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Scopoletin from *Lasianthus lucidus* Blume (Rubiaceae): A potential antimicrobial against multidrug-resistant *Pseudomonas aeruginosa*

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ABSTRACT

A bioassay-guided study of stem bark lipophilic extracts led to the isolation of a bioactive compound from *Lasianthus lucidus* Blume species related to traditional medicine for treatment of wound infections, bleeding, and fevers. Scopoletin (7-hydroxy-6-methoxycoumarin) could be isolated from stem bark extract yielding 1.4 µmol/g by dry weight. The minimum inhibitory concentrations of extracts and scopoletin proved to be effective against both strains of *Pseudomonas aeruginosa* ATCC 27853 (AmpC β-lactamase producing strain) and *P. aeruginosa* DMSC 37166 (clinical strain). They revealed the highest antibacterial effect at 128 µg/ml and morphological changes on bacterial cells were demonstrated by Field Emission Scanning Electron Microscope. The lipophilic extracts and the purified scopoletin from *L. lucidus* have clear antibacterial activities, especially acting against *P. aeruginosa* strains. They produce cell lysis, inflated swelling cell walls and cell walls sinking into cells in the same way.

INTRODUCTION

Scopoletin is a phenolic coumarin isolated from many plants, known as an important compound of the phytoalexin group (Tal and Robeson, 1985). It has a yellow crystalline structure with a molecular weight of 192 and a melting point of 204–206°C (Vasconcelos *et al.*, 1998). Its various biological activities have been reported through a number of investigations. Booth et al. (2004) reported that such compound produced specific biological activities and possible health implications for humans in food and medicine. In clinical uses, scopoletin and the substance class of coumarins were described and tested for treating anticonvulsant properties, cardiovascular and neuromuscular symptoms (Adesina, 1982) as well as an anti-diabetic agent, use in alleviating insulin resistance and anticoagulant (Chang *et al.*, 2015). For infectious diseases, coumarins and scopoletin were described as potentially exhibiting antibacterial activity against bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Escherichia coli* (Souza *et al.*, 2005 and Deng *et al.*, 2007). In an animal model study, Panda and Kar (2006) demonstrated that scopoletin at a low dose (1 mg/kg) had the potential to regulate hyperthyroidism and hyperglycemia. Obasi *et al.* (1996) and Moon *et al.* (2007) suggested the possible role of dietary scopoletin in some disorders of blood clotting and lipid metabolism in animals and effects on inflammation acting on mast cells. Scopoletin has served an important role for a long time

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in traditional medicine in Africa, Asia, and Europe. Several plant families, e.g. Aceraceae, Asteraceae, Euphorbiaceae, Fabaceae, Rubiaceae, Combretaceae, Meliaceae, Rutaceae, Solanaceae etc. contain high yields of scopoletin and are used as medicine for convulsion symptoms and rheumatic pain. The fruit and seeds of *Tetrapleura tetraptera* (Fabaceae) are used in Nigeria and Ghana, while the fruit of *Physalis alkekengi* (Solanaceae) is used to reduce inflammation in Colombia (Ojewole and Adesina, 1983; Xia *et al.*, 2007). The juice from the fruits and leaves of *Morinda citrifolia* (Rubiaceae), namely known as "Noni" in the Asia Pacific, are used for the treatment of diabetes, regulation of blood pressure and as a poultice on wounds (Handy *et al.*, 1934; McClatchey, 2002). In 2003, the official journal of the European Union reported that the European Commission approved that "Noni" fruit juice was a novel and safe health food in Europe. In addition, Nawrot *et al.* (2013) and Dai *et al.* (2018) reported that scopoletin isolated from the stem bark of *Cedrelopsis rakotozafyi* Cheek & Lescot (Rutaceae) used as febrifuges or reduce fevers. Additionally, the new coumarins were discovered from the roots of *Terminalia trophophylla* H. Perrier (Combretaceae) and the stem bark of *Astrotrichilia sp.* (Meliaceae) revealed potentially activities against A2780 human ovarian cancer cell line (Dai *et al.*, 2018).

