

## ฤทธิ์ปรับภูมิคุ้มกัน ต้านออกซิเดชัน และต้านจุลชีพของสารสกัดด้วยน้ำของเลี้ยงเดง

### Immunomodulating, Antioxidant and Antimicrobial Activities of Aqueous Extract of *Bauhinia penicilliloba* Pierre ex Gagnep.

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### Abstract

The aqueous extract of *Bauhinia penicilliloba* Pierre ex Gagnep root, traditionally used as a tonic and appetizer, was investigated for immunomodulating, antioxidant and antimicrobial activities. The immunomodulating activity of aqueous extract was evaluated using mouse splenocytes proliferation by BrdU assay. The results showed that *B. penicilliloba* had an immunostimulating activity. The extract alone exhibited stimulating activity at the concentration range of 12.5-400  $\mu$ g/ml with the maximum activity at 200  $\mu$ g/ml (P.I. = 2.95; control = 1.00). In the presence of pokeweed mitogen (PWM), the extract significantly increased lymphocyte proliferation at concentration range of 12.5-100  $\mu$ g/ml with the strongest activity at 50  $\mu$ g/ml (P.I. = 5.68; control = 4.25). The effect of aqueous extract on phagocytic activity of mouse peritoneal macrophage was evaluated by NBT assay. The aqueous extract at the range of 100-400  $\mu$ g/ml gave non-significant stimulating effects. The concentration of extract exhibiting maximum activity was 400  $\mu$ g/ml (NBT reduction index = 1.14; control = 1.00). The effect of *B. penicilliloba* extract on cytokine production was determined by ELISA kits. The extract with concanavalin-A (ConA) augmented IFN- $\gamma$  production at concentration of 100  $\mu$ g/ml (1076.20 pg/ml; control = 981.20 pg/ml). But the extract alone showed inhibitory effects on IL-4 production. The aqueous extract of *B. penicilliloba* also exhibited potent antioxidant activity based on DPPH and FRAP assay with the EC<sub>50</sub> and FRAP values of 11.34  $\mu$ g/ml and 2,159.72  $\mu$ M, respectively. The total phenolic compounds determined by Folin-Ciocalteau assay was 249.96  $\mu$ g/mg. For antimicrobial activity, *B. penicilliloba* aqueous extract was more active against *S. aureus* than *B. cereus*, *B. subtilis* and *S. sonnei* with inhibition zone of 10.82, 8.36, 7.33 and 7.03 mm, respectively.

**Keywords:** *Bauhinia penicilliloba*, Immunomodulating activity, Antioxidant activity, Antimicrobial activity

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## บทคัดย่อ

การวิจัยนี้เป็นการศึกษาฤทธิ์ปรับภูมิคุ้มกัน, ฤทธิ์ต้านออกซิเดชั่น และฤทธิ์ต้านจุลชีพของสารสกัดตัวยน้ำของรากเลี้ยงแดง ซึ่งหม้อพื้นบ้านใช้เป็นยาอย่างวัฒนธรรมและเจริญอาหาร เมื่อวัดการกระตุ้นการเพิ่มจำนวนของเซลล์ลิมโฟซึ่งของม้าหมูทั้งในสภาวะที่มีตัวกระตุ้น และไม่มีตัวกระตุ้นด้วยวิธี BrdU พบว่าสารสกัดเลี้ยงแดงเมื่อไม่มีตัวกระตุ้น ที่ความเข้มข้น 12.5-400  $\mu\text{g}/\text{ml}$  มีฤทธิ์กระตุ้นภูมิคุ้มกัน โดยสารสกัดความเข้มข้น 200  $\mu\text{g}/\text{ml}$  ให้ผลตอบสนองที่ดีที่สุด (P.I. = 2.95; ตัวควบคุม = 1.00) ส่วนสารสกัดที่มี Pokeweed mitogen (PWM) เป็นตัวกระตุ้น ที่ความเข้มข้น 12.5-100  $\mu\text{g}/\text{ml}$  มีฤทธิ์กระตุ้นภูมิคุ้มกันอย่างมีนัยสำคัญ โดยสารสกัดความเข้มข้น 50  $\mu\text{g}/\text{ml}$  ให้ฤทธิ์กระตุ้นภูมิคุ้มกันดีที่สุด (P.I. = 5.68; ตัวควบคุม = 4.25) เมื่อวัดฤทธิ์สนับสนุนการกลืนกินลิ้งแบลกลอลของแมโคโรฟจากด้วยวิธี NBT พบว่า สารสกัดความเข้มข้น 100-400  $\mu\text{g}/\text{ml}$  มีฤทธิ์สนับสนุนความสามารถแต่ไม่มีนัยสำคัญ โดยสารสกัดความเข้มข้น 400  $\mu\text{g}/\text{ml}$  ให้ฤทธิ์ดีที่สุด (ดัชนี = 1.14; ตัวควบคุม = 1.00) เมื่อวัดความสามารถของสารสกัดในการกระตุ้นการหลังสารซัยโดยไนโคน์ด้วยเทคนิค ELISA พบว่าสารสกัดในสภาวะที่มี Concanavalin-A (Con A) เป็นตัวกระตุ้น สามารถกระตุ้นการสร้างสารซัยโดยไนโคนนิด IFN- $\gamma$  (ที่ความเข้มข้นสารสกัด 100  $\mu\text{g}/\text{ml}$ ; IFN- $\gamma$  = 1,076.20 pg/ml; ตัวควบคุม = 981.20 pg/ml) แต่สารสกัดไม่มีฤทธิ์กระตุ้นการสร้าง IL-4 นอกจากนี้ เมื่อทดสอบฤทธิ์ต้านออกซิเดชั่นของสารสกัดด้วยวิธี DPPH และ FRAP พบว่าสารสกัดมีฤทธิ์ต้านออกซิเดชั่น โดยมีค่า  $\text{EC}_{50}$  และ FRAP value เท่ากับ 11.34  $\mu\text{g}/\text{ml}$ , 2,159.72  $\mu\text{M}$  ตามลำดับ และมีปริมาณสารประกอบพื้นออลรวม เมื่อวัดด้วยวิธี Folin-Ciocalteau เท่ากับ 249.96  $\mu\text{g}/\text{mg}$  ส่วนฤทธิ์ต้านจุลชีพ สารสกัดตัวยน้ำของเลี้ยงแดงสามารถยับยั้งเชื้อ *S. aureus*, *B. cereus*, *B. subtilis* และ *S. sonnei* ได้โดยมีค่าเส้นผ่าศูนย์กลางของโซนใส เท่ากับ 10.82, 8.36, 7.33 และ 7.03 มม. ตามลำดับ  
**คำสำคัญ:** *Bauhinia penicilliloba* ฤทธิ์ปรับภูมิคุ้มกัน ฤทธิ์ต้านออกซิเดชั่น ฤทธิ์ต้านจุลชีพ

