Original Article

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Phytochemistry and DNA-based authentication of medicinal *Lasianthus stipularis* blume (Rubiaceae) species against human pathogenic bacteria

Tiwtawat Napiroon[1], Sutee Duangjai[2], Srunya Vajrodaya[3], Markus Bacher[4], Henrik Balslev[5], Wichai Santimaleeworagun[6]

[1]Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Pathum Thani, Thailand, [2]Department of Forest biology, Faculty of Forestry, Kasetsart University, Bangkok, Thailand, [3]Department of Botany, Faculty of Science, Kasetsart University, Bangkok, Thailand, [4]Division of Chemistry of Renewable Resources, University of Natural Resources and Life Sciences (BOKU), Vienna, Austria, Wien, [5]Department of Bioscience, Faculty of Science and Technology, Aarhus University, Aarhus, Denmark, [6]Department of Pharmacy, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand

Corresponding Author:
Tiwtawat Napiroon, Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Pathum Thani, Thailand.
E-mail: napiroont@gmail.com/
Wichai Santimaleeworagun, Department of Pharmacy, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand.
E-mail: swichai1234@gmail.com

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ABSTRACT

Lasianthus stipularis Blume is a medicinal plant found in the tropical rainforests of peninsular Thailand and Malaysia. Its leaves are used in traditional medicine to treat a condition called "noise in the head" (i.e., ear infections). As such infections are often caused by Gram-negative bacteria; this species of *Lasianthus* may have antibacterial effects. In this study, we examined the non-polar fractions from the leaves and stem bark of *L. stipularis* using thin-layer chromatography and high-performance liquid chromatography (HPLC). The non-polar fractions exhibited a high coumarin content, while stem bark extracts showed a high concentration of 7-hydroxy-6-methoxycoumarin when isolated by column chromatography. The antibacterial activity of these extracts was evaluated by disk diffusion and broth microdilution against seven Gram-negative bacteria include *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* DMSC 37166, *P. aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC–BAA 1705, *K. pneumoniae* ATCC–BAA 1706, *Acinetobacter baumannii* ATCC 19606, and *A. baumannii* DMSC 3487. The DNA of *L. stipularis* was sequenced to support species identification and authentication. The minimum inhibitory concentration of fractions and pure compounds against pathogenic bacteria ranged from 100 to 200 µg/ml. Both strains of *P. aeruginosa* revealed the highest antibacterial activity at 100 µg/ml and 400 µg/ml concentration effect on *Acinetobacter baumannii*. In conclusion, leaf and stem bark non-polar extracts of *L. stipularis* have clear antibacterial activities.

Keywords: Biotechnology, DNA, infectious diseases, *Lasianthus*, medicinal plants

INTRODUCTION

Lasianthus stipularis Blume (Rubiaceae), also known as Stipulate Wool flower and Ubat barah in Malaysia,[1,2] and Kham Sri in Thailand, is widely distributed throughout the tropical rainforests of peninsular Thailand and Malaysia in the southeastern Asian archipelago. In Thailand, *L. stipularis* is found in the southern provinces Phang-nga, Ranong, Surat Thani, Trang, and Nakhon Si Thammarat.[3] It has glabrous to glabrescent branches, membranaceous, oblanceolate to oblong and glabrous leaf blades, broadly triangular to ovate stipules that cover the flowers. Its flowers are sessile, and the calyx is campanulate and hirsute. The fruits are drupaceous, glossy, and dark blue when ripe.[4] On the Malay peninsular, people in the Sarawak state use a liquid extract from the plant's leaves by boiling them with the leaves of *Lindera selangorensis* (Lauraceae) to treat a condition called "noise in the head,"[1,2,5,6] although the therapeutic basis has yet to be studied.

