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Brief communication (Original)

Bioactivity of plant extracts against *Burkholderia pseudomallei*

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Background: *Burkholderia pseudomallei* are causative agents of melioidosis, a disease found mostly in South-East Asia and Northern Australia. Recent reports of a reduced susceptibility of *B. pseudomallei* to antibiotics, especially ceftazidime, have indicated need for further research into new antimicrobial substances from plants.
Objectives: We tested antimicrobial activity of 10 plant extracts; *Barringtonia acutangula* (L.) Gaertn., *Cleome gynandra* Linn., *Luffa acutangula* (Linn.) Roxb., *Limnophila geoffrayi* Bonati, *Centella asiatica* (L.) Urban, *Piper sarmentosum* Roxb., *Tamarindus indica*, *Cyperus rotundus* Linn., *Cassia fistula* Linn., and *Allium sativum* Linn.
Materials and Methods: Crude extracts were tested for their antimicrobial activity by the standard disc diffusion assay and micro-dilution assay. Methanol, ethyl acetate, ethanol, hexane, and water were used as solvents for extraction.
Results: The methanolic extract of *B. acutangula* (L.) Gaertn. showed the best antimicrobial results against *B. pseudomallei* with an inhibition zone of 18 mm and minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of 4 mg/ml. The chemical structure of crude methanolic extracts of *B. acutangula* (L.) Gaertn. was primarily elucidated by nuclear magnetic resonance spectroscopy (NMR). The results showed that the chemical constituent was in the group of steroids.
Conclusions: It is concluded that *Barringtonia acutangula* (L.) Gaertn. may play an active part in the inhibition of the growth of *B. pseudomallei*.

Keywords: Antimicrobial activity, *B. pseudomallei*, disc diffusion, plant extracts

Burkholderia pseudomallei, a causative agent of melioidosis, is an important pathogen in tropical regions of Northern Australia and South-East Asia, including Thailand [1]. Several case have been reported from other parts of the world [16], including Central and South America, Middle East, Pacific nations, several African countries, and a recent case in Spain of a diabetic immigrant who had visited West Africa during the rainy season [16-19]. It is an intracellular pathogen that can give rise to latent or dormant infections in tropical regions. The bacteria can be transmitted via inoculation, inhalation of contaminated soil, and/or surface water. Clinical manifestations can vary from acute infection and chronic localized pathologic symptoms to latent infection that may reactivate decades later. In northern Thailand, the disease accounts for 20% of all community-acquired septicemias and causes death in 40% of treated patients. The high mortality rate is usually due to septic shock [2, 3]. Several factors may explain the remarkably high incidence of melioidosis and the immunosuppressive predisposing factor [3]. The disease is found more in people with an underlying risk factor such as diabetes and renal disease [4]. *B. pseudomallei* is intrinsically resistant to many antibiotics, including penicillin, and first- and second-generation cephalosporins, macrolides, rifamycins, colistin, and aminoglycosides [1, 3]. Currently, the antibiotic of choice for the treatment of acute melioidosis is ceftazidime [5]. Moreover, resistance to ceftazidime was recognized soon after this antibiotic was widely used for the treatment of severe melioidosis.

Antimicrobial resistance is a subject of great concern to public health and in the design of strategies for current therapeutic protocols. New drugs, including those necessary for a reserve armamentarium and exhibiting fewer side-effects, deserve attention.

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In Thailand, a number of natural products, mainly from plants, have been used to study activity against *B. pseudomallei*. The aim of this study was to screen the antimicrobial activity of 10 plant extracts; *Barringtonia acutangula* (L.) Gaertn., *Cleome gynandra* Linn., *Luffa acutangula* (Linn.) Roxb., *Limnophila geoffrayi* Bonati, *Centella asiatica* (L.) Urban, *Piper sarmentosum* Roxb., *Tamarindus indica*, *Cyperus rotundus* Linn., *Cassia fistula* Linn., and *Allium sativum* Linn.