## Introduction

*Bauhinia penicilliloba* Pierre ex Gagnep (Fabaceae), known as Sieo daeng, is a tendrilled shrub found in Northeastern part of Thailand, Cambodia, Western Laos, and Vietnam (Smitinand and Larsen, 1984). Aqueous extract of the root is applied as a tonic and appetizer (Prasitpuriprecha et al., 2005). In our previous work, some indigenous medicinal plants having ethnomedical application in the treatment of chronic diseases and immunological disorders by the local medicine practitioners of Ubon Ratchathani were initially investigated for lymphocyte proliferation activities by colorimetric MTT assay (Prasitpuriprecha et al., 2006). *B. penicilliloba* was found to have immuno-

stimulating activity. However, prior to our work, there have been no published studies on the pharmacological, toxicological activities of *B. penicilliloba*.

As a continuation of the study, we will study on the effects of this plant on the immune response such as cytokines production and macrophages activity in order to explain its immunomodulating activity as claimed by the folklore doctors. Various concentrations of the aqueous extracts were tested on in vitro mouse splenocyte proliferation by BrdU assay, macrophage phagocytosis and cytokine release. Moreover, the present study also revealed antioxidant and antimicrobial activities, which

could be related to the traditional usage. It may be possible to obtain a potent and efficacious extract with minimal side effects for practical applications such as immunotherapy of cancer and treatment of infectious and immunologic diseases.

## Materials and methods

### 1. Plant material and extraction

Root of *B. penicilliflora* was collected from the forest of Ubon Ratchathani in April 2005 under the guidance of the local practitioners. It was verified for the botanical identity by staffs of the Faculty of Pharmaceutical Sciences, Ubon Ratchathani University and also by comparing with the authentic specimens at the Herbarium of the Department of Agriculture and Department of Forestry, Ministry of Agriculture, Bangkok. Voucher specimen (No.ch02/0019) was deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Ubon Ratchathani University.

The extract was made by decoction as described by the local traditional practitioners. Briefly, the root was washed and dried at 50-60 °C until constant weight. One hundred grams of dried ground plant material was soaked in 300 ml of distilled water for 30 min at room temperature, boiled in soxhlet apparatus for 8 hr and collected the content. The residue was re-extracted for 3 times. Then pooled content was lyophilized. It was kept at - 80 °C in tight and light-protected containers until use. Its yield presented as percentage of dried extracts after being lyophilized compared with dried plant weights was 3.33% w/w.

