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Antimicrobial Activity of Spermine Alkaloids From *Samanea Saman* against Microbes Associated with Sick Buildings

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Abstract. Bioassay-guided fractionation and isolation of compounds with antimicrobial activity were performed on the 80% methanolic extract of a legume tree (*Samanea saman*) leaves yielding two known macrocyclic spermine alkaloids, pithecolobines 1–2. The structures of these compounds were elucidated by spectral analyses and compared with literature data. The antimicrobial activity of these compounds was evaluated using a disk diffusion method against one Gram-positive bacterium (*Bacillus subtilis*) and four filamentous fungi (*Aspergillus flavus*, *Aspergillus niger*, *Penicillium oxalicum* and *Cladosporium oxysporum*) that were isolated from sick enclosed buildings. The minimum inhibitory concentration (MIC) for each compound against the highly sensitive microorganisms: *B. subtilis* and *P. oxalicum* was determined using a two-fold serial dilution assay. The MIC values of alkaloid (2) were in the range of 0.019 – 0.625 mg/ml, whereas alkaloid (1) only inhibited *B. subtilis* with MIC of 0.312 mg/ml. These results provided evidence that the isolated compounds, especially pithecolobine 2, might be potential plant-based formulations for management of microbes in sick enclosed buildings.

Keywords: Antimicrobial activity, MIC, *Samanea saman*, Pithecolobine, Sick-building syndrome, Biocide

1. Introduction

Various species of bacteria and fungi have been found growing on all building materials and surfaces [1, 2]. These organisms cause corrosion and degradation of materials and components of building materials and thereby load the indoor air environment with harmful spores and substances, thus leading to poor indoor air quality, sick building syndrome and building-related diseases [1, 3, 4]. Treatment of decay in buildings by synthetic biocides has now been restricted due to their harmful effects to the environment, residual toxicity and carcinogenic nature [5]. Moreover, the constant use of chemicals may induce resistance in a target organism. This concern has encouraged researchers to search for efficient method to prevent and cure the harmful effects of microbes in a more eco-friendly manner. Biocides derived from plants are safer, more effective and environment-friendly alternatives for microbial infection because they are rich in bioactive secondary metabolites such as alkaloids, flavonoids, terpenes, coumarins and saponins. Several investigators have demonstrated potential of some plants as possible source of anti-airborne microbial compounds [6, 7]. In our efforts to screen plants for antimicrobial activity, we demonstrated the potential of methanolic leaves extract of *Samanea saman* as an anti-airborne microbial agent using *B. subtilis, Cladosporium oxysporum, Penicillium oxalicum, Aspergillus flavus* and *A. niger* isolated from sick buildings [8].

Samanea saman (Jacq.) Merr. (syn. *Pithecolobium saman*) (Fabaceae), commonly known as "Rain tree", is a widely distributed and cultivated legume in the tropical and subtropical countries [9]. It is a folk remedy

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for cold, diarrhea, headache, intestinal ailments, stomach cancer, sore throat and stomachache. The antimicrobial activity of this plant was reported against some human and plant pathogenic bacteria and fungi [10-12]. Rain tree is also known to have antiplasmodial, antioxidant and cytotoxic properties [13-15]. Previous phytochemical studies revealed the occurence of alkaloids, saponins, flavonoids, tannins, terpenoids, cardiac glycosides and steroidal compounds [11, 16-18]. As a follow up of our earlier studies to determine the compounds active against these microorganisms we carried out activity guided fractionations that led to identification of the alkaloids as the compounds responsible for activity. These alkaloids had previously been isolated and characterized [16]. In this paper we report the *in vitro* antimicrobial activities of these alkaloids.

2. Materials and Methods

2.1. General Experimental Procedures

Mass spectra were recorded on a high resolution mass spectrometer Bruker 7. OT. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker 300 MHz Ultrashield spectrometer equipped with a 5 mm BBI inverse spectra gradient probe. Silica gel column chromatography was carried out using silica gel (0.060-0.200 mm pore diameter ca 6 nm Across organics). Thin layer chromatography (TLC) was conducted with Merck silica gel 60 F_{254} on plates. Spots on TLC were visualized by spraying with Dragendorff's reagent.

2.2. Plant Material

The leaves of *S. saman* were collected from Universiti Sains Malaysia Campus, Malaysia in June, 2009. The botanical identification was made by comparing with authentic herbarium specimen at the Herbarium, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. A voucher specimen (No.11020) was deposited at the Herbarium.

