ANTIBACTERIAL PROPERTY TESTING OF TWO SPECIES OF TROPICAL PLANT *LASIANTHUS* (RUBIACEAE)

Tiwtawat Napiroon[1,6], Henrik Balslev[2], Sutee Duangjai[3], Duangchai Sookchaloem[3], Kongkanda Chayamarit[4], Wichai Santimaleeworagun[5] and Srunya Vajrodaya[1]

[1]Phyto-Chemodiversity and Ecology Research Unit, Department of Botany, Faculty of Science, Kasetsart University, Bangkok, Thailand; [2]Department of Bioscience, Faculty of Science and Technology, Aarhus University, Aarhus, Denmark; [3]Department of Forest Biology, Faculty of Forestry, Kasetsart University, Bangkok; [4]Department of National Parks, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok; [5]Department of Pharmacy, Faculty of Pharmacy, Silpakorn University, Nakhon Phathom; [6]The Center for Advanced Studies in Tropical Natural Resources (CASTNaR), Kasetsart University, Bangkok, Thailand

Abstract. Two tropical plant species, *Lasianthus pilosus* and *Lasianthus stipularis* are used in traditional medicine but there have been no published studies of the extracts of these plants against bacteria. In this study, we aimed to determine the antimicrobial activities of the above two plants against two gram-positive and seven gram-negative bacterial strains to determine the potential of these two plant species for possible antimicrobial drug development. The antibacterial activities of the lipophilic extracts of these plants were evaluated by disk diffusion and broth microdilution methods. The zone diameters and minimum inhibitory concentrations (MIC) for these plant extracts exhibited their highest antibacterial activity against *Pseudomonas aeruginosa,* followed by *Staphylococcus aureus* and *Acinetobacter baumannii*, respectively. The MIC of these extracts against *P. aeruginosa* (ATCC 37166 and ATCC 27853) varied from 50 to 200 μg/ml. Thin layer chromatography and detection using different specific reagents revealed the presence of terpenoids, phenolic compounds and iridoid. Cell lysis due to the effect of the lipophilic extracts of these tested plants was demonstrated using scanning electron microscopy. In conclusion, the bioactive compounds of these plants should be studied further to develop potential antimicrobial agents.

Keywords: *Lasianthus,* medicinal plants, pathogenic bacteria

Correspondence: Wichai Santimaleeworagun, Department of Pharmacy, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom 73000, Thailand.
Tel: +66 (0) 34 4255800; Fax: +66 (0) 34 255801
E-mail: swichai1234@gmail.com
Srunya Vajrodaya, Department of Botany, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand
Tel: +66 (0) 2562 5555; Fax: +66 (0) 2940 5627
E-mail: fscisyv@ku.ac.th

INTRODUCTION

Lasianthus stipularis Blume, known as Stipulate Wool flower in Malaysia and Kham Sri in Thailand (Wiart, 2006), is distributed in the tropical rain forest from peninsular Thailand down the Malay peninsula to the southeastern Asian archipelago (Zhu *et al*, 2012). On the Malay peninsula, people use the leaf of *L. stipularis* to treat a condition they called "noises in

the head" (or tinnitus) (Wiart, 2006) which corresponds to inner ear damage, earwax build-up or middle ear infection. People in Sarawak use the liquid extract from the leaves of *L. stipularis* mixed with the leaves of *Lindera selangorensis* (Lauraceae) to treat tinnitus (Werner, 2002; Quattrocchi, 2012), but the therapeutic efficacy of this extract has not been studied. Preparation of the congener *L. pilosus* Wight, has also not been reported, but this species of medicinal Rubaceous plant has potential antioxidant activity (Ahmad *et al*, 2010) and it is called Pad Khon Khang in Thailand. There are no published studies regarding the effect of the extracts of these two plants against bacteria; however, methanol extracts of *L. laevigatus*, *L. furcatus* and *L. obscurus* have been reported to have antibacterial activity against *Staphylococcus aureus* (Puwantoro *et al*, 2010).

Staphylococcus aureus, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* are causes of serious morbidity in humans (Boucher *et al*, 2009; Otter and French, 2010; Hotez, 2014). These bacteria have developed resistance to many antimicrobials, causing some to describe the world as entering the post-antibiotic era (Aksoy and Unal, 2008). New antimicrobial agents are needed to combat these bacteria (Lee *et al*, 2011; Bassetti *et al*, 2013).

We investigated the antibacterial activity of extracts of *L. stipularis* and *L. pilosus* plants to determine their suitability for possible developing into antimicrobial agents.