Phytochemically, several species of *Lasianthus* have been studied but there are no previous reports on the isolated compounds and bioactivity of *L. stipularis* or its potential antibacterial effect. Iridoid glycosides were previously isolated from the related *Lasianthus wallichii* Wight and Arn.[7] while alkaloids, steroids, and flavonoids were isolated from *Lasianthus fordii* Hance, *Lasianthus gardneri* (Thw.) Hook., and *Lasianthus acuminatissimus* Merr.[8] Subsequently, 47 chemical constituents were isolated from different species of *Lasianthus*, including bioactive compounds such as anthraquinones, alkaloids, sesquiterpene, secolupane derivatives, as well as iridoid and noniridoid glycosides, for which the chemical structures were described using spectroscopic analysis.[9] Iridoid glycosides, asperuloside, and deacetyl asperuloside were isolated from leaf extracts of *L. acuminatissimus*, a plant commonly used to treat rheumatoid arthritis.[10] The biological activity of *Lasianthus oblongus* was studied using methanolic leaf extracts and screening them for antioxidant and nitric oxide inhibitory activities.[11] Interestingly, many *Lasianthus* extracts display substantial antibacterial activity against Gram-negative pathogenic bacteria.[12,13]

Pathogenic bacterial infections remain a serious concern worldwide, particularly those caused by Gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae, Acinetobacter baumanii*, and *Pseudomonas aeruginosa.*[14-16] These microorganisms infect various organs including skin and soft tissues, the respiratory tract, urinary tract, bloodstream, and intra-abdomen.[17-19] Moreover, many of these pathogenic bacteria have developed resistance to mainstream antimicrobials; the world is in a post-antibiotic era. In the past 30 years, the treatment of pathogenic bacterial infections has been more problematic than ever due to multidrug-resistant organisms.[20] Effective infectious control of resistant bacterial strains and the appropriate use of available antibacterial agents remains the best strategy to combat infectious diseases in this era of antibiotic resistance. To improve treatments within the community and hospital settings alike, scientists need to discover new antimicrobial agents to combat multidrug antibiotic-resistant bacteria.[21,22]

In this study, we investigate the phytochemical characters, DNA characterization and antibacterial activity of *L. stipularis*, commonly used as a traditional treatment in South East Asia against pathogenic bacterial strains. We hypothesized that some compounds in this plant inhibit the growth of potentially dangerous pathogenic bacteria. We specifically examined the effect of *L. stipularis* leaf and stem bark extracts on strains of various Gram-negative bacteria (*E. coli*, *P. aeruginosa,* and *Acinetobacter baumannii*).

MATERIALS AND METHODS

Plants Material

Mature leaves and stem bark of *L. stipularis* [Figure 1] were collected from Sri Phang-nga National Park, peninsular Thailand. Voucher specimens of *L. stipularis* (Napiroon 037) were deposited at the Forest Herbarium (BKF) and Aarhus University Herbarium (AAU). The plant samples were taxonomically identified by Dr. Tiwtawat Napiroon and compared with type specimens (isotype K!000763942).

Figure 1: *Lasianthus stipularis* Blume; A. Plant habit (top view) and leaves, B. Stem bark

Preparation of Plant Extractions

Mature leaves and stem bark were separated and dried under shade, then chopped into small pieces and powdered using an electronic mill. The powder was macerated with methanol (CH_3OH) for 7 days in the dark at room temperature. Subsequently, the extracts were filtered through Whatman No.1 filter paper, and then concentrated by rotary evaporation at 37–39°C until the crude extracts were semi-solid. The concentrated crude extracts were partitioned into a polar-fraction in distilled water and non-polar fraction in chloroform. The non-polar fractions were concentrated and stored at below –20°C.

Phytochemical Analysis

The non-polar fraction were placed on thin layer chromatography (TLC) pre-coated 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 and 254 nm). The relative front (Rf) values of each fluoresced spot were determined as a TLC pattern. After development in the solvent system, the TLC plates were sprayed with a detecting reagent for screening major secondary metabolites using different reagents. Anisaldehyde sulfuric acid was used to detect terpenoids. After spraying the TLC plate, the plate was heated at 120°C until maximal visualization of the spots was achieved. Dragendorff's reagent was used to detect alkaloids and vanillin sulfuric acid reagent for higher alcohols, steroids, phenols, and essential oils, while UV light at 365 nm and 254 nm wavelengths was used to detect fluorescent and non-fluorescent compounds.

Samples of 10 mg/mL of non-polar fraction and scopoletin isolated from stem bark extract were dissolved in methanol (CH_3OH; HPLC grade, Merck). The samples were then filtered through a 0.45-µm nylon mesh. The solvent system, which included a methanol (HPLC grade; Merck) and an aqueous buffer (0.015 M ortho-phosphoric acid, pH 3; 0.015 M tetrabutyl ammonium hydroxide), was used as suitable mobile phase for coumarins detection.[23-25] The HPLC analysis was performed on PerkinElmer (Flexar series) and detected with UV photodiode arrays detector with wavelengths of 230, 254, and 280 nm.