2.3. Source of Microorganisms

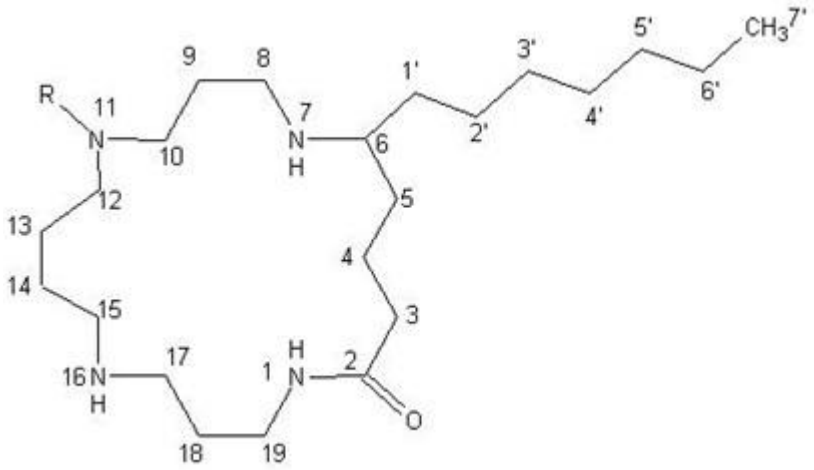
Four species of fungi and one species of bacteria isolated during a series of indoor samplings in air-conditioned sick buildings [19] were obtained from the culture collection unit of the Plant Pathology Laboratory, School of Biological Sciences, Universiti Sains Malaysia. The microorganisms were identified as *Bacillus subtilis* (BP1BA), Aspergillus *flavus* (AP22BA), *A. niger* (AP29BA), *Penicillium oxalicum* (PP1BA) and *Cladosporium oxysporum* (CPIBA) and kept in 15% glycerol at -86 ℃ [20]. The work cultures of bacterium and fungi were maintained at 4 ℃ on Nutrient Agar (NA) and Potato Dextrose Agar (PDA) slants, respectively.

2.4. Extraction and Isolation

The dried and ground plant material (1.5 kg) was exhaustively extracted with a mixture of MeOH: H_2O (8: 2) in a Soxhlet apparatus and concentrated *in vacuo* to afford 349.5 g of extract. The extract was then treated with 5% HCL to pH~2 and extracted with $CHCL_3$ (3 × 800 ml). The $CHCL_3$ extract was concentrated to dryness to give the acid extract NCSS (6.96 g). The aqueous residue was basified with 25% NH_4OH to pH~10 and extracted repeatedly with $CHCL_3$ (3 × 800ml). The combined $CHCL_3$ was washed with distilled water, dried over anhydrous (Na_2SO_4), filtered and evaporated to dryness giving the basic extract CSS (6.67 g). The antimicrobial activity of NCSS and CSS was determined. The basic extract CSS, that was proven to be active against all the tested microorganisms, was subjected then to column chromatography (75.5 cm x 3.5 cm) on silica gel (154.28 g) eluting with $CHCL_3$, $CHCL_3$: MeOH (98: 2, 96: 4, 94: 6, 90: 10, 80: 20) and finally with MeOH affording 39 fractions. The same fractions were combined according to TLC analysis to yield compounds **1** (226 mg) and **2** (394 mg) (Figure 1).

Pithecolobine (**1**), $C_{22}H_{46}N_4O$, was obtained as brown oil. EIMS m/z [M^+] 382 (100), 381 (7.3), 370 (9.5), 369 (44.5), 354 (13.0), 353 (62.1), 317 (2.3), 301 (3.4), 229 (2.9).^{1}H-NMR (DMSO): δ 0.85 (3H, t, *J*= 6.18 Hz, H-7'), 1.25 (12H, *m*, H-1' to H-6'), 1.6-2.1 (8H, *m*, H_2-7, H_2-11, H_2-12, H_2-16), 3.1 (1H, *m*, H-6), 3.34 (1H, *m*, H-17α), 3.4 (1H, *m*, H-17b), 7.6 (1H, s, N-H).^{13}C-NMR (DMSO) δ 167.9 (C-2), 38.79 (C-3), 22.4 (C-4), 31.7 (C-5), 57.7 (C-6), 46.7 (C-8), 29.4 (C-9), 50.6 (C-10), 48.5 (C-12), 28.7 (C-13), 22.3 (C-14), 67.6 (C-15), 66.1 (C-17), 23.5 (C-18), 37.0 (C-19), 33.6 (C-1'), 27.2 (C-2'), 29.4 (C-3'), 29.2 (C-4'), 29.1 (C-5'), 22.7 (C-6'), 13.1 (C-7').