### 2. Immunomodulating activity

#### 2.1. Splenocytes proliferation

##### *Preparation of mouse splenocytes*

Inbred male BALB/c mice, 6-8 weeks old with a weight range of 20-25 g were purchased from National Laboratory Animal Center, Mahidol University, Thailand. They were housed under standard conditions at 25 °C and fed with standard pellets and tap water. The animals were sacrificed by cervical dislocation and spleens were removed aseptically. The single cell suspension was prepared in completed RPMI-1640 medium (RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum) (Gibco, USA). After centrifugation at 1,000 rpm for 10 min at 4 °C, erythrocytes were lysed and the cell pellets were washed twice with completed medium. The cells were re-suspended and the cell number was adjusted to  $4 \times 10^6$  cell/ml. The viability of splenocytes was determined by the trypan blue dye exclusion technique (Brousseau et al., 1998).

##### *Proliferation assay by BrdU assay*

Splenocytes proliferation activity was measured by using BrdU assay (Messele et al., 2000). Commercial cell proliferation enzyme-linked immunosorbent assay (ELISA) BrdU kits (Roche, Germany) were used as instructed by the manufacturer. Briefly, the plant extracts were serially diluted to make the final concentration of 12.5-800 µg/ml. Twenty-five microlitres of diluted extracts, 25 µl of mitogen (Phytohemagglutinin [PHA] 1:100 or Pokeweed mitogen [PWM] 1 µg/ml) and 50 µl of fresh cells were added to flat-bottom 96-well plate (TPP, Switzerland). After incubation in 5% humidified CO<sub>2</sub> incubator at 37 °C for 48 hr, the process of detection was

started. Ten microlitres of BrdU labeling solution was added and the plate was reincubated for 24 hr. After centrifugation, the solution was removed and the cells were dried. Two hundred microlitres of FixDenat solution was added. The plate was incubated at room temperature for 30 min and then removed the solution. One hundred microlitres of anti-BrdU-POD working solution was added. The plate was incubated for 90 min and removed the solution again. The plate was rinsed three times with 200  $\mu$ l of washing solution, then added with 100  $\mu$ l substrate solutions and incubated for 30 min. Twenty-five microlitres of 1M  $H_2SO_4$  was added for stopping reaction. The absorbance was read at 450 nm within 5 min with a microplate reader (Biotek, ELX 808, USA). Medium was used as a control during sample (extract) test. The data were expressed in term of lymphocytes proliferation index (P.I.).

$$P.I. = \frac{\text{absorbance of sample}}{\text{absorbance of control}}$$

## 2.2. Phagocytic activity

### *Preparation of peritoneal macrophages*

Three percents of brewer thioglycollate medium (Merck, Germany) were injected intra-peritoneally into mice as a stimulant to elicit peritoneal macrophages. Three days after injection, the mice were killed by euthanization. The peritoneal cells were harvested by peritoneal lavage with completed RPMI-1640 medium. The exudates were centrifuged at 1,000 rpm, 4 °C for 10 min. The erythrocytes were lysed. After centrifugation, the cells were washed twice and re-suspended in completed medium. The cell number was adjusted to  $3 \times 10^6$  cell/ml. The trypan blue dye exclusion techniques were used

to determine the viability of macrophages. (Kang et al., 2002)

### ***Phagocytosis assay on nitroblue tetrazolium (NBT) reduction in mouse macrophages***

The NBT reduction assay was carried out according to the method previously described by Rainard et al. (1986). Briefly, 50  $\mu$ l of various concentrations of the plant extract were added to 50  $\mu$ l of the cells in flat bottom 96-well plate. After incubation for 24 hr at 37 °C in an incubator with humidified 5%  $CO_2$ , 50  $\mu$ l of 0.1 mg/ml opsonised zymosan ( $5 \times 10^6$  particles/ml) (Sigma-Aldrich, Germany) and 50  $\mu$ l of 0.5 mg/ml Nitroblue tetrazolium (NBT) (Sigma-Aldrich, Germany) solution in phosphate buffer solution (PBS) were added. Completed medium was used as control. The cultures were incubated for further 60 min. Then the plates were centrifuged at 2,500 rpm, 25 °C for 15 min. The supernatants were pipetted off and washed 3 times with 200  $\mu$ l methanol to eliminate the unreduced NBT dye. The cell pellets were air-dried. Amounts of 120  $\mu$ l of 2M KOH and 140  $\mu$ l of DMSO (dimethyl sulfoxide) were added consecutively. The absorbance of turquoise blue solution was measured at 570 nm by a microplate reader (Biotek, ELX 808, USA). The index of NBT reduction was calculated by the following equation:

$$\text{NBT reduction index} = \frac{\text{absorbance of sample}}{\text{absorbance of control}}$$

## 2.3 Cytokines production assay

### *Culture condition*

Mouse splenocytes ( $5 \times 10^6$  cell/ml) were cultured in 24-well tissue culture plate

(TPP, Switzerland) for 24 hr in the presence or absence of the various concentrations of extracts (12.5 - 800 µg/ml). Concanavalin A (Con A) (Sigma-Aldrich, Germany) (5 µg/ml) was used as stimulator of cytokines production. Culture supernatants were harvested and stored at -80 °C until tested.

