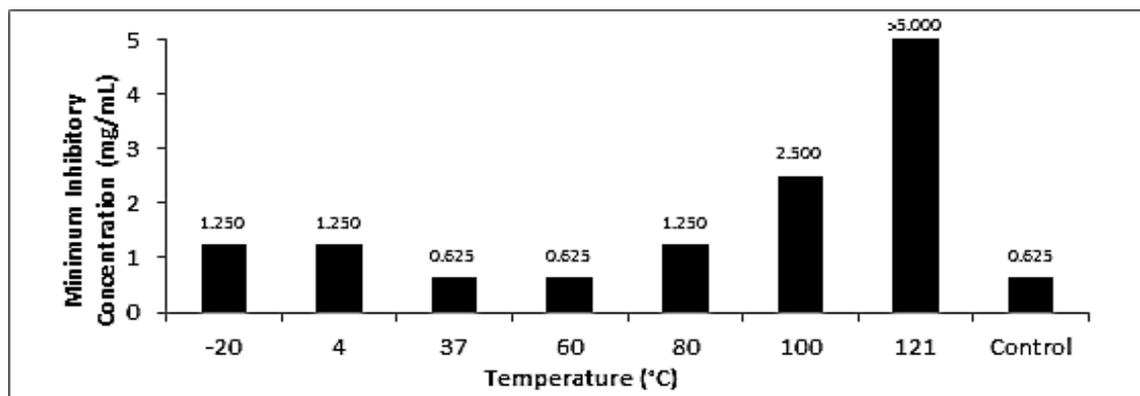


Figure 2: MIC of PS(14) CCFS against MRSA (ATCC 700699) after treatment at different temperature for 15 min, CCFS incubated at room temperature was used as control.

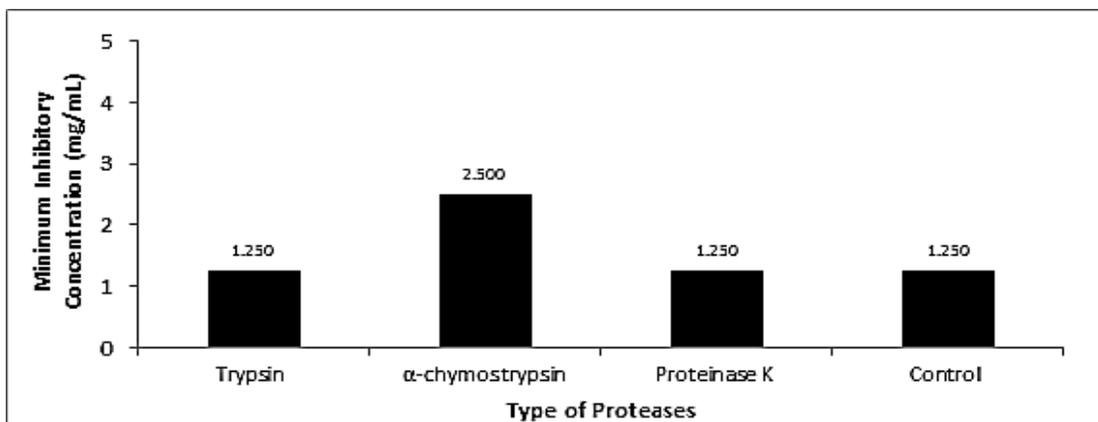


Figure 3: MIC of PS(14) CCFS against MRSA (ATCC 700699) after treatment with proteolytic enzymes for an hour. Untreated CCFS was used as control.