Isolation

Both lipophilic extracts of the leaf and stem bark at a concentration of 300 mg in 3 ml of methanol were selected

for isolation. Silica gel (25–40 μm) was used as an absorbent in a glass column measuring 80 cm long and 1.7 cm diameter for four-step gradient elution of 50 ml $CHCl_3$ in MeOH (95, 90, 85, and 80% v/v). In each fraction, 25 ml per gradient was collected. All eight fractions were tested on the TLC plate and observed under UV irradiation (wavelength 365 nm). Compounds were examined before their combination into four fractions of the same TLC and HPLC profiles. Those fractions were evaporated and their respective physical characteristics were examined under their solid form. As only Fraction 2 showed a yellowish crystalline, we recrystallized those crystals using diethyl ether before collecting them in diethyl ether at −45°C before examining their structure in more detail using HPLC and nuclear magnetic resonance (NMR).

NMR Structure Elucidation

The structures of isolated compounds were verified using 1D- (^{1}H, ^{13}C) and 2D- (COSY, HSQC, HMBC) NMR information. The NMR spectra data were recorded on a Bruker Avance II 400 (resonance frequencies 400.13 MHz for ^{1}H and 100.63 MHz for ^{13}C) installed with a 5-mm observation broadband probe head with z-gradients at room temperature with a standard Bruker pulse program at the Institute of Chemistry of Renewable Resources, University of Natural Resources and Life Sciences, Vienna. Samples were dissolved in 0.6 ml CD_3CN (99.8% D, Euriso-top, Saint-Aubin Cedex, France). Chemical shifts were recorded in ppm unit, referenced to residual solvent signals (CD_3CN: 1.94 ppm for ^{1}H).

Antibacterial Activity

For the antimicrobial assays, non-polar fractions and pure compound were examined using pathogenic bacterial strains from the Department of Medical Science of the Thai Ministry of Public Health, which included seven gram-negative bacteria (*E. coli* ATCC 25922, *P. aeruginosa* DMSC 37166, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC–BAA 1705, *K. pneumoniae* ATCC–BAA 1706, *A. baumannii* ATCC 19606, and *A. baumannii* DMSC 3487).

Screening of the extracts for antibacterial activity determined the zone of inhibition using the disk (6-mm diameter) diffusion method. The obtained microorganism strains were inoculated in Muller-Hinton broth at 37°C for 18 h. Bacterial suspensions were then adjusted to the turbidity with a 0.5 McFarland standard. The standard inoculums were spread uniformly on Muller-Hinton agar. After drying, disks of paper containing 200 μg of fraction and pure compound were placed 30 mm apart on Muller-Hinton agar with the test organism. The agar plate was incubated at 37°C for another 24 h. According to the quality control of the Clinical and Laboratory Standards Institute,[26] *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 strains were used as control species with a standard antibiotic disk including ceftazidime (30 μg), ciprofloxacin (5 μg), imipenem (10 μg), meropenem (10 μg), colistin (10 μg), amikacin (30 μg), gentamicin (10 μg), piperacillin (100 μg), vancomycin (30 μg), and tigecycline (15 μg). These antibiotics were obtained from the standard laboratory of the Department of Pharmacy, Faculty of Pharmacy, Silpakorn University, Thailand.

Minimum inhibitory concentrations (MIC) of leaf and stem bark extracts were determined using the broth microdilution method. Stock solutions of 40 mg/mL (prepared by dissolving each extract in dimethyl sulfoxide, DMSO-Sigma-Aldrich, USA) were serially diluted with 2-fold concentrations. Each well of the 96-well plate was filled with standard inoculum of test bacteria in Mueller-Hinton broth (MHB-Oxoid, Basingstoke, UK). DMSO was used as control. The organisms were cultured at 37°C for 18 h. The MIC was then documented as the lowest concentration of extract that inhibited visible growth.

DNA Sequencing

Silica gel dried leaves of *L. stipularis* were ground under liquid nitrogen into a fine powder using a pestle. DNA extraction followed the cetyl trimethyl ammonium bromide extraction method[27] and total genomic DNA was determined qualitatively on 0.8% agarose gel electrophoresis stained by ethidium bromide and visualized under UV light. A 900-bp Sharp DNA Marker (Thermo Scientific) was used as standard molecular size. The extracted DNA was stored at –20°C and used for PCR amplification.