Lasianthus lucidus Blume species of the coffee family (Rubiaceae) is a shrub native to tropical and subtropical regions of Asia, especially in tropical regions of China, India and Southeast Asia. The species grows in primary forests, with occasional records from disturbed forests or forest edges (Robbrecht, 1988; Cai *et al.*, 2005; Zhu *et al.*, 2012). Interestingly, the leaves and stem bark extracts have been reported for its potentially antibacterial property against *Pseudomonas aeruginosa* (Rai and Lalramnghinglova, 2011; Napiroon *et al.*, 2017a; Napiroon *et al.*, 2017b). *Pseudomonas aeruginosa* is one pathogenic bacterium in the list of antimicrobials resistant or superbugs for surveillance and monitoring by the World Health Organization (WHO) reports (WHO, 2017). Developing novel antimicrobial agents against *P. aeruginosa* is a much more difficult task because of the presence of decrease membrane permeability and multidrug efflux pumps (Morita *et al.*, 2014).

Lasianthus lucidus was currently studied in our laboratories and was used in traditional medicine to stop bleeding from wounds and applied to reduce fever which caused by infections (Rai and Lalramnghinglova, 2011). Consequently, we hypothesize that the bioactive compounds from *L. lucidus* extract may have antibacterial effects. To test this hypothesis, we did plant extraction and isolation of a pure compound found in the species and finally tests their anti-pseudomonad action.

MATERIALS AND METHODS

Plant material

Mature leaves and stem bark of *L. lucidus* were collected during the fruiting stage between November and December 2016, from hill evergreen forests in Thailand. Voucher specimens of *L. lucidus* (collector no. *NT015*) were deposited at the Department of Botany, Kasetsart University, Bangkok Forest Herbarium (BKF), and Aarhus University Herbarium (AAU). Comparisons were made with type specimens of the species at the K and L herbaria, using photographs available on the web and related literature (Zhu *et al.* 2012). Plant samples were observed under a stereo microscope. Herbarium acronyms follow Index Herbariorum (sweetgum.nybg.org/science/ih/).

Plant extractions

Mature leaves and stem bark were separated and dried under shade, chopped into small pieces and powdered using an electronic mill. 200 g of the powder was macerated with methanol (CH_3OH) for seven days in the dark at room temperature. Subsequently, extracts were filtered through Whatman No.1 filter paper and then concentrated by rotary evaporation at 37° to 39°C, until the crude extracts were semi-solid. The concentrated crude extracts were partitioned to a hydrophilic extract in distilled water and lipophilic extract in chloroform. The lipophilic extracts were stored at a temperature below −45°C.

The lipophilic extracts were placed on Thin Layer Chromatography (TLC) precoated silica gel 60 F_{254} plates (20 × 20 cm; Merck) using a solvent system of hexane:ethyl acetate (7:3 v/v) and detected under UV irradiation (365 nm and 254 nm). The Rf values of each fluorescent spot were determined as a TLC pattern. For high-performance liquid chromatography (HPLC) analysis, samples of 10 mg/mL of lipophilic extracts and the pure compounds in methanol (CH_3OH; HPLC grade, Merck) were prepared and filtered through a 0.45 µm nylon filter using a solvent system, 60% v/v methanol gradient 60 to 100% (HPLC grade; Merck) in 40% v/v aqueous buffer (0.015 M orthophosphoric acid pH 3 and 0.015 M tetrabutylammonium hydroxide) that followed suitable mobile phase for coumarins detection (Vajrodaya 1998 and Vajrodaya *et al.* 1998). The HPLC analysis was performed with Agilent 1100 series and was detected by UV photodiode arrays detector with wavelengths of 230 nm, 254 nm, and 280 nm, respectively.