Pithecolobine (**2**), $C_{22}H_{46}N_4O_2$, was obtained as yellow oil. EIMS *m/z* [M^+] 398 (17.7), 382 (23.3), 381(100), 369 (44.5), 354 (13), 353 (62.1), 317 (2.3), 301 (3.4), 229 (2.9).^{1}H-NMR (DMSO) δ 0.8 (3H, t, *J*= 5.17 H_z, H-7'), 1.28 (12H, *m*, H-1' to H-6'), 1.6-2.2 (8H, *m*, H_2-7, H_2-11, H_2-12, H_2-16), 3.2 (1H, *m*, H-6), 3.25 (1H, *m*, H-17α), 3.3 (1H, *m*, H-17b), 5.3 (1H, N-O-H) , 8.5 (1H, s, N-H) .^{13}C-NMR (DMSO): 174.2 (C-2), 38.8 (C-3), 22.37 (C-4), 31.7 (C-5), 55.8 (C-6), 46.8 (C-8), 29.4(C-9), 54.9 (C-10), 48.5 (C-12), 29.0 (C-13), 22.3 (C-14), 55.7 (C-15), 55.8 (C-17), 23.48 (C-18), 37.9 (C-19), 33.6 (C-1'), 26.7 (C-2'), 29.37 (C-3'), 29.1 (C-4'), 29.0 (C-5'), 22.9 (C-6'), 13.0 (C-7').



Pithecolobine **1**: R = H
Pithecolobine **2**: R = OH
Fig. 1: Spermine alkaloids from *Samanea saman*.

2.5. Antimicrobial activity test

All samples dissolved in dimethylsulfoxide (DMSO) were filter-sterilized through Millipore 0.2 µm filters under a laminar hood. *In vitro* antimicrobial activity tests were carried out by disc diffusion method [21] with a slight modification in volume and concentration of tested compounds. Briefly, 0.1 ml of suspension containing 10^8 CFU/ml of bacterium and 10^4 spore/ml of fungi was spread on nutrient agar (NA Hi Media Pvt. Ltd.) and potato dextrose agar (peeled potato 250 g, dextrose 20 g, agar 15 g, distilled water 1 Liter), respectively. Sterilized Whatman AA discs (6 mm in diameter) containing 20 µl of each sample at 100 mg/ml concentration (2 mg/disc) were placed on the inoculated agar. Discs prepared with only the corresponding volume of DMSO were used as negative control. The plates were incubated at room temperature (28 ± 2°C) for 48 h in case of bacterium and 3 - 5 days for the fungi isolates. Each sample was tested against each organism in triplicate. The cultures were examined for areas of no growth around the disc (zone of inhibition). The microorganisms that were susceptible to antimicrobial agents were inhibited at a distance from the disc whereas the resistant strains grew up to the edge of disc. Measurement of the inhibition zones around the discs were done using rulers and expressed in millimeter (mm) unit.

2.6. Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the isolated active alkaloids against the highly sensitive microorganisms i.e. *B. subtilis* and *P. oxalicum* were determined by agar dilution method [22, 23] with a slight modification. In treated plates, the amount of the isolates was dissolved in 1 ml sterile distilled water and then mixed thoroughly with 9 ml of molten agar at 50 ℃ in pre-labeled sterile Petri dishes to obtain a series of final concentrations of compounds between 0.625 and 0.0097 mg/ml for *B. subtilis* and 5 – 0.312 mg/ml for *P. oxalicum*. The negative control was prepared using sterile distilled water only. The surfaces of the media were allowed to dry before streaking with 20 µl of suspension containing 10^8CFU/ml of bacterium and 10^4spore/ml of fungi. The plates were incubated at room temperature (28 ± 2°C) for 48 h in case of *B. subtilis* and 72 h for *P. oxalicum*. The lowest concentration of an antimicrobial agent at which there was no visible growth of a microorganism after incubation was taken as MIC [22, 24, 25].