#### **Determination of mouse IL-4 and IFN-γ**

Commercial enzyme-linked immunosorbent assay (ELISA) kits, mouse IL-4 ELISA assay kit (BD OptEIA™, USA) and mouse IFN-γ ELISA assay kit (Endogen, USA) were used to measure IL-4 and IFN-γ concentrations, respectively, in culture supernatant as described by the manufacturer. The absorbance was read at 450 nm within 30 min by using ELISA plate reader (Biotek, ELX 808, USA). The concentration of IL-4 and IFN-γ in culture supernatant was calculated by using standard curve.

#### **2.4 Statistical analysis**

Each experiment was performed triplicate and the results were expressed as mean ± S.E.M. ANOVA was used to analyze statistical significance of the differences between the control and the treated values. Differences at  $p<0.05$  were considered significant.

### **3. Antioxidant activity**

#### **3.1. DPPH assay**

DPPH (1,1-diphenyl-2-picrylhydrazyl) (Sigma-Aldrich, Germany) was used to determine the free radical-scavenging activity of the extract by a slight modifications of McCune and Johns (2002). Briefly, 1 mg/ml of aqueous stock solutions of plant extract was prepared. Then,

1.6 ml of 0.1 mM DPPH in methanol was added to 0.4 ml of various concentrations of samples to produce the final sample concentrations of 1-100 µg/ml. The mixture was shaken vigorously and allowed to stand under protection from light at room temperature for 20 min. The absorbance was measured at 517 nm by using a spectrophotometer (Jenway 6305, USA). Ascorbic acid (vitamin C) (Reidel, Germany) and Trolox® (vitamin E analog) (Fluka, USA) were included in experiment as positive controls. Methanol was used as a blank. All determinations were performed triplicately. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The percentage inhibition of the DPPH radical by the samples was calculated as follows:

$$\% \text{ inhibition} = \frac{(\text{OD control} - \text{OD sample})}{\text{OD control}} \times 100$$

The antioxidant activity of plant extract was expressed as an efficient concentration or  $\text{EC}_{50}$  which defined as the concentration of extracts required to inhibit the formation of DPPH radicals by 50%. The  $\text{EC}_{50}$  value was calculated by the linear regression plots on the percent of antiradical activity against the concentration of the tested samples (Godjevac et al., 2004).

#### **3.2. FRAP assay**

FRAP assay (ferric reducing antioxidant power assay) was performed according to the modified method of Benzie and Strain (1996). Briefly, the FRAP reagent was prepared by mixing 50 ml of 300 mM acetate buffer pH 3.6, 5 ml of 10 mM 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) (Acros, Belgium) in 40 mM hydrochloric

acid and 5 ml of 20 mM ferric chloride hexahydrate (Carlo, Italy) in distilled water. All solutions were freshly prepared on the day of assay. Various concentrations (50-500  $\mu$ M) of standard aqueous  $\text{FeSO}_4$  (Carlo, Italy) solutions were added to the FRAP reagent. The absorbance was measured at 593 nm for 4 min by using a spectrophotometer (Jenway 6305, USA). The standard curve of  $\text{FeSO}_4$  was plotted. Analyzed sample was diluted to fit within the linearity range. The sample was then added to the FRAP reagent in the same procedure. The relative antioxidative activities of samples were assessed by calibration curve. The final results were expressed as FRAP values, which are the concentrations of antioxidants having a ferric ability equivalent to standard  $\text{FeSO}_4$  solution. Trolox® and ascorbic acid were also used as positive controls. Distilled water was used as a blank control.

### 3.3. Total phenolic compounds

Total phenol concentration was determined according to the method of Singleton (Singleton et al., 1999). Briefly, 0.5 ml of the prepared samples in distilled water at various concentrations (50-200  $\mu$ M) were mixed with 0.25 ml of 1N Folin-Ciocalteu reagent (Sigma, USA) (previously diluted with water 1:1 (v/v)) and 1.25 ml of 20% sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) solution. The mixture was intensively shaken and was then allowed to stand for 40 min at room temperature. The absorbance was measured using spectrophotometer at 725 nm. Tannic acid (Pharma, Thailand) was used as a standard. The standard curve of tannic acid was plotted. The total phenolic contents were determined as tannic acid equivalents. The

results were expressed in mg of total phenolic compounds per 1 g of plant extract in term of mean  $\pm$  SD.