Isolation

The bioactive compound was evaluated using medium pressure liquid chromatography (MPLC) technique. Stem bark lipophilic extract (670 mg dissolved in 6 ml MeOH) was applicated onto a glass column (400 × 40 mm, Büchi) filled with silica gel 25 to 40 µm (Merck LiChroprep Silica gel 60, 25-40 µm) as an absorbent using a flow rate of 12 ml/min. A pressure of about 4 bars was used (pump model 1-10 bar, Fluid Metering, Chromatographic pump from Combiflash, USA). Fractions of 50 ml were collected with the following solvent system: First, 30% ethyl acetate in hexane was collected in 7 fractions (fractions 1 to 7). Next, 50% ethyl acetate in hexane was collected in 9 fractions (fractions 8 to 16) followed by 70% ethyl acetate in hexane collected in 9 fractions (fractions 17 to 25). The column was washed and calibrated with methanol (Analytic grade, Merck) before and after each run for 30 min with the pressure set to 4 bars and a flow rate of 12 ml/min.

All fractions were checked for purity with thin layer chromatography (TLC) and UV detection (wavelength 365 nm). Fractions with similar composition (fraction 14-20) were combined and recrystallized with diethyl ether to give 54 mg of pure scopoletin in form of yellow crystals and then 10 mg of scopoletin was dissolved in DMSO for bioassay tests.

Antibacterial activity

For antimicrobial assays, plant extracts were evaluated using two pathogenic bacterial strains from the Department of Medical Science, Ministry of Public Health, Thailand: *Pseudomonas aeruginosa* ATCC 27853 (AmpC β-lactamase producing strain) and *P. aeruginosa* DMSC 37166 (Clinical strain). The quality control of the Clinical and Laboratory Standards Institute (CLSI 2017) for standardized sensitivity test, *P. aeruginosa* ATCC 27853 was used as positive control species with standard antibiotic including; AMK (Amikacin 30 µg), CIP (Ciprofloxacin 5 µg) and PIP/TAZ (Piperacillin/Tazobactam 100/10 µg).

The minimum inhibitory concentration (MIC) of extracts and pure compound were determined using the broth microdilution method. Stock solutions of 10 mg/mL which corresponds to 0.91% w/w (prepared by dissolving each extract and pure compound in dimethyl sulfoxide (DMSO-Sigma-Aldrich, USA) was serially diluted with 2-fold concentrations. Standard inocula of test bacteria in Mueller-Hinton broth (MHB-Oxoid, Basingstoke, UK) were placed in separated wells of a 96-well plate using DMSO as the control. The organisms were cultivated at 37ºC for 18 hours. The MIC was documented as the lowest concentration of extracts that inhibited visible growth.

Field Emission Scanning Electron Microscopy (FE-SEM)

For field emission scanning electron microscopy (FE-SEM), the suspensions of *Pseudomonas aeruginosa* ATCC 27853 (AmpC β-lactamase producing strain) and *P. aeruginosa* DMSC 37166 (Clinical strain) of 105 CFU/mL in lipophilic extracts and scopoletin at the lowest MIC concentrations combined with Mueller-Hinton Broth (MHB) were incubated at 37ºC for six hours and then filtered through 0.22 µM filter paper. The bacteria on the filter paper were fixed with 2.5% glutaraldehyde in 0.2 M sodium phosphate saline buffer (pH 7.2) for 12 hours at 4ºC. The fixed bacteria were washed three times with 0.2 M sodium phosphate saline buffer (pH 7.2) for about 15 minutes each time and then post-fixed with 1% osmium tetroxide (OsO4) in distilled water for one hour applying the method of cell fixation from Cardozo *et al.* (2013). After that, the filter paper was cleaned up using three cycles (15 minutes) of distilled water. The fixed samples were dehydrated in gradient acetone concentrations (20, 40, 60, 80 and 100%) and then dried at a critical point of CO2 (Polaron Range SC7620 Sputter Coater & CA7625 Carbon Accessary). Finally, the filter paper on the carbon tape was coated with platinum (AUTOLAB, Spin coater) and observed under an FE-SEM (HITACHI FE-SEM SU8010 Ultra-High Resolution (1.0 nm); Japan).