3. Results

Table 1 shows the results of antimicrobial activities of fractions and alkaloids isolated. The total crude alkaloid fraction (CSS) showed significant antimicrobial activities against all of the five test organisms with zone of inhibition ranging from 16.67 ± 0.57 mm to 46 ± 1.00 mm at a concentration of 100 mg/ml whereas the acid partitionate (NCSS) remained insensitive to the microorganisms at the test concentration. Based on these findings, the re-fractionation of (CSS) fraction on silica gel led to the isolation of two active alkaloids, Pithecolobine **1** and **2**.

Table 1: The diameter of inhibition zones of spermine alkaloids (1-2) and fractions

Material	Diameter of inhibition zone (mm) *				
	Bacillus subtilis	Aspergillus niger	Aspergillus flavus	Cladosporium oxysporum	Penicillium oxalicum
NCSS	-	-	-	-	-
CSS	46.00 ± 1.00	17.33 ± 0.57	16.67 ± 0.57	18.33 ± 1.52	19.00 ± 1.00
Pithecolobine 1	20.00 ± 0.00	-	-	-	-
Pithecolobine 2	30.67 ± 1.15	10.00 ± 0.00	8.00 ± 0.00	8.33 ± 0.57	10.33 ± 0.57

Annotation: * refers to inhibition zone including the diameter of disk paper (6 mm); - refers to no inhibition zones; NCSS (non basic $CHCL_3$ extract), CSS (basic $CHCL_3$ extract); values are mean inhibition (mm) ± S.D of three replicates.

These compounds were identified by direct comparison of their ^{1}H and ^{13}C NMR and mass spectra with those previously found and described by Wiesner *et al*. [16, 26, 27]. Pithecolobine **2** presented the widest antimicrobial activity by inhibiting all the tested microorganisms with the highest activity against the bacterium than fungi, whereas pithecolobine **1** was only active against *B. subtilis* (Table 1). MIC values of compounds (**1**-**2**) were determined against the highly sensitive microorganisms i.e. *B. subtilis* and *P. oxalicum* using agar dilution method. The results (Table 2) showed that compound **2** was the most potent against *B. subtilis* with MIC of 0.019 mg/ml.

Table 2: MIC values (mg/ml) of spermine alkaloids (1-2)

Material	*Bacillus subtilis*	*Penicillium oxalicum*
Pithecolobne 1	0.312	-
Pithecolobne 2	0.019	0.625

Annotation: - not active

4. Discussion

As part of a program of studying Malaysian local flora for antimicrobial activity, it was observed that an 80% MeOH leaves extract of *S. saman* showed inhibitory activity against the more common microorganisms isolated from sick buildings. This activity has been traced to the alkaloids pithecolobine **1** and **2** using systematic fractionation guided by antimicrobial assay.

To our knowledge, no reports are available on the antimicrobial activity of pithecolobines. The macrocyclic spermine alkaloids (pithecolobine **1** and **2**) were first isolated and characterized from the bark of *S. saman* [16]. A series of macrocyclic spermine alkaloids (budmunchiamines) belong to the pithecolobine class of alkaloids have been reported from plants of *Albizia* genus, a close taxonomic relatives of *Samanea saman i*n the family Fabaceae [28- 32]. Alkaloids of this structural type have been found to possess antibacterial, antifungal, antiplasmodial and cytotoxic properties [30-31]. In the current study, we demonstrated for the first time that the two substances **1** and **2** isolated from *S. saman* might serve as the main components responsible for *in vitro* antimicrobial activity seen in 80% MeOH extract. In the literature, antimicrobial activity of plant secondary metabolites against the common microorganisms in the indoor environment has been reported. *Pinus sylvestris* essential oil showed inhibitory activity against a variety of

airborne microorganisms including *Chaetomium globosum, Cladosporium cladosporioides, Aspergillus versicolor, A. niger, Aureobasidium pullulans, Paecilomyces variotii, Penicillium chrysogenum, Phoma* sp., *Rhizopus stolonifer, Stachybotrys chartarum, Trichoderma viride, Ulocladium atrum, Rodococcus* sp., *Bacillus* sp., *Candida lipolytica* and *Geotrichum candidum* isolated from the human environment [7]. Volatile oils obtained from *Juniperus communis, Abies alba, Foeniculum vulgare, Thymus vulgaris, Thymus serpyllum* and *Pinus sylvestris* were active against four types of moulds isolated from two buildings: two species of *Alternaria, Penicillium* sp., and *Auerobasidium* sp. [33].

In conclusion, within the large reservoir of natural biocides that exist in plants, it is reasonable that bioactive compounds can serve as safe and effective alternatives to synthetic biocides.

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6. References

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