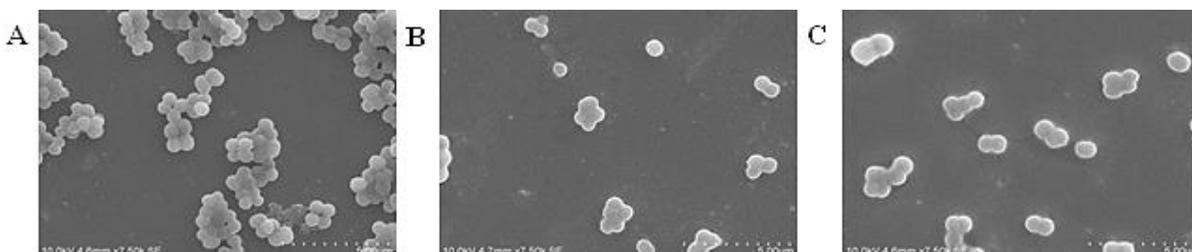


Figure 4: SEM images of MRSA (ATCC 700699) showing (A) negative control, (B) treated with 1 mg/mL chloramphenicol (positive control) and (C) treated with MIC (1.250 mg/mL) of PS(14) CCFS. Images were taken under 7500x magnification at 10 kV.

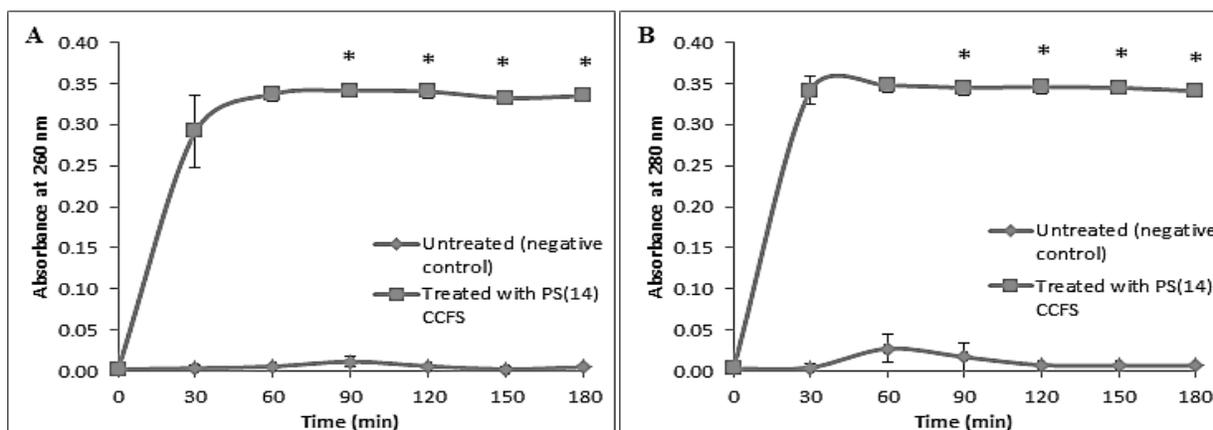


Figure 5: Leakage of (A) protein (260 nm) and (B) nucleic acid (280 nm) from cell suspensions of MRSA (ATCC 700699) exposed to MIC (1.250 mg/mL) of PS(14) CCFS. The absorbance was reported in mean with error bars of \pm 1 standard deviation. Asterisk above the line graph represents significant difference ($p < 0.05$) between the untreated (negative control) and treated with PS(14) CCFS.

CONCLUSIONS

An antimicrobial producing bacteria was successfully isolated from the Southeast Pahang tropical peat swamp soil, identified as *Burkholderia* spp. via 16S rRNA PCR with a maximum identity of 99%. It was

found that the antimicrobial produced had broad spectrum antimicrobial activity against Gram positive bacteria, Gram negative bacteria and yeast. The Antimicrobial activity of PS(14) CCFS was stable at a pH range from 1 to 11, temperatures of -20 °C to 80 °C and after treatment with several proteolytic enzymes: α -chymotrypsin, proteinase K and trypsin,

indicating that the antimicrobial produced might not be proteinaceous. It was hypothesized that the PS(14) CCFS was able to affect the cellular membrane, leading to nucleic acid and protein leakage from MRSA (ATCC 700699) but further studies with the pure compounds are required to determine the actual mode of action. However, this preliminary study had shown that tropical peat swamp forests are indeed potential locations for antimicrobial compounds to combat infections.

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