Materials and methods

Plant materials and preparation of extracts

The 10 plant materials used in this study were collected from various locations in Thailand (**Table 1**). *B. acutangula*, *C. gynandra*, *L. acutangula*, *L.geoffrayi*, *C, asiatica*, *P. sarmentosum* were collected from Roi-ed province. *T. indica*, *A. sativum* and *C. fistula* were collected from Khon Kaen province. *C. rotundus* was collected from Ubon Ratchathani province. All plants were identified according to a Thai Forest Bulletin [20, 21]. Approximately 500 g of dried and ground plant material was extracted by maceration with hexane, methanol, ethyl acetate, ethanol, and water. Organic extracts were concentrated, and aqueous extracts were freeze-dried. All extracts were stored in dark bottles at 4°C until use. Stock solutions were prepared from concentration of 100 mg/ml from the following: *B. acutangula*, *T. indica, A. sativum,* and *C. gynandra.* Concentrations of 50 mg/ml of stock solution were prepared for *L. geoffrayi*, *C. asiatica*, *C. fistula, C. rotundus* and *P. sarmentosum*, and a concentration of 40 mg/ml of stock solution was prepared for *L. acutangula*. For organic extracts, dimethyl sulfoxide (DMSO) was used as a solvent. Aqueous extracts were prepared in distilled water and sterilized by filtration through a 0.22 μm membrane (Satorins, Minisart ®). Blank paper discs (6 mm diameter) were loaded with 50 μL of the prepared stock solutions to obtain the desired concentration per disc and allowed to air dry at room temperature. The air-dried discs were prepared before use.

Bacterial strains and culture conditions

B. pseudomallei used in all experiments (strain G207 and A2) were isolated from a septic shock patient in Srinagarind Hospital in Khon Kaen province, Thailand where melioidosis is endemic. The organism was identified by biochemical tests and immunoreactivity with polyclonal and monoclonal antibodies [6, 7]. The LD_{50} of G207 and A2 were 20 CFU/mouse. For experimental bacterial preparation, an overnight shaken culture of *B. pseudomallei* in tryticase soy broth (TSB) (Criterion, Santa Maria, USA, Hardy Diagnostics) was sub-cultured into 2%TSB, incubated at 37°C with shaking, and then diluted in phosphate buffered saline (pH 7.2) to the desired concentration ($1X10^8$ CFU/ml). The exact number of viable bacteria in the suspension was determined by plating them on nutrient agar for bacterial counts after 30 to 48 hours of incubation at 37°C and expressed as CFU/ml. All solutions used were sterilized, and all procedures described were carried out in a biosafety hood (Dwyer Mark II, Michigan, DW Yer Instruments, Inc.).

Table 1. Plant data

Scientific name	Family	Parts of plant sampling
Barringtonia acutangula (L.) Gaertn	Barringtoniaceae	leaves
Cleome gynandra Linn.	Capparidaceae	whole
Luffa acutangula (Linn.) Roxb.	Cuphorbiaceae	leaves
Limnophila geoffrayi Bonati	Scrophlariaceae	whole
Centella asiatica (L.) Urban	Umbeliferae (Apiaceae)	leaves
Piper sarmentosum Roxb.	Piperraceae	leaves
Tamarindus indica	Fabaceae	fruit
Cyperus rotundus Linn.	Cyperaceae	whole
Cassia fistula Linn.	Leguminosae-Caesalpinioideae	leaves
Allium sativum Linn.	Alliaceae	fruit

Antimicrobial susceptibility testing [15]

Disc diffusion method

Muller Hinton (MH) Agar (Criterion, Santa Maria, USA, Hardy Diagnostics) was used as a media and poured into a sterilized petri dish and left to solidify. The bacterial suspension was adjusted to $1.0X10^5$ CFU/ml. MH agar plates were streaked evenly with a swab dipped into the bacterial suspension. Lids were left ajar for three minutes in a laminar flow cabinet to allow for any excess surface moisture to be absorbed into the agar before the impregnated discs were applied. Discs containing the test agents were applied to the surfaces of inoculated plates. Plates were incubated at 37°C for 24 hours. Inhibition zone diameters were measured in millimeters (mm). All isolates were run in triplicate, and the standard deviations were determined. The assay was based on the Kirby-Bauer method. Ceftazidime (30µg/disc) and solvent were used as a control.