RESULTS

Chromatographic analyses of stem bark extracts and pure compound

The HPLC profiles showed four dominant peaks in leaf extracts while only one dominant peak appeared in stem bark extracts. The results showed that the chemical characters of stem bark lipophilic extract present similar characteristics to the pure compound at an approximate retention time of 3.7 min in the same mobile phase. HPLC chromatograms of both stem bark lipophilic extract and the pure compound are provided in Figure 1. Pure scopoletin had a retention time of approximately 3.7 min, the UV spectra, and HPLC chromatogram is shown in Figure 2.

After fractionation combination of fraction 14 to 20 appeared as fluorescent light blue spots on developed TLC plates (solvent system, hexane:ethyl acetate (7:3 v/v)), when observed under UV irradiation, at wavelength 365 nm. The fractions produced the most intense fluorescent light blue spot. The stem bark extract of *L. lucidus* and the fractions also produced the same spot with a relative front (Rf) of 0.5 (Figure 1).

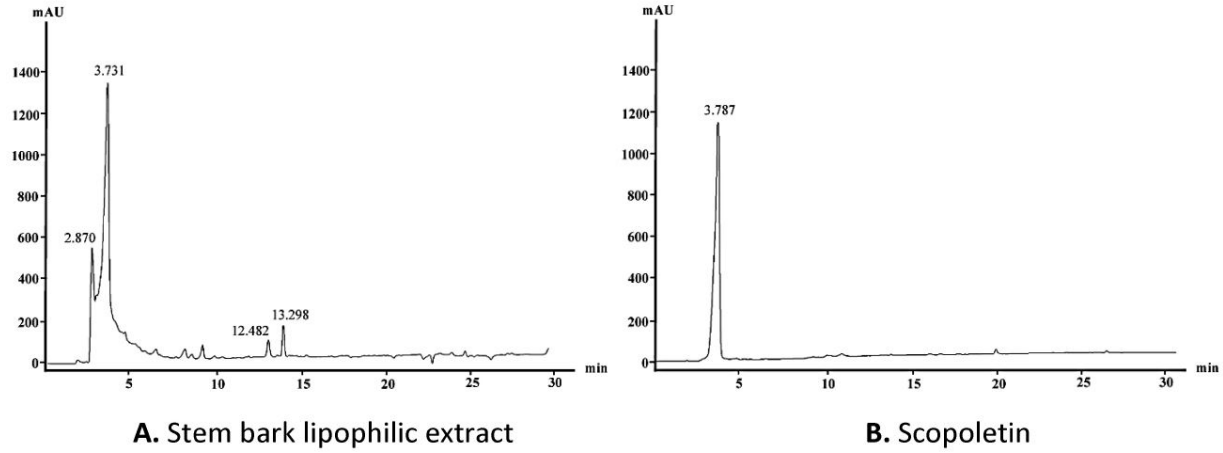

Fig. 1: HPLC chromatograms of stem bark lipophilic extract and scopoletin isolated from stem bark lipophilic extracts of *L. lucidus* in 10 mg/mL concentration (mobile phase; aqueous buffer: methanal, 40:60 v/v).

Pure compound and Nuclear Magnetic Resonance (NMR) structure elucidation

The yellowish crystalline illuminated blue fluorescence under UV light (wavelength 365 nm) and showed the physical property using 1D (^{1}H, NOE) and 2D (HSQC) NMR as following; ^{1}H NMR (400 MHz, AcCN) δ: 3.90 (3H, s, OMe), 6.18 (1H, d, J = 9.5 Hz, H-3), 6.82 (1H, s, H-8), 7.09 (1H, s, H-5), 7.75 (1H, d, J = 9.5 Hz, H-4). The ^{1}H NMR spectrum of the pure compound

