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**Original Research Article**

# Bactericidal, Bacteriolytic, and Antibacterial Virulence Activities of *Boesenbergia pandurata* (Roxb) Schltr Extract against *Streptococcus pyogenes*

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## Abstract

***Purpose:*** *To determine the anti-Streptococcus pyogenes activity of the chloroform extract of Boesenbergia pandurata (Roxb.) Schltr. (Zingiberaceae) and investigate its possible antibacterial mechanisms of action.*
***Methods:*** *Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were investigated against 47 clinical isolates of S. pyogenes. Time-kill study was performed to determine how quickly the extract acts on S. pyogenes. The ability of the extract to damage bacterial cell wall and effects on S. pyogenes virulence factors including protease enzyme and haemolysin were investigated.*
***Results:*** *The extract exhibited good antibacterial activity against all of the clinical isolates of S. pyogenes with similar MIC and MBC ranging from 3.91-62.50 µg/ml. Complete killing of the bacterial cells by the extract at concentrations of 4MIC, 2MIC, and MIC occurred within 4, 8, and 12 h, respectively. Treatment of the bacterial cells with the extract at 2MIC and 4MIC caused cell lysis. All the test concentrations (1/32 - 1/2MIC) produced no effects on protease and haemolysin enzymes.*
***Conclusion:*** *Boesenbergia pandurata extract has powerful in vitro activity against S. pyogenes. The ability of the extract to lyse the bacterial cells suggests that the mechanism of action may be associated with cell wall and cell membrane damage.*

***Keywords:*** *Antibacterial, Bacteriocidal, Bacteriolytic, Virulence, Boesenbergia pandurata, Streptococcus pyogenes.*

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## INTRODUCTION

*Boesenbergia pandurata* (Roxb.) Schltr. (Syn. *Kaempferia pandurata*, *Boesenbergia rotunda*) is an edible medicinal plant and spice from the ginger family (Zingiberaceae). This medicinal plant is generally used as a traditional medicine in Southeast Asia. The rhizome of this plant is widely used to treat a number of ailments such as gastrointestinal [1], oral, respiratory [2,3], and skin diseases [4]. The antimicrobial activities of this plant have been reported by several research groups. The oil from this plant showed inhibitory activities against bacteria [5], fungi, and yeasts [6]. Pinostrobin and red oil from this plant exhibited anti-*Helicobacter pylori* activity [7]. The chloroform extract of this plant was found to be effective against Gram-positive bacteria with high activity against *S. aureus* [8]. Panduratin A from this plant possessed a very

good antibacterial activity against a number of staphylococci and enterococci clinical isolates and its activity was more powerful than many commonly used antibiotics [9,10]. Isopanduratin A from this plant demonstrated antibacterial activity against *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus sanguinis*, and *Streptococcus salivarius* [11].

*Streptococcus pyogenes* (group A streptococci), an important species of Gram-positive bacterial pathogens, can colonize the throat or skin and responsible for a wide variety of diseases in humans. *S. pyogenes* is the most common cause of bacterial pharyngitis and is the cause of scarlet fever and impetigo. In severe cases this bacterium can cause invasive diseases such as cellulitis, septicemia, necrotizing fasciitis, and streptococcal toxic shock syndrome. *S. pyogenes* infections may leads to the delay sequelae of rheumatic fever and glomerulonephritis [12]. Increased antibiotic resistance in *S. pyogenes* [13] and antibiotic treatment failure for *S. pyogenes* infections [14] have been reported and become a serious clinical problem. The relationship between antibiotic consumption and resistance rate in *S. pyogenes* has been extensively investigated in previous study [15]. Thus, the discovery of novel agents for the treatment of *S. pyogenes* infections is required in the near future. Many medicinal plants have been studied for their antibacterial property and some plants have a strong activity and good potential to be developed into an effective drug. Such plants may substitute antibiotic consumption or decrease antibiotic resistant bacteria. A previous study found that *B. pandurata* extract produced good antibacterial activity against *S. pyogenes* [16]. However, the previous study reported the activity against only a single *S. pyogenes* isolate. Therefore, this present study was aimed to evaluate antibacterial activity of this effective plant against various clinical isolates of *S. pyogenes* and examine its mechanisms of action.