MATERIALS AND METHODS

Plant sample preparation

The mature leaves and stem bark of *L. pilosus* and *L. stipularis* were collected between July and December, 2015, from a tropical rain forest in peninsular Thailand. Voucher specimens of *L. pilosus* (Napiroon 036) and *L. stipularis* (Napiroon 037) are deposited at the Department of Botany, Kasetsart University, Bangkok Forest Herbarium (BKF), and Aarhus University Herbarium. The samples were identified and comparable to *L. pilosus* (holotype K!000031668) and *L. stipularis* (isotype K!000763942) at KEW Herbarium.

The collected samples, 200 grams of each part, were then dried in the shade, chopped into small pieces and powdered. The powder was macerated with methanol for seven days in the dark at room temperature. The extract was concentrated by rotary evaporation at 37ºC. The extract was divided into hydrophilic extract in distilled water and lipophilic extract in chloroform. The lipophilic extract was stored at -45ºC until used.

Sources of bacterial strains. To determine the antimicrobial activity of the extracts, the studied bacteria were obtained from the Department of Medical Science, Ministry of Public Health, Thailand. These bacteria were: methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 25923, *S. aureus* ATCC43300, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 37166, *P. aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC–BAA 1705, *K. pneumoniae* carbapenemase-positive controls, *K. pneumoniae* ATCC–BAA 1706, *Acinetobacter baumannii* ATCC 19606 and *A. baumannii* ATCC 3487.

Antimicrobial testing

The antibacterial efficacy of the studied extracts was determined by the disk diffusion method. The bacterial strains were inoculated into Muller-Hinton broth (MHB) (Oxoid, Hamshire, UK) and adjusted to a turbidity of 0.5 McFarland standard and then plated thoroughly on Muller-Hinton

agar (MHA) (Oxoid). Paper disks (6 mm diameter) were soaked in the extract until the disk contained 200 μg of the studied extract; the disks were placed 30 mm apart on MHA containing the test organism. The MHA plate was then incubated at 37°C for 24 hours. Following quality control recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2014), *Escherichia coli* ATCC 25922 and *S. aureus* ATCC 25923 strains were used as control species with standard antibiotic disk for ceftazidime (30 μg), ciprofloxacin (5 μg), imipenem (10 μg), meropenem (10 μg), colistin (10μg), amikacin (30 μg), gentamicin (10 μg), piperacillin (100 μg), vancomycin (30 μg) and tigecycline (15 μg).

The minimum inhibitory concentration (MIC) of each tested extract was determined using the broth microdilution method. Stock solutions of the tested extract at 40 mg/ml dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO) were serially diluted and placed in the wells of a 96-well plate. A standard inoculum of the test bacteria in MHB was also added to each well. DMSO was used as a control. The organisms were cultured at 37°C for 18 hours. The MIC was documented as the lowest concentration of extract that inhibited visible growth. The tests were performed in duplicate.

Evaluation of the extracts

The lipophilic extracts of the studied plants were placed on Thin Layer Chromatography (TLC) pre-coated silica gel 60 F_{254} plates (20×20 cm; Merck, Dermstade, Germany) using a solvent system of hexane:ethyl acetate (7:3 v/v) and detected under UV irradiation (365 nm and 254 nm). The Relative front (Rf) values for each fluoresced spot were determined as a TLC pattern. The TLC plates were sprayed with detecting reagent to screen for secondary metabolites using different reagents: 1) Anisaldehyde sulfuric acid reagent to detect terpenoids, 2) Dragendorff's reagent to detect alkaloids, 3) Vanillin sulfuric acid reagent to detect phenolic compounds and 4) the Weiffering test was used to detect iridoid compound (Wieffering, 1966; Merck, 1980).

Scanning electron microscopy

The pathogenic bacteria (10^5 CFU/ml) were suspended in lipophilic extract at the MIC for that bacterium in MHB and incubated it at 37°C for six hours and then filtered it through 0.22 μM filter paper. The bacteria were then fixed as described by Cardozo *et al* (2013). The bacteria on the filter paper were then dehydrated with increasing concentrations of acetone (20%, 40%, 60%, 80% and 100%) and then dried with CO_2 (Polaron Range SC7620 Sputter Coater & CA7625 Carbon Accessary). Finally, the filter paper was placed on carbon tape and coated with platinum (AUTOLAB, Spin coater, Utrecht, the Natherlands) and observed under a scanning electron microscope (HITACHI SEM S-2500, Tokyo, Japan).

RESULTS

Antibacterial activity

The disk diffusion test and MIC test revealed studied extracts exhibited the greater antimicrobial activity against *P. aeruginosa* followed by *S. aureus* and *A. baumannii* (Table 2). The leaf and stem bark lipophilic extracts of *L. stipularis* and *L. pilosus* showed the best antibacterial activity against *P. aeruginosa*. The MIC for *L. pilosus* leaf extract was 50 μg/ml and 100 μg/ml against *P. aeruginosa* ATCC 37166 and ATCC 27853, respectively.