## 4. Antimicrobial activity

### 4.1 Microorganism strains

The aqueous extract was tested against a panel of microorganisms, including Gram positive bacteria : *Staphylococcus aureus* ATCC25927, *Staphylococcus epidermidis* ATCC12228, *Bacillus subtilis* DMST6633, *Bacillus cereus* DMST11778, Gram negative bacteria: *Shigella sonnei* DMST7126, *Escherichia coli* DMST4741, *Salmonella typhimurium* DMST0562, *Pseudomonas aeruginosa* DMST27853, *Propionibacterium acnes* DMST14916, fungi : *Trichophyton rubrum* DMST21147, *Microsporum gypseum* DMST21146 and yeast : *Candida albicans* DMST5851. All strains were obtained from Culture collection for medical micro-organism, Department of Medical Sciences, Thailand. *P. acnes* was cultured overnight at 37 °C in Thioglycollate agar (Difco, USA) under anaerobic conditions using Anaerobic Jar HP11 (Oxoid, UK). The other bacteria were cultured overnight at 37 °C in Mueller Hinton agar (MHA) (Difco, USA). Fungi and yeast were cultured at 30 °C in Potato dextrose agar (PDA) (Difco, USA) and Sabouraud dextrose agar (SDA) (Difco, USA), respectively.

### 4.2 Antimicrobial assay

The dried plant extracts were dissolved in distilled water to a final concentration of 50 mg/ml and sterilized by filtration by 0.45 nm Millipore filters. Antimicrobial assay was performed by agar disc diffusion method (Jorgensen et al., 1999) using 100  $\mu$ l of

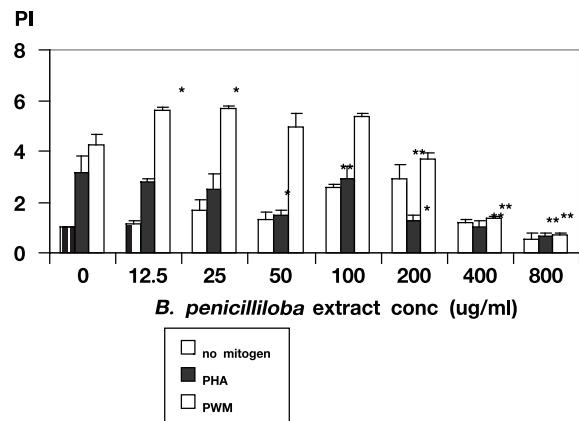
suspension containing  $10^8$  CFU/ml of bacteria,  $10^6$  CFU/ml of yeast, and  $10^4$  spore/ml of fungi spread on MHA, SDA, and PDA, respectively. The discs (6mm in diameter) were impregnated with 50  $\mu$ l of the extracts (2,500  $\mu$ g/disc) at the concentration of 30 mg/ml and placed on the inoculated agar. Ampicillin (Oxoid, UK) 10  $\mu$ g/disc, sulbactam/ cefoperazone (Oxoid, UK) 105  $\mu$ g/disc, tetracycline (Oxoid, UK) 30  $\mu$ g/disc and amphotericin (Squibb, Italy) 25  $\mu$ g/disc were used as positive reference standards to determine the sensitivity of each microbial species tested. The inoculated plates were incubated at 37 °C for 24 hr for bacterial strains, 48 hr for yeast, and 72 hr for fungi isolates. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. Each assay in this experiment was repeated triplicate.

## Results

### 1. Immunomodulating activity

#### 1.1 Splenocytes proliferation

Effects of the aqueous extracts of *B. penicilliloba* on mouse splenocyte proliferation with or without mitogens (PHA or PWM) by BrdU assay were shown in Figure 1. *B. penicilliloba* significantly stimulated lymphocyte proliferation both with and without PWM. The extract alone exhibited stimulating activity at the concentration range of 12.5-400  $\mu$ g/ml with the maximum activity at 200  $\mu$ g/ml (P.I. =  $2.9510 \pm 0.5170$ ; control = 1.000). In the presence of PWM, the extract significantly increased lymphocyte proliferation at concentration range of 12.5-100  $\mu$ g/ml with the strongest activity at 25  $\mu$ g/ml (P.I. =  $5.6879 \pm 0.6401$ ; control =  $4.2577 \pm 0.6379$ ).



**Figure 1** Effects of aqueous extracts of *B. penicilliloba* on mouse splenocytes proliferation activity with or without mitogen (PHA 1:100, PWM 1  $\mu$ g/ml) by BrdU assay. [Data are mean  $\pm$  S.E.M. (n=3). Statistics are ANOVA. \* =  $p < 0.05$ , \*\* =  $p < 0.01$  compared with the medium]

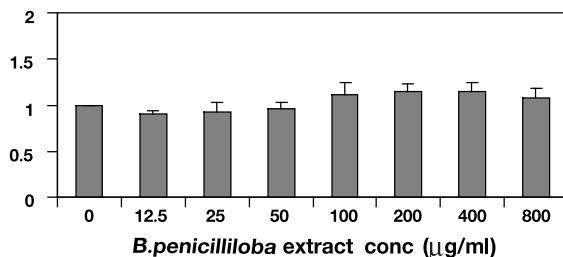
#### 1.2. Phagocytic activity

Effects of aqueous extracts of *B. penicilliloba* on the reduction of NBT dye in mouse macrophages were demonstrated in Figure 2. Higher concentrations of plant extracts did not show significant stimulation. The aqueous extract at the range of 100-400  $\mu$ g/ml gave the most effective NBT dye reduction, approximately 20% higher than that of control. The concentration of extract exhibiting maximum activity was 400  $\mu$ g/ml (NBT reduction index =  $1.14 \pm 0.13$ ; control = 1.00).