showed two doublets at δ 6.18 ppm and δ 7.75 ppm (each, 1H, d, J = 9.5 Hz), characteristic of H-3 and H-4 protons of the pyrone ring. In addition, two singlets at δ 6.82 ppm and δ 7.09 ppm were assigned to H-5 and H-8, respectively, a three proton singlet at δ 3.90 ppm was attributed to 6-O-methyl group and melting point of 204–206°C. The NMR data are in agreement with those of reported ones for scopoletin. NMR spectra were recorded on a Bruker Avance II 400 (resonance frequencies 400.13 MHz for ^{1}H and 100.63 MHz for ^{13}C) equipped with a 5 mm observe broadband probe head (BBFO) with z-gradients at room temperature. The sample was dissolved in 0.6 ml of CD_3CN (99.8 % D). Chemical shifts are given in ppm, referenced to residual solvent signals (1.94 ppm for ^{1}H).

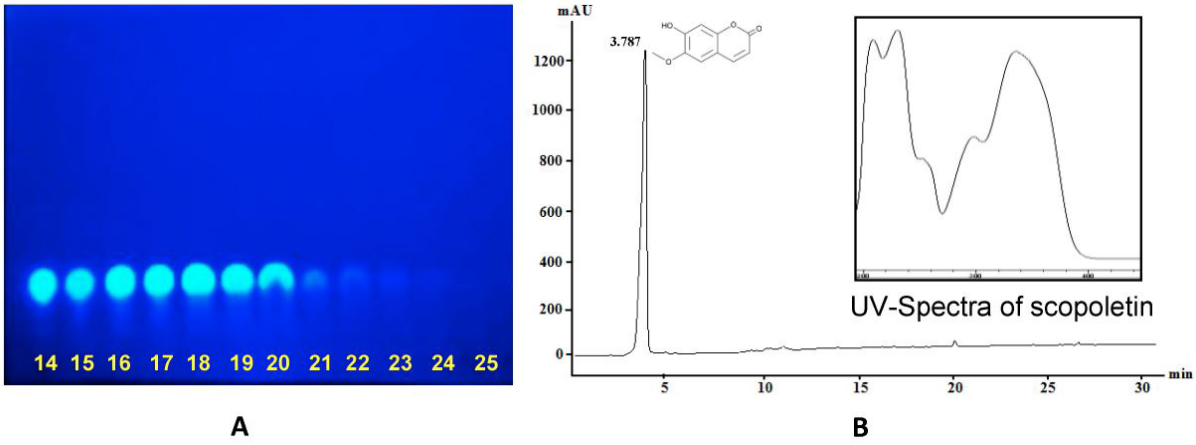

Fig. 2: A. TLC chromatogram of scopoletin isolated from *L. lucidus*, dissolved with methanol in mobile phase; hexane:ethyl acetate (7:3 v/v) **B.** HPLC chromatogram and UV spectra of scopoletin (combined fractions 14 to 20), dissolved with methanol in mobile phase; aqueous buffer:methanol (40:60 v/v).

The chemical structure of scopoletin (7-hydroxy-6-methoxy coumarin) is shown in Figure 3.

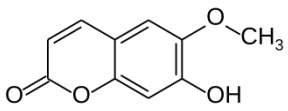

Fig. 3: Scopoletin (7-Hydroxy-6-methoxycoumarin).

Antibacterial activity

The MIC tests of the plant extracts exhibited the highest efficacy against both strains of *P. aeruginosa* (DMSC 37166 and ATCC 27853) (Table 1). The MICs of *L. lucidus* leaf extract, stem bark extract, and scopoletin were 256, 128 and 128 µg/ml, respectively. The lipophilic stem bark extract and pure scopoletin had equal effective inhibitory effects on *P. aeruginosa* ATCC 27853 (AmpC β-lactamase producing strain) at 128 µg/ml as shown in Table 1.