Chromatography analyses

The results on the TLC plates appeared

Table 1
The inhibition zone diameters (mm) with the studied extracts and antimicrobial controls.

Bacterial strains	Diameter of inhibition zones in mm							
	L. pilosus		*L. stipularis*		Antimicrobial agents			
	Leaf	Stem bark	Leaf	Stem bark	AMP	PIP	GEN	CIP
Gram-positive								
S. aureus (methicillin-susceptible)	14	12	12	13	-	-	-	-
S. aureus (methicillin-resistant)[a]	14	10	10	15	28	28	21	23
Gram-negative								
E. coli (ATCC 25922)[b]	6	6	6	6	20	26	23	32
P. aeruginosa (ATCC 37166)	17	16	18	21	-	-	-	-
P. aeruginosa (ATCC 27853)	17	17	18	19	-	-	-	-
K. pneumoniae (BAA 1705)	6	8	6	8	-	-	-	-
K. pneumoniae (BAA 1706)	8	6	9	9	-	-	-	-
A. baumannii (ATCC 19606)	7	7	10	9	-	-	-	-
A. baumannii (ATCC 3487)	12	12	10	12	-	-	-	-

[a]*S. aureus* ATCC 25923 and [b]*E. coli* ATCC 25922 strains were used as control species; the clear zone diameter for each control antibiotic was within the quality control ranges set by the CLSI (2014). AMP, ampicillin; PIP, piperacillin; GEN, gentamicin; CIP, ciprofloxacin.

Table 2
Minimum inhibitory concentration of studied extracts against studied bacteria with the microdilution method.

Pathogenic bacteria strains	MIC (μg/ml)			
	Lasianthus pilosus		*Lasianthus stipularis*	
	Leaf	Stem bark	Leaf	Stem bark
S. aureus (methicillin-susceptible)	400	800	1,600	800
S. aureus (methicillin-resistant)	400	800	800	800
P. aeruginosa (ATCC 37166)	50	100	200	100
P. aeruginosa (ATCC 27853)	100	200	200	200
A. baumannii (ATCC 19606)	400	800	1,600	1,600
A. baumannii (ATCC 3487)	800	400	400	400

as violet and grey-green bands for detection of terpenoids using anisaldehyde sulfuric acid as the spraying reagent. Orange bands of alkaloids appeared after spraying with Dragendorff's reagent when detected with vanillin sulfuric acid for the higher alcohols, phenolic compounds, steroids, and essential oils and heated with dilute hydrochloric acid, violet-grey bands presented in leaf and stem bark extracts were presented, indicating a positive test. The leaf lipophilic extracts of the two species respond positively