## EXPERIMENTAL

### Plant material

The rhizome of *B. pandurata* was collected during 2007 by Dr Oratai Neamsuvan, an ethnobotanist of Traditional Thai Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand, where a voucher specimen (NPRC0007) was deposited in the herbarium. The chloroform extract of rhizome of *B. pandurata* was prepared according to a previous study [16]. Briefly, the rhizomes of the plant were cut into small pieces and dried at 60 ˚C. The dried rhizomes were crushed in a mechanical mortar and soaked with chloroform for 7 days. The extract was completely dried and dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) before use.

### Bacterial isolate and culture condition

Forty-seven clinical isolates of *S. pyogenes* (NPRC 101-147) from patients with tonsillitis or pharyngitis were obtained from Department of Microbiology and Natural Products Research Center, Faculty of Science, Prince of Songkla University. All isolates were susceptible to penicillin G (MIC range, < 0.015 - 0.062 µg/ml) and erythromycin (MIC range, <0.015 - 0.125 µg/ml). All isolates were stored in brain heart infusion (BHI) broth containing 20 % glycerol at -70 ˚C until use. *S. pyogenes* DMST 17020 was purchased from the National Institute of Health (NIH), Department of Medical Sciences, Ministry of Public Health, Thailand. All strains were routinely grown in BHI broth or blood agar (BA) plates and incubated with 5 % $CO_2$ at 37 $^{o}$C for 24 h.

### Antibacterial activity

The minimum inhibitory concentration (MIC) values were determined by a broth microdilution method according to Clinical and Laboratory Standards Institute Guidelines [17]. The MIC of the extract was defined as the lowest concentration that produced a complete suppression of visible growth. Aliquots (20 µl) from the broth with no visible growth were cultured onto BA plates and incubated with 5 % $CO_2$ at 37 ˚C for overnight. The minimum bactericidal concentration (MBC) was recorded as the lowest concentration completely preventing bacterial growth. All tests were performed in triplicate independent experiments.

### Time-kill assay

A time-kill assay was used to investigate the bactericidal activity of the extract. The bacterial cultures ($10^5$ cfu/ml) in BHI broth supplemented with the extract at concentrations equivalent to 1/2MIC, MIC, 2MIC, and 4MIC were incubated at 37 ˚C. Surviving bacteria were observed every 2 h intervals until 24 h by culturing on BA plates with 5 % $CO_2$ at 37 ˚C. A control with 1 % DMSO was applied with the same conditions. All assays were carried out in duplicate.

### Bacteriolysis

A modified method according to Carson *et al*. [18] was used to determine the bacteriolytic activity of the extract. Briefly, the suspensions of *S. pyogenes* (1.5 × $10^8$ cfu/ml) in 0.85 % normal saline solution were mixed with the extract at concentrations equivalent to 1/2MIC, MIC, 2MIC, and 4MIC. Then the optical density (OD) at 620 nm was measured every 2 h intervals until 12 h and 24 h. Bacterial cell lysis was indicated by a decrease in OD 620 nm. Corresponding dilutions of the extract were used as blanks. A control with 1 % DMSO was applied with the same conditions. The results were expressed as a ratio of the OD at each time interval versus the OD at 0 min (in %). All assays were carried out in triplicate.