Field Emission Scanning Electron Microscopy (FE-SEM)

FE-SEM analysis showed significant morphological changes in *P. aeruginosa* ATCC 27853 after exposure to lipophilic extracts and scopoletin regarding its MIC value. The extracts clearly produced cellular lysis, an inflated swelling appeared on cell walls and cell elongation when compared with untreated bacteria (control) (Figure 4).

DISCUSSION

The separation of extracts was done by using MPLC technique with a suitable mobile phase, TLC and HPLC were used to determine and investigate the purity of the isolated compounds (Kupiec, 2004 and Lukaza and Minika, 2009). Our HPLC process was confirmed in triplicate testings that showed all the similar UV spectra information of scopoletin. This result showed the similar UV spectrum (λ max) as the information of scopoletin from the previous report of Ferdinal *et al.* (2015).

Table 1: Inhibitory effects (MIC, µg/ml) of extracts and scopoletin from *L. lucidus* against *Pseudomonas aeruginosa*.

Compounds*	MIC µg/mL	
	***P. aeruginosa* ATCC 27853 (AmpC β-lactamase producing strain)**	***P. aeruginosa* DMSC 37166 (Clinical strain)**
Leaf extract	256	256
Stem bark extract	128	128
Scopoletin (µM)	0.66	0.66

*Each sample was tested in triplicate.

Of our study, both chromatographic techniques helped to discover scopoletin from stem bark lipophilic extract of *L. lucidus*. The profiles may provide prediction tools of accumulation trends which can be expected in yet unstudied, but closely related species within a genus, thus facilitating the investigation for bioactive compounds (Murray *et al.*, 1982). Our study also detected coumarins and the firstly isolated scopoletin from stem bark lipophilic extract of L. lucidus (Rubiaceae). Similarly, Murray *et al.* (1982) collected information of the coumarin group found in Rubiaceae such as the species in genus *Morinda*, *Hymenodictyon* and *Hedyotis* etc. (Gnonlonfin *et al.*, 2012). These species contained scopoletin (7-hydroxy-6-methoxycoumarin) either in leaves, stem bark and roots. It has been found in several other plants revealing different concentrations in each plant part (Ishikura *et al.*, 1979). In 1979 Ishikura *et al.* described for the first time the occurrence of scopoletin in *Lasianthus* by isolating it from *L. japonicus*. We isolated scopoletin from *L. lucidus* yielding 1.4 µmol/g from stem bark, (200 g dry weight), that gave the highest yield of scopoletin

when compared with related reports in other plants, i.e. the report of yields from Gnonlonfin *et al.* (2012).

Regarding the role in defense, scopoletin has shown potential antibacterial properties, i.e. coumarins and scopoletin, detected in *Morinda citrifolia* (Rubiaceae) or Noni, inhibiting the growth of certain pathogenic bacteria, such as *S. aureus, P. aeruginosa, Morganella morgaii, Bacillus subtilis, Escherichia coli* and *Salmonella* spp. (More *et al.*, 2012). Similarly, extracts from several plants in the genus *Lasianthus* (Rubiaceae) have inhibited pathogenic bacteria especially in strains of *P. aeruginosa* and which one related to traditional infectious diseases (Napiroon *et al.*, 2017a; Napiroon *et al.*, 2017b). Thus, these authors have explained that scopoletin seems to be an effective antimicrobial as proved by the bioassays. From cytotoxicity reports, scopoletin was relatively nontoxic on McCoy fibroblast cell lines, showing its IC_{50} of 132.50 µg/mL (Hayes and Orr, 1983).