Determination of minimum inhibitory and bactericidal concentrations

The minimal inhibitory concentration (MIC) values were determined by micro-dilution assay [8] with dilution ranging from 0.125 to 128 mg/ml. The MIC was defined as the lowest concentration of the compound to inhibit the growth of *B. pseudomallei* and the minimal bactericidal concentration (MBC) was defined as the lowest concentration of the compound to kill the *B. pseudomallei*. The MIC and MBC values were performed on the compounds, which expressed the inhibition zone by disc diffusion method. All assays were performed in duplicate.

Nuclear magnetic resonance spectroscopy (NMR)

^{1}H NMR spectra were recorded on Bruker AVANCE (300 MHz) spectrometers. The residue of the non-denatured solvent was used as an internal standard which was related to tetramethylsilane with δ = 7.26 ppm for $CDCl_3$. ^{13}C NMR spectra were recorded on a Bruker AVANCE (75 MHz) with the residue of the non-deuterated solvent peak as the internal standard, δ = 77.00 ppm for $CDCl_3$.

Results

Antibacterial activity of plant extracts against B. pseudomallei

Ten plant extracts were examined for antimicrobial activity against *B. pseudomallei*. Plant material was extracted by maceration with hexane, methanol, ethyl acetate, ethanol, and water. Only the crude extracted from methanol was active against *B. pseudomallei* by disc diffusion. The results showed that four extracts could effectively inhibit the growth of *B. pseudomallei*. Among those, the extracts of *B. acutangula* and *L. acutangula* showed strong inhibition effects (zone of inhibition ≥11 mm) as shown in **Table 2**. *C. fistula* showed inhibition zone 12 mm against to *B. pseudomallei* strain A2 and 8 mm for to *B. pseudomallei* strain G207. Interestingly, *B. acutangula* showed promising antibacterial activity similar to cefatzidime against *B. pseudomallei*. Subsequent experiments were conducted to determine inhibitory concentrations of plant extracts, which expressed inhibition zones by the disc diffusion. *B. acutangula* showed the greatest antimicrobial effect. The MIC and MBC values against both organisms were equal (4 mg/ml). In addition, *L. acutangula* also showed antimicrobial effects against *B. pseudomallei* with a MIC of 64 mg/ml and a MBC of 128 mg/ml. The remaining plant extracts had no detectable activity and required a high concentration to inhibit and kill *B. pseudomallei* (>128 mg/ml) (**Table 3**).

Table 2. Disc diffusion data of methanolic extracts of plant

Plant material	Concentration (mg/disc)	Diameter of the inhibition zones (mm)	
B. pseudomallei		A2	G207
Barringtonia acutangula (L.) Gaertn	5.0	18	16
Cleome gynandra Linn.	5.0	NC	NC
Luffa acutangula (Linn.) Roxb.	2.0	11	14
Limnophila geoffrayi Bonati	2.5	8	8
Centella asiatica (L.) Urban	2.5	NC	NC
Piper sarmentosum Roxb.	2.5	NC	NC
Tamarindus indica	5.0	NC	NC
Cyperus rotundus Linn.	2.5	NC	NC
Cassia fistula Linn.	2.5	12	8
Allium sativum Linn.	5.0	NC	NC
Ceftazidime (positive control)	30 (µg/disc)	13	16
Methanol (negative control)	5.0	NC	NC

NC = no clear zone

Table 3. MIC and MBC of crude extracts

Plant material/ *B. pseudomallei*	MIC (mg/ml)		MBC (mg/ml)	
	A2	G207	A2	G207
Barringtonia acutangula (L.) Gaertn	4	4	4	4
Luffa acutangula (Linn.) Roxb.	64	64	128	128
Cassia fistula Linn.	>128	>128	>128	>128
Limnophila geoffrayi Bonati	>128	>128	>128	>128
Ceftazidime	0.25	0.25	0.25	0.25

Chemical constituents

The study of chemical constituents of crude extracts from *B. acutangula* was performed by ^{1}H NMR and ^{13}C NMR spectroscopy. The ^{1}H NMR and ^{13}C NMR spectra showed signals in an aliphatic region with the pattern of steroid.