#### 1.3. Cytokines production

The effects of various concentrations of *B. penicilliloba* aqueous extracts on IL-4 production of mouse splenocytes were shown in Figure 3. The extracts alone (12.5-800  $\mu$ g/ml) showed significant inhibition effects with dose response relationship (IL-4 range = 13.64 -3.41 pg/ml; control = 34.32 pg/ml). In Con A

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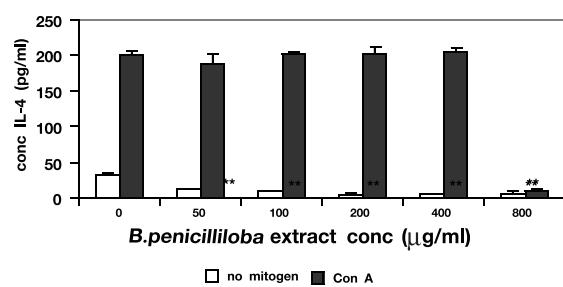
**Figure 2** Effects of aqueous extracts of *B. penicilliloba* on mouse macrophage phagocytic activity by NBT assay. [Data are mean  $\pm$  S.E.M. ( $n=3$ ). Statistics are ANOVA.]

stimulation, *B. penicilliloba* extract at the concentration range of 12.5-400  $\mu$ g/ml showed indifferent effects (IL-4 range = 188.86-205.45 pg/ml; control = 200.45 pg/ml). At higher concentration (800  $\mu$ g/ml), it exhibited the suppressive effect (10.68 pg/ml).

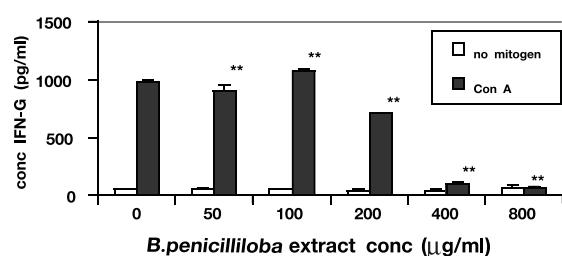
The effects of *B. penicilliloba* aqueous extracts on IFN- $\gamma$  production of mouse splenocytes were shown in Figure 4. Stimulated with Con A, 100  $\mu$ g/ml of *B. penicilliloba* extract significantly augmented the IFN- $\gamma$  production (1,076.20 pg/ml; control = 981.20 pg/ml). At higher concentration (200-800  $\mu$ g/ml), it exhibited the significantly inhibitory effects.

## 2. Antioxidant activity

The antioxidant capacities of aqueous extracts from the root of *B. penicilliloba* were determined. As displayed in Table 1, the aqueous extract of *B. penicilliloba* exhibited antioxidant activity based on DPPH and FRAP assay with the EC<sub>50</sub> and FRAP value of 11.34  $\mu$ g/ml and 2,159.72  $\mu$ M, respectively. The total phenolic compound was 249.96  $\mu$ g/mg.



**Figure 3** Effects of aqueous extracts of *B. penicilliloba* on IL-4 production of mouse splenocytes stimulated with Con A (5  $\mu$ g/ml) by IL-4 ELISA assay. Data are mean  $\pm$  S.E.M. ( $n=3$ ). Statistics are ANOVA. (\*\*) =  $p<0.01$  compared with the medium)



**Figure 4** Effects of aqueous extracts of *B. penicilliloba* on IFN- $\gamma$  production of mouse splenocytes stimulated with Con A (5  $\mu$ g/ml) by IFN- $\gamma$  ELISA assay. Data are mean  $\pm$  S.E.M. ( $n=3$ ). Statistics are ANOVA. (\*\*) =  $p<0.01$  compared with the medium)

## 3. Antimicrobial activity

Antimicrobial activities of *B. penicilliloba* aqueous extracts against tested microorganisms were shown in Table 2. This extract was more active against *S. aureus* than *B. cereus*, *B. subtilis* and *S. sonnei* with inhibition zone of 10.82, 8.36, 7.33 and 7.03 mm, respectively. It had no activity against some bacteria such as *E. coli*, *S. typhimurium*, *P. aeruginosa*, *P. acnes*, *S. epidermidis* and all tested fungi including *T. rubrum*, *M. gypseum* and *C. albicans*.