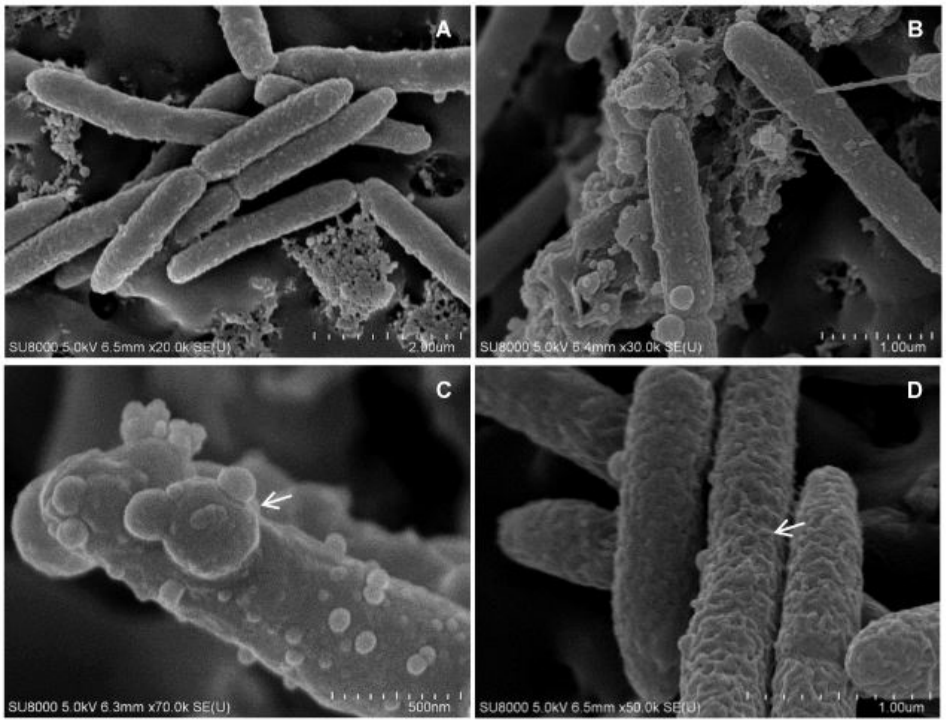

Fig. 4: FE-SEM images of *Pseudomonas aeruginosa* (AmpC β-lactamase producing strain); **A.** Cells in the absence of compounds (control), **B.** Cells in stem bark extract showed cell lysis and inflated swelling on cell walls**, C.** and **D.** Cells after treatment with scopoletin isolated from stem bark extracts. Note: cell walls showed inflated swelling (C) and cell walls sinking into cells (D).

Our study revealed the effect *L. lucidus* lipophilic extracts and its pure compound, scopoletin, from stem bark extract on *P. aeruginosa* for the first time. The stem bark lipophilic extracts and scopoletin had activity against *P. aeruginosa* even at low concentration (128 µg/ml) which therefore can be used as an effective source of antibacterial agents. In addition, we found morphological cellular changes documented by electron microscopy. Inflated swelling cell walls, prolonged cells and the leakage of content were observed. These phenomena seem to be similar to the mechanisms of antibiotics in β-lactam group, which inhibit penicillin-binding proteins leading to deformed cell wall structure such as elongation, lesions, and lysis (Hayes and Orr, 1983; Cardozo *et al.*, 2013). Thus, scopoletin might serve as a hopeful antibacterial agent when further developed to use as antibiotic sources.

CONCLUSION

The scopoletin isolated from stem bark lipophilic extracts of *L. lucidus* showed significant antibacterial properties in a similar manner; from this action morphological changes could be observed on bacterial cells after treating with compounds. The lipophilic extracts showed pronounced anti-pseudomonas activity against *P. aeruginosa*, which could explain the use of the plant in treating fevers and wound infections caused by bacteria such as *P. aeruginosa.* The biological activity of *L. lucidus* found in this study was attributed to its high concentration of scopoletin 1.4 µmol/g from stem bark (200 g dry weight). The MIC results of scopoletin in this study showed activity as an anti-Pseudomonas agent. The analytical methods used in this study showed also reproducible compound patterns useful to identify scopoletin and other coumarins in *Lasianthus*.

PATENT

This work has received the petty patent no. 14069 from Department of Intellectual property, Thailand in July 2018.

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CONFLICT OF INTERESTS

No potential conflict of interest was reported by the authors.

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