Primers were used based on the *rps16*, *trnTF*, internal transcribed spacer (ITS), and external transcribed spacer (ETS) region. The *rps16* intron was amplified with the PCR primers rpsF and rpsR2[28] and sequenced using the same primers. The *trnTF*-region was amplified using 1F[29] and *Lasianthus*-trnT1300R primers. The ITS region was amplified using the primers P17[30] and P25R.[31] For amplification and sequencing of ETS, the primers Erit-F[32] and 18S-E[33] were used [Table 1]. PCR reactions were started with a 10-min initial denaturation at 95°C to ensure complete separation of DNA strands, followed by 35 cycles of a double-stranded template DNA denaturation (at 94°C for 30 s), single-stranded template DNA primer annealing (at 56–58°C for 40 s), and primer extension (at 68°C for 45 s). The solution was stored at 20°C for further sequencing. The PCR products were examined on 0.8% agarose gel electrophoresis. The gels were stained using ethidium bromide solution for 15 min, de-stained in deionized water for 10 min, and the DNA was photographed under UV light. PCR products were then cleaned and purified by incubation with phosphatate and exonuclease enzymes (Thermo Scientific) and determined by Molecular Informatics Laboratory of Macrogen Company, Korea.

Table 1 Primers used for PCR and sequencing on *L. stipularis*.

DNA regions	Primers (5' to 3')
rps16	
rpsF	GTGGTAGAAAGCAACGTGCGACTT
rpsR2	TCGGGATCGAACATCAATTGCAAC
trnTF	
a1F	ACAAATGCGATGCTCTAACC3
Lasianthus-trnT1300R	ATTAATTCCAGGGTTTCTCTGA
ITS	
P17	CTACCGATTGAATGGTCCGGTGAA
P25	GGGTAGTCCCGCCTGACCTG
ETS	
Erit-F	CTTGTATGGGTTGGTTGGA
18S-E	GCAGGATCAACCAGGTAGCA

L. stipularis: *Lasianthus stipularis*

About 900-bp sequences per sample were obtained and consensus sequences were analyzed [Supplementary Table 1] using the ClustalX program.[34] Multiple sequence alignments were evaluated using the Macclade program and aligned by eye in colored cells. The file was then saved in the nexus format using PAUP* to examine nucleotide sequences. The percentages of guanine and cytosine (% CG) and DNA length were calculated using the Geneious 9.0.4 program (AAU Herbarium, Aarhus, Denmark), which takes a sequence in each species and compares it with data in Genbank. The amplified partial sequences were included in BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi), which takes a sequence query and matches it against GenBank. We used the percentage of average maximum identity, the percentage of average coverage, and that of average pairwise identity to measure the efficiency of species identification. Phylogenetic relationship of *Lasianthus* was performed using the maximum parsimony (MP) and Bayesian inference (BI).[24]

RESULTS

Qualitative Chromatography Analyses

The results on the TLC plates appeared as violet and gray-green bands for the detection of terpenoids using anisaldehyde sulfuric acid as a spraying reagent. Orange bands of alkaloids appeared after spraying with Dragendorff's reagent. Bands that attained maximum color intensity appeared after spraying with vanillin sulfuric acid reagent to detect higher alcohols, phenols, steroids, and essential oils. The TLC profile of leaf extracts showed both similarities and differences with that of stem bark extracts when deciding by Rf values. The results of the qualitative tests performed on lipophilic extracts from leaves and stem bark are shown in Table 2.

The HPLC profiles showed the three dominant peaks in the leaf extracts of *L. stipularis*, while only one dominant peak appeared in stem bark. The retention time and UV spectra of comparable signals in the lipophilic extracts of leaves and stem bark are shown in Figure 2. The results showed that the chemical characteristics of leaf and stem bark lipophilic extracts were similar to those of the pure compound (scopoletin) which was isolated from the stem bark extracts at an approximate retention time of 3.7 min in the mobile phase in Table 3.