**Table 1** Antioxidant activities of *B. penicilliloba* aqueous extract

Test sample	EC <sub>50</sub> (ug/ml) (SD) of DPPH assay	FRAP value (μM) (SD)	Total phenolic compound (μg/mg) (SD)
<i>Bauhinia penicilliloba</i>	11.34 (1.50)	2,159.72 (5.65)	249.96 (36.63)
Ascorbic acid	3.26 (0.53)	15,876.67 (635.69)	N
Trolox®	2.98 (0.19)	7,166.11 (2,230.66)	N

N = not tested

**Table 2** The antimicrobial activities of *B. penicilliloba* aqueous extract

microorganism	Diameter of clear zone (mm) ± SD				
	<i>B. penicilliloba</i> extract 2,500 μg	Ampicillin 10 μg	Tetracycline 30 μg	Sulbactam/ cefoperazone 105 μg	Amphotericin 25 μg
<i>S. aureus</i>	10.82±0.11	30.40±0.72	29.27±0.49	34.83±0.20	NT
<i>B. subtilis</i>	7.33±0.20	27.10±0.26	28.33±0.65	31.17±0.66	NT
<i>B. cereus</i>	8.36±0.07	11.00±0.05	31.60±0.19	25.00±0.22	NT
<i>S. sonnei</i>	7.03±0.05	11.11±0.01	25.60±0.27	23.50±0.14	NT
<i>E. coli</i>	X	22.23±0.49	22.87±0.23	24.13±0.55	NT
<i>S. typhimurium</i>	X	29.07±0.50	24.67±0.64	32.07±0.20	NT
<i>P. aeruginosa</i>	X	27.37±0.70	29.10±0.17	28.33±0.75	NT
<i>P. acnes</i>	X	23.40±0.09	18.30±0.11	31.00±0.03	NT
<i>S. epidermidis</i>	X	10.00±0.06	22.20±0.01	23.30±0.36	NT
<i>T. rubrum</i>	X	NT	NT	NT	9.03±1.15
<i>M. gypseum</i>	X	NT	NT	NT	9.87±0.76
<i>C. albicans</i>	X	NT	NT	NT	9.70±0.01

X = negative or no clear zone

NT = not tested

## Discussion and conclusion

Our findings indicated that the aqueous extract of *B. penicilliloba* had an immuno-stimulating activity. It stimulated lymphocytes proliferation both with and without mitogen. There are some differences on mitogens that PHA specifically activated T-lymphocytes

proliferation (Nakamura et al., 1986) while PWM activated B-lymphocytes proliferation (Schreck et al., 1982). *B. penicilliloba* showed higher activity in the presence of PWM than that of with PHA, suggesting specificity toward B-cell proliferation. It also augmented the IFN-γ production and showed inhibitory effects of IL-4 production. This finding suggests that its

majority mechanism would be a cell-mediated immune response (CMIR), acting through Th1 lymphocytes. CMIR are critical for intracellular microorganism infection, foreign grafts, tumor and delayed type hypersensitivity reactions (Miller, 1991). The results of this study support our previous study and the remedies of herbal medicine practitioners as a tonic and appetizer.

The aqueous extract from the root of *B. penicilliloba* also showed potent antioxidant activity with the EC<sub>50</sub> of DPPH assay and FRAP value of 11.34 µg/ml and 2,159.72 µM, respectively. A correlation between antioxidant activity obtained from DPPH assay and FRAP assay implied that this extract was capable of scavenging the DPPH free radical and reducing ferric ions. The result of antioxidant activity of this extract was correlated to the content of phenolic compounds (249.96 µg/mg). Generally, extracts that contain a high amount of polyphenols also exhibit high antioxidant activity (Wong et al., 2006). Kumar et al (2005) reported the antioxidant and antimicrobial activities of *Bauhinia racemosa*, a plant in this genus. A methanol extract of *B. racemosa* stem bark had EC<sub>50</sub> value of DPPH assay of 152.29 µg/ml and the amount of total phenolic compounds of 64.7 µg/mg. Our results supported that *B. penicilliloba* had potent antioxidant activity as same as the plants in this genus.

Phenolic compounds such as flavonoids, alkaloids have been reported to have immunomodulating activities. The phenolic compounds can stimulate or suppress the immune system due to the hydroxyl groups in the structure. These groups can affect the enzyme or electron-transferring system, resulting in an immunomodulating property such as

phagocytic activity. Therefore, the phenolic compounds in these plants might be assumed to be responsible for the immunomodulating activities found in this study. Other plant constituents which can stimulate immunity are polysaccharides, sesquiterpines, diterpene, triterpene, and alkaloids (Rudi, 1993). Thus, the medicinal claims of the plant being used as tonic may be in part due to the antioxidant activity. Further isolation of antioxidant constituents is warranted.

The substances that have the immunomodulating activity can belong to some molecular structure classes. The natural organic compounds in plants that have immunostimulating activities are associated with lipophilic compounds and polar fraction (Rudi, 1993). Our process of extraction was decoction or hot water extraction. It has been used to extract monosaccharides, tannins, gums, starches and color matter. But other solvent such as ethanol have been used to extract polysaccharide, glycoprotein, phenolic, terpenoid, waxes, fats, some resin and portion of wood gum (Harborne and Barter, 1993; Arnason et al., 1995). More studies are needed to identify the active ingredients in this extract and to investigate for the immunological activity of other solvent extracts.