### Enzyme and toxin inhibition assay

A method from Nostro *et al*. [19] was modified and used in this experiment. The productions or activities of protease enzymes and haemolysins in the presence of the plant extract were estimated on 1% skim milk-BHI agar and BA, respectively. The extract was mixed with the melted test agar in order to obtain 1/2MIC, 1/4MIC, 1/8MIC, 1/16MIC, and 1/32MIC. Five µl of the bacterial culture diluted to $10^8$ cfu/ml were spot inoculated in radial patterns on the plate surfaces with and without extracts and incubated with 5 % $CO_2$ at 37 ˚C for 24 h. All determinations were performed in duplicate including 1 % DMSO as control. The diameter of bacterial growth and clear zones (protease activities) on 1% skim milk-BHI agar were recorded and calculated as the degree of hydrolysis. The diameter ratio of haemolysis zone and colonies was calculated as the degree of haemolysis. Inhibition of productions or activities of protease enzymes and streptolysins were evaluated as total when the halo was absent and partial when the halo was ≥50 % compared with the control.

## RESULTS

### Antibacterial activity

Antibacterial activities of the extract against *S. pyogenes* were presented as MIC and MBC values. As shown in Table 1, the chloroform extract of *B. pandurata* displayed a good anti-*S. pyogenes* activities with similar MIC and MBC ranges, 3.91 - 62.50 µg/ml. Both $MIC_{50}$ and $MIC_{90}$ values of the extract on *S. pyogenes* were 7.81 µg/ml.

**Table 1:** The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of chloroform extract of *Boesenbergia pandurata* against 47 clinical *S. pyogenes* isolates

| Botanical name | Anti-*S. pyogenes* activity (µg/ml) | | | |
|---|---|---|---|---|
| | [a]$MIC_{50}$ | [b]$MIC_{90}$ | MIC range | MBC range |
| *Boesenbergia pandurata* | 7.81 | 7.81 | 3.91-62.50 | 3.91-62.50 |

[a]*MIC at which 50 % of the isolates were inhibited ($MIC_{50}$);* [b]*MIC at which 90 % of the isolates were inhibited ($MIC_{90}$)*

### Time-kill

Time kill assay of the chloroform extract from *B. pandurata* against *S. pyogenes* is demonstrated in Figure 1. Complete killing of *S. pyogenes* cells treated with 4MIC, 2MIC, and MIC (MBC) of the extract occurred within 4, 8, and 12 h after treatment with the extract, respectively. The growth rate of 1/2MIC treated culture was lower than the control until 16 h and subsequently reached the same level as the control.

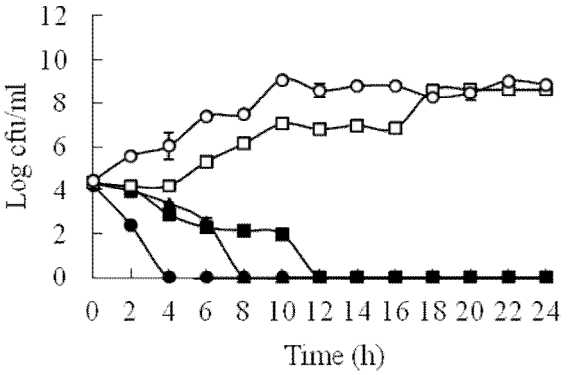


**Figure 1:** Time-kill curves of *Streptococcus pyogenes* NPRC 101 after treatment with the extract of *Boesenbergia pandurata*. 1/2MIC (□), MIC (■), 2MIC (▲), 4MIC (●), and 1 % DMSO was used as a control (○). Each symbol indicates the mean ± SD.

### Bacteriolysis

*S. pyogenes* cell lysis activity of the chloroform extract from *B. pandurata* is presented in Figure 2. The treatment of the bacterial cells with the extract at 2MIC and 4MIC caused cell lysis. After 2 h at 2MIC and 4MIC of *B. pandurata* extract induced the cell lysis around 44% and 76%, respectively. After 4 h until 24 h, the bacterial cells were lysed approximately 36-49% and 76-98% when treated with the extracts at 2MIC and 4MIC, respectively.