Chemical Structure Elucidation by NMR

The yellow crystals were isolated from non-polar fraction which radiated blue fluorescence when detected with UV light (wavelength 365 nm). These crystals displayed the physical properties using 1D (1H, NOE) and 2D (HSQC) NMR information as following; 1H 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), and 7.75 (1H, d, J = 9.5 Hz, H-4). The 1H NMR spectrum of the crystals showed two doublets at δ 6.18 ppm and δ 7.75 ppm (each, 1H, d, J = 9.5 Hz), H-3 and H-4 protons showed the character of pyrone ring. 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 a melting point of 204–206°C. Its ^{13}C NMR (100 MHz, AcCN) spectrum exhibited important signals including oxygenated methyl group (OMe) at δ 57.10 and four methine groups at δ: 103.8 (C-8), 110.0 (C-5), 113.6 (C-3), and 114.7 (C-4). The crystal sample was dissolved in 0.6 ml of CD3CN (99.8 % D). The chemical shifts are provided in ppm, referenced to residual solvent signals (1.94 ppm for 1H). The NMR data are in agreement with those of reported ones for scopoletin.

Antibacterial Activity

The results of zone diameters and MIC of plant extracts exhibited the highest antibacterial activity against *P. aeruginosa*, followed by *A. baumannii*. In the clear zone of the lipophilic extract, the leaf and stem bark lipophilic extracts of *L. stipularis* showed the best antibacterial activity against *P. aeruginosa* DMSC 37166 and ATCC 27853, respectively, [Table 4 and Supplementary Figure 1]. Similarly, the MIC of *L. stipularis* leaf and stem bark extracts was recorded at 200 and 100 μg/ml for *P. aeruginosa* DMSC 37166, respectively. For *A. baumannii* DMSC 3487, the extracts were able to inhibit bacterial activity at 400 μg/ml [Table 5].

Molecular Characterization

The sequence length and GC content of the four loci (*rps16*, *trnTF*, ITS, and ETS) were obtained from the Geneious 9.0.4 and Clustal X alignment results [Table 6]. The GC content

Table 2: Relative front (Rf) values of the accumulated compounds in positive test of leaf and stem bark lipophilic extracts from *L. stipularis*

Reagent/UV wavelength	Relation front (Rf) values of extracts	
	Lasianthus stipularis	
	Leaf	Stem bark
Anisaldehyde sulfuric acid	0.14, 0.25, 0.40, 0.43, 0.94	0.14, 0.25
Dragendorff's reagent	NT*	NT*
Vanillin sulfuric acid	0.16, 0.26, 0.40, 0.45	0.16, 0.26
10% NaOH in ethanol	0.4	0.4
UV 254 nm	0.26	0.15
UV 365 nm	0.4	0.15, 0.4