The aqueous extract established moderate antibacterial activity against *S. aureus*, *B. cereus*, *B. subtilis* and *S. sonnei*. It may support the folkloric use in the prophylaxis of infection as antimicrobial agents. The results of this study supported previous study. Although the findings might provide a scientific basis for the applicability and therapeutic actions of *B. penicilliloba*, further purification of the

immunologically active extract would be essential before it could be exploited in modern medicine.

In conclusion, the results obtained in the present study indicated that aqueous extract of *B. penicilliflora* root can be a potential source of natural immunostimulator, antioxidant and antimicrobial agent. From the history of using *B. penicilliflora* as a tonifying drug, and with the recent study, it may play a new role in modern medicine. It can be added to the ever-growing

list of herbal medicines that have the potential to modulate immune responses. More studies are needed to identify the active ingredients in these extracts and to investigate for the immunological activity of other solvent extracts. The other possible roles in the immune function of them remain to be elucidated. Further systematic investigations into the chemical constituents, pharmacological actions, and toxicity of the plant material will be needed to improve medicinal value.

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## References

Arnason TJ, Mata R, Romeo TJ. 1995. *Phytochemistry of medical plants: recent advances in phytochemistry*, vol.29. New York: Plenum press: 4-15.

Benzie IF, Strain JJ. 1996. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: The FRAP assay. *Anal Biochem* 239: 70-76.

Brousseau P, Payette Y, Tryphonas H, et al. 1998. *Manual of immunological methods*, CRC press : 27-28.

Godjevac D, Vajs V, Menkovic N, et al. 2004. Flavonoids from flowers of *Cephalaria pastricensis* and their antiradical activity. *J Serb Chem Soc* 69(11): 883-886.

Harborne BJ, Barter H. 1993. *Phytochemical dictionary: a handbook of bioactive compounds from plants*. Washington DC: Taylor & Francis: 9744.

Jorgensen J, Turnidge J, Washington J. 1999. Antibacterial susceptibility tests: Dilution and disk diffusion methods. In Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH. Editors. *Manual of clinical microbiology*, 6<sup>th</sup> ed. Washington DC: ASM Press: 1526-1543.

Kang N, Park S, Lee K, et al. 2002. Modulation of macrophage function activity by ethanolic extract of larvae of *Holotrichian diomphalia*. *J Ethnopharmacol* 79: 89-94.

Kumar RS, Sivakumar T, Sunderam RS, et al. 2005. *Braz J Med Biol Res* 38(7): 1015-24.

McCune LM, Johns T. 2002. Antioxidant activity in medicinal plants associated with the symptoms of diabetes mellitus used by the Indigenous Peoples of the North American boreal forest. *J Ethnopharmacol* 82: 197-205.

Messele T, Roos MT, Hamann D, et al. 2000. Nonradioactive techniques for measurement of *in vitro* T-cell proliferation: alternatives to the [<sup>3</sup>H] thymidine incorporation assay. *Clin Diagn Lab Immunol* 7(4): 687-692.

Miller LE. 1991. *Manual of laboratory immunology*, Lea and Febiger, London, pp1-18.

Nakamura A, Nagai K, Suzuki S, et al. 1986. A novel method of screening for immunomodulating substances, establishment of an assay system and its application to culture broth of microorganisms. *J Antibio* 8: 1148-54.

Prasitpuriprecha C, Sripanidkulchai B, Lulitanond V, et al. 2005. Studies on the Utilization of Medicinal Plants as Immunomodulators in Ubon Ratchathani Province, Thailand. *KKU research Journal* 1: 31-41.

Prasitpuriprecha C, Sripanidkulchai B, Lulitanond V, et al. 2006. Studies on Evaluation of immuno-modulating activity of selected Thai medicinal plants by lymphocytes proliferation assay. *IJPS* 1: 53-62.

Rainard P. 1986. A colorimetric microassay for opsonins by reduction of NBT in phagocytosing bovine polymorphs". *J Immunol Methods* 90: 197-201.

Rudi L. 1993. Immunomodulatory compounds. In: Steven, Mc. And Russel, M (Eds.), *Bioactive natural products: detection, isolation and structure determination*, CRC Press, London: pp279-317.

Schreck C, Lamberson H, Davey F. 1982. Characterization of the B lymphocytes response to pokeweed mitogens. *Annals Clin Lab Sci* 12(6): 455-462.

Singleton VL, Orthofer R, Lamuela-Raventos RM. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteau reagent. *Methods Enzymol* 299: 152-178.

Smitinand T, Larsen K. 1984. *Flora of Thailand*. vol 4, The TISTR PRESS, Bangkok : 33.

Wong SP, Leong LP, Koh JHW. 2006. Antioxidant activities of aqueous extracts of selected plants. *Food chemistry* 99(4): 775-783.