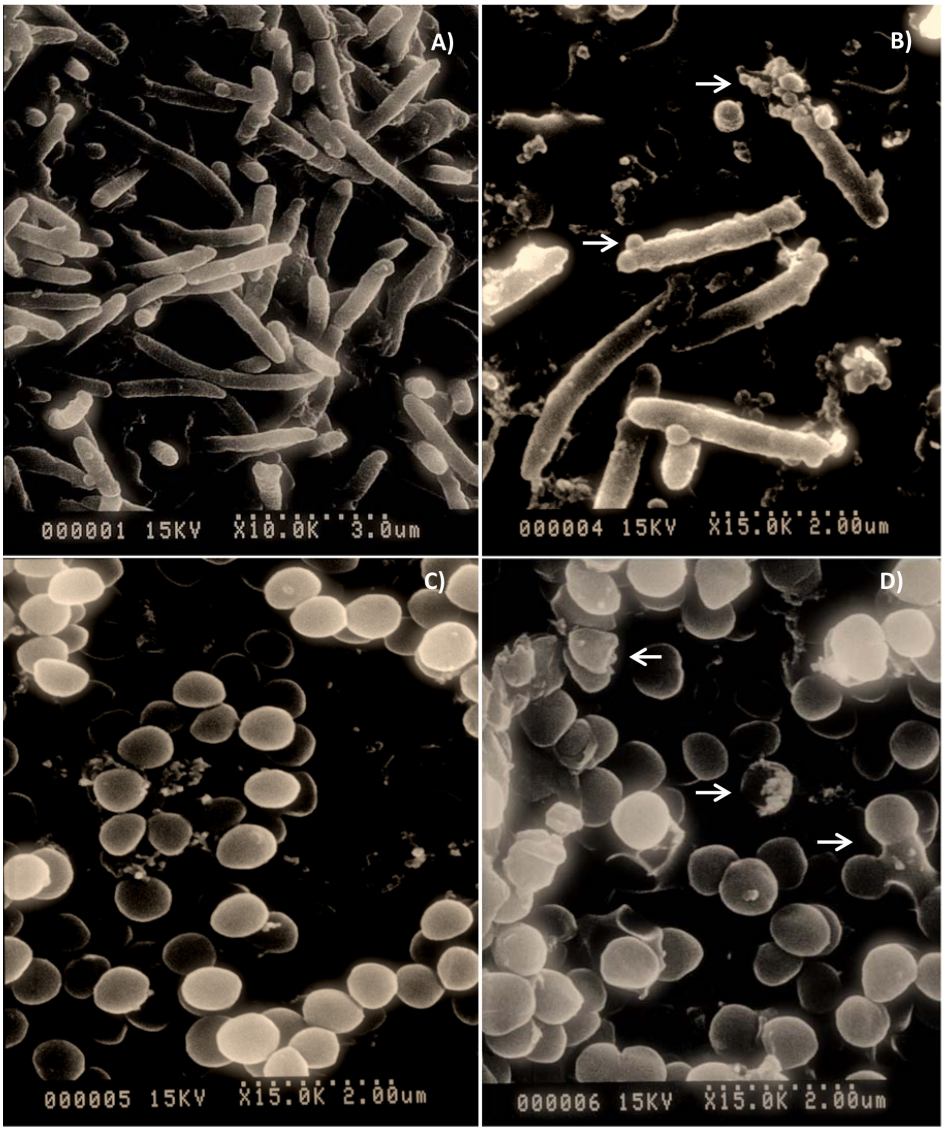

Fig 1–Scanning electron microscope images of studied bacteria: A, and (B) *P. aeruginosa* ATCC 37166; C, D *S. aureus* ATTC 25923. A and C were controls without treatment; B and D were treated with the studied extract. Note the morphological alternations in B and broken cells with leakage in D.

to the Wieffering test, giving blue-green coloration, which indicates an iridoid. The TLC profile of leaf extracts were similar among themselves but differed from the stem bark extracts based on their Rf values and the qualitative comparison of the bands or spots in each reagent.

Scanning Electron Microscopy (SEM)

SEM showed significantly morphological changes in *S. aureus* ATTC 25923 and *P. aeruginosa* ATCC 37166 after exposure to the lipophilic extract at the MIC, suggesting the extract produced cellular lysis. We observed the cell wall sinking

into the bacterial cell (Fig 1D). Some cells were deformed and there appeared to have been cell fluid leakage (Fig 1B and 1D). The untreated cells of *P. aeruginosa* ATCC 37166 and *S. aureus* ATTC 25923 appeared unharmed; the cell wall was not deformed or changed (Fig 1A).

DISCUSSION

Of the TLC screening, the match in Rf values, color, size and the shape of the detection zones among samples from *L. pilosus* and *L. stipularis* which were preliminary evidence to support the same compound in the leaf as the stem bark. Many iridoid glucosides have been detected in *Lasianthus* species and in the family Rubiaceae (Takeda *et al*, 2002, 2004). Typically, iridoid screening was positive to direct testing using the Weiffering test and this is most common with member species of the genus *Lasianthus* such as *L. wallichii* (Takeda *et al*, 2002). Then after screening for iridoids using the Weiffering reagent, a positive test resulted in the leaf extracts but not in the stem bark extracts. Three chemical compound groups were found in our study extracts; it is unclear which compounds had antimicrobial effects. These compounds need to be separated to determine their individual antimicrobial effects prior to developing further as an antimicrobial drug.

Our study extracts had antimicrobial activity against *P. aeruginosa, A. baumannii* and *S. aureus*. Dinda *et al* (2007) reported several iridoids from Rubiaceous plants that had antibacterial and antiviral activity. Moreover, we firstly revealed the cellular morphological alterations via electron microscope among bacteria treated with lipophilic extracts. The elongated cell was found in *S. aureus* and cell lysis and fluid leakage in *P. aeruginosa.* These phenomena were similar to the appearance of bacteria treated with β-lactam which inhibits the various penicillin binding proteins leading to irregularities in cell wall structure such as elongation, and lysis (Hayes and Orr, 1983). The mechanism of the antimicrobial effect of our studied extracts needs further study.

In summary, we studied the antimicrobial effects of the lipophilic leaf and stem bark extracts from *Lasianthus pilosus* and *L. stipularis* plants and found them to have antibacterial activity against *P. aeruginosa*, *A. baumannii* and *S. aureus*. The compounds of these extracts need to be separated and studied for their potential to be developed into antimicrobials.

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