Discussion

Melioidosis accounts for 20% of all community-acquired septicemias and is the most common cause of mortality is severe sepsis. Although ceftazidime is the drug of choice, the mortality rate in treated patients has still been found to be more than 40% [3]. With the increasing development of antibiotic resistance of antibiotics to this organism, it is necessary to search for novel anti-infective agents. In the present study, 10 plant extracts were examined for antimicrobial activity against *B. pseudomallei*. Plant material was extracted by maceration with hexane, methanol, ethyl acetate, ethanol, and water. Only the crude extracted from methanol was active against *B. pseudomallei* by disc diffusion. This study is in accord with that of Ghasemzadeh and co-workers who studied solvent for extraction to examine the antibacterial and antioxidant activity. The results showed the highest activities were found in methanol extracts, and methanol was recommended to extract phenolic compounds from young ginger [9]. Moreover, the methanolic extract was found to be the most effective high antioxidant activity [10.

The methanolic extracts showed that four extracts could effectively inhibit the growth of *B. pseudomallei* by disc diffusion. However, only *B. acutangula* and *L. acutangula* can inhibit growth of *B. pseudomallei* with an inhibition zone of more than 11 mm in both strains. Subsequent experiments were conducted to determine inhibitory concentrations of all antimicrobial effect by disc diffusion. *B. acutangula* showed the greatest antimicrobial effect. The MIC values against both strains of *B. pseudomallei* were equal (4 mg/ml) and MBC values were equal too (4 mg/ml). These results suggested that *B. acutangula* extract could possibly act as a bactericidal agent to *B. pseudomallei*. However, *B. acutangula* showed the highest concentration to inhibit and kill when compared to ceftazidime activity. *L. acutangula* showed inhibited growth of *B. pseudomallei* but MIC values were 64 mg/ml and required a high concentration to kill *B. pseudomallei* (MBC values were 128 mg/ml). The remaining two plant extracts also showed poor antimicrobial effect against *B. pseudomallei* with MIC and MBC of >128 mg/ml.

The crude methanolic extracts from *B. acutangula* were further analyzed by ^{1}H NMR and ^{13}C NMR spectroscopy. The ^{1}H NMR and ^{13}C NMR spectra showed signals in an aliphatic region with the pattern of steroid. The structure showed the same pattern as a water extract of dried bark of *B. acutangula* [11] but further research is required. However, there are several reported antimicrobial actions of compounds containing steroid. Fresh fruiting bodies of *Fomitopsis pinicola* extracted with methanol showed antimicrobial activity against *Bacillus subtilis* in TLC bioassay and crude extracts showed the pattern of steroids [12]. In addition, studies of antimicrobial activity indicated that crude extracts containing steroids showed significant activity against various strains of *Staphylococcus aureus*, *Enterococcus faecalis*, and *Escherichia coli* [13]. Moreover, crude extracts isolated from *Chromolaena squalid* from leaves and stems containing steroids showed significant activity against gram positive and gram negative bacteria, mainly against gram positive bacteria [14].

Conclusion

Steroids may be active against the inhibition of bacterial growth because of their antimicrobial effects. However, *B. acutangula* was active against *B. pseudomallei* only in the case of methaloic extract and showed an inhibition zone not different from ceftazidime by standard disc diffusion. However, the test of inhibition concentration was less active when compared to ceftazidime. The result showed the inhibition concentration of methanolic extract from *B. acutangula* was 15 times higher than ceftazidime. It is possible the crude extracts may have a synergism effect but further investigation is required in the search for the active part for the inhibition of the growth of *B. pseudomallei*.

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