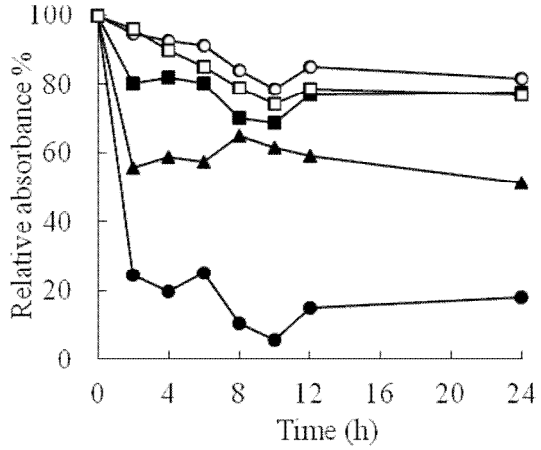


**Figure 2:** Bacteriolytic activity of *Boesenbergia pandurata* extract against *Streptococcus pyogenes* NPRC 101. 1/2MIC (□), MIC (■), 2MIC (▲), 4MIC (●), and 1% DMSO (○).

### Enzyme and toxin inhibition

The productions or activities of protease enzymes and streptolysins in the presence of the plant extract were calculated as degree of hydrolysis and haemolysis, respectively. For protease inhibition assay, at all concentrations of the extract, all isolates demonstrated degree of hydrolysis ranging from 1.39 - 1.65. The degree of hydrolysis in the control agar was 1.42 - 1.61. None of extracts produced total (the halo was absent) or partial inhibition (the halo was ≥50 %) when compared with the control (Figure 3). The streptolysin inhibition assay showed similar results (Figure 4).

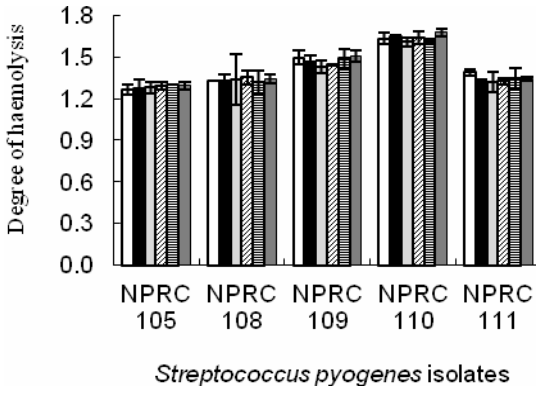


**Figure 3:** The effect of *Boesenbergia pandurata* extract on streptolysins production by *Streptococcus pyogenes*. 1/2MIC (□), 1/4MIC (■), 1/8MIC (□), 1/16MIC (▨), 1/32MIC (▤), and 1 % DMSO (■) was used as control. The degree of hydrolysis is indicated by the mean ± SD.

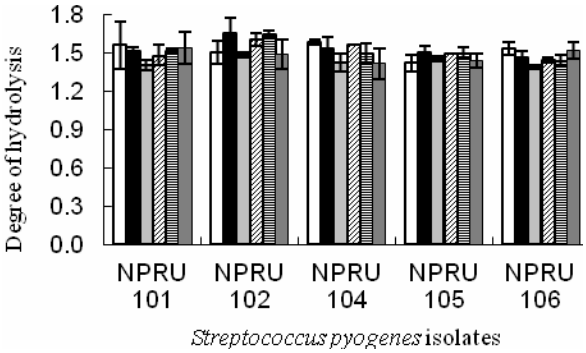


**Figure 4:** The effect of *Boesenbergia pandurata* extract on protease enzymes production by *Streptococcus pyogenes*. 1/2MIC (□), 1/4MIC (■), 1/8MIC (□), 1/16MIC (▨), 1/32MIC (▤), and 1%DMSO (■) was used as control. The degree of hydrolysis is indicated by the mean ± SD. Degree of hydrolysis

## DISCUSSION

The rhizome of *B. pandurata* has long been used as traditional Thai medicine for bacterial infections. From the antibacterial screening test against *S. pyogenes* by disc diffusion assay, the chloroform extract of *B. pandurata* produced a very narrow inhibition zone around 7 - 8 mm (data not shown). However, it generated very good MIC and MBC values (3.91 - 62.50 µg/ml). This finding suggests that the active components against *S. pyogenes* in this plant are most likely to be slightly or non-polar molecules. Extract from non-polar solvent and oil from this plant have been reported to be effective against many bacteria [5,8], fungi, and yeasts [6].

Isopanduratin A, an active compound from this plant, demonstrated antibacterial activity against many streptococci including *S. mutans*, *S. sobrinus*, *S. sanguinis*, and *S. salivarius* [11]. The MIC of isopanduratin A on *Streptococcus* spp. from the previous study was 4 µg/ml. The crude extract from this study showed 7.81 µg/ml ($MIC_{90}$) on *S. pyogenes*. The MIC values of isopanduratin A and the crude extract are not significantly different. However, it is rather difficult to compare the inhibitory activity of this compound and the crud extract as the studies differ in the bacterial species used. Hence, the isolation for active compounds that responsible for anti-*S. pyogenes* is needed.

To determine how quickly the extract from *B. pandurata* acts on *S. pyogenes* time-kill study was performed. Time-kill study is defined as the rate of killing by a fixed concentration of an antimicrobial agent and is one of the methods for determining tolerance. The extract of *B. pandurata* at concentrations of 4MIC, 2MIC, and MIC (MBC) demonstrated a ≥ 3 $\log_{10}$-cfu killing

(99.9 %) at 4, 8, 12 h, respectively. This extract exhibited a concentration-dependent killing profile. The time-kill activity of rhodomyrtone, an isolated active compound from *Rhodomyrtus tomentosa*, has been studied against *S. pyogenes* [20]. At the concentrations equivalence to 2MBC and 4MBC, the killing rate of *B. pandurata* extract was faster than rhodomyrtone.

Recently, many studies have attempted to explain the mechanisms of action of some medicinal plant extracts on pathogenic bacteria. The essential oils from oregano, rosewood, thyme [21], and tea tree [18] have been reported to cause bacterial membrane damage and induce cell lysis. The essential oil from *B. pandurata* has been reported to altered permeability of the membrane of *Escherichia coli* [5]. Isopanduratin A from this plant has been demonstrated to damage the cell membrane and cell wall of *S. mutans* [11]. This present study revealed that treatment of *S. pyogenes* cells with *B. pandurata* extract resulted in cell lysis. A possible mechanism of action may be associated with cell wall and membrane damage.

Proteases have long been considered as virulence factors for organisms as they are able to facilitate the spread of organism. *S. pyogenes* produces, or at least have the potential to produce, a number of different proteolytic enzymes that can play important roles during infections [12]. Some medicinal plant extracts including, *Helichrysum italicum* and *Nepeta cataria* showed inhibitory effects on some bacterial enzymes that contribute to the pathogenic properties [19]. However, this present study revealed that the extract of *B. pandurata* at sub-inhibitory concentrations had no effect on the protease enzymes of *S. pyogenes*.

*S. pyogenes* secretes two haemolysins, streptolysin O and streptolysin S. Both streptolysin O and S are classified as virulence factors of *S. pyogenes*. Abuharfeil *et al.* [22] reported that date fruit (*Phoenix dactylifera*) neutralized the haemolytic activity of the streptolysin O. The neutralization property of this extract was most probably due to stabilization of the erythrocyte membrane and inhibition of the streptolysin O enzyme. Nevertheless, the results from this study have demonstrated that all tested concentrations of the extract of *B. pandurata* had no effect on *S. pyogenes* haemolysin.

## CONCLUSION

In summary, the extract of *B. pandurata* rhizome demonstrated good activity against all clinical isolates of *S. pyogenes*. The bacteriolytic activity of the extract indicated that a possible mechanism of action may be associated with cell wall and membrane damage. Our research is ongoing to investigate the active compounds in this plant as well as its antibacterial mechanisms. The isolated active compounds may have potential to be used as therapeutic agent against streptococcal infections.

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