*NT refer to Negative test. *L. stipularis*: *Lasianthus stipularis*

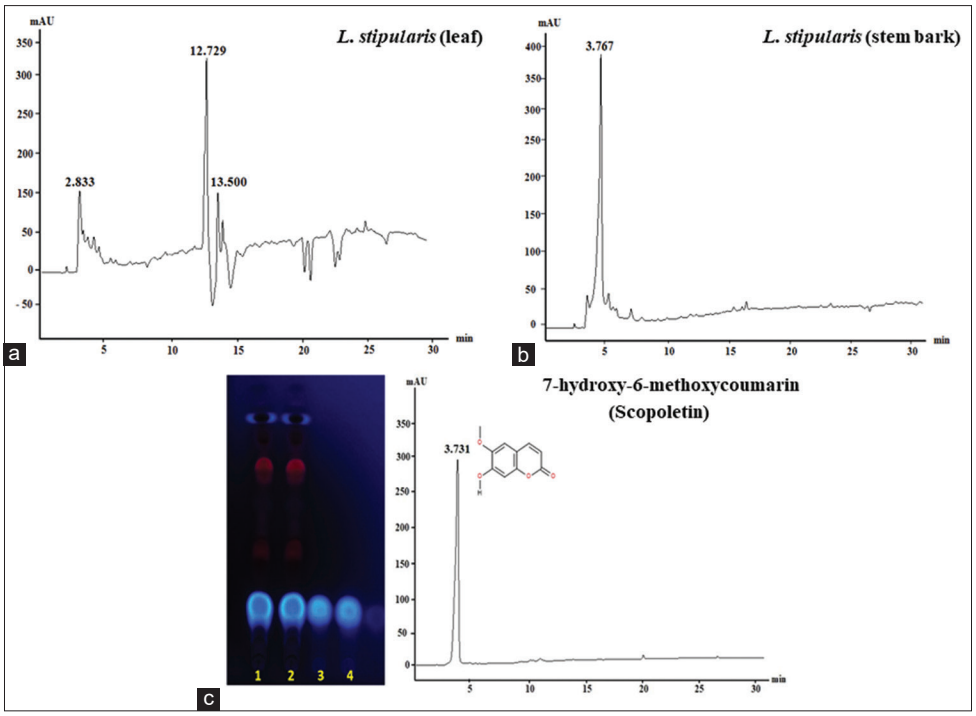

Figure 2 : Chromatogram characters; A. leaf lipophilic extract, B. stem bark lipophilic extract and C. Scopoletin (blue spots) contained in fraction no. 1-2 (leaf extracts), no. 3-4 (stem bark extracts). All samples dissolved with methanol (mobile phase; aqueous buffer: methanal, 40:60 v/v).

Table 3: The retention time and UV spectra of comparable signals in lipophilic extracts andscopoletin from *L. stipularis*

Botanical name	Retention time (min) and UV spectra (λ max, nm)		Stem bark		Scopoletin	
	Leaf					
	Retention time (min)	λ max (nm)	reTention time (min)	λ max (nm)	Retention time (min)	λ max (nm)
Lasianthus stipularis	2.833	283	3.767	205	3.731	205
	12.729	230				
	13.500	285				

L. stipularis: *Lasianthus stipularis*

Table 4: The inhibition zones diameter (mm) of leaf and stem bark lipophilic extract against bacterial strains

Bacterial strains	Diameter of inhibition zones (mm)ǂ			Standard antibiotics			
	L. stipularis						
	Leaf	Stem bark	Scopoletin	AMP	PIP	GEN	CIP
Gram–positive							
S.aureus (methicillin-resistant)*	10.67±1.15	15.66±0.57	6.33±0.58	28	28	21	23
Gram-negative							
E. coli (ATCC 25922)**	6.33±0.58	7.67±0.57	6.33±0.58	20	26	23	32
P. aeruginosa (ATCC 37166)	18.00±1.00	21.67±0.57	9.00±1.00	NT	NT	NT	NT
P. aeruginosa (ATCC 27853)	18.33±0.58	18.67±0.57	8.67±0.58	NT	NT	NT	NT
K. pneumoniae (BAA 1705)	7.00±1.00	8.33±1.15	6.33±0.58	NT	NT	NT	NT
K. pneumonia (BAA 1706)	9.00±1.00	9.00±1.00	6.33±0.58	NT	NT	NT	NT
A. baumannii (ATCC 19606)	11.33±1.15	8.67±0.58	8.67±1.15	NT	NT	NT	NT
A. baumannii (ATCC 3487)	9.67±0.58	12.00±1.00	8.33±0.58	NT	NT	NT	NT

ǂEach sample was tested in triplicate. **S. aureus* ATCC 25923 and ***Escherichia coli* ATCC 25922 strain were used as control species; the diameter (mm.) of clear zone in each antibiotic was within quality control ranges based on CLSI 2019. (AMP (Ampicillin), PIP (Piperacillin), GEN (Gentamicin), CIP (Ciprofloxacin). NT abbreviation refer to not tested. *L. stipularis*: *Lasianthus stipularis. P. aeruginosa*: *Pseudomonas aeruginosa*, *A. baumannii*: *Acinetobacter baumannii*

Table 5: MIC of lipophilic extracts against bacteria strains by microdilution method

Pathogenic bacteria strains	MIC (μg/ml)		
	L. stipularis		
	Leaf	Stem bark	Scopoletin
P. aeruginosa (ATCC 37166)	200	100	100
P. aeruginosa (ATCC 27853)	200	200	100
A. baumannii (ATCC 19606)	1600	1600	200
A. baumannii (ATCC 3487)	400	400	200

L. stipularis: *Lasianthus stipularis*, *P. aeruginosa*: *Pseudomonas aeruginosa*, *Acinetobacter baumannii*: *Acinetobacter baumannii*, MIC: Minimum inhibitory concentration

Table 6: DNA sequences data and BLAST analysis of *L. stipularis* compared within the genus *Lasianthus*

Accession no.	Molecular information					
	L. stipularis		BLAST analysis*			
	GC content (%)	DNA length (bp)	Average pairwise identity (%)	Average maximum identity (%)	Average GC content (%)	Number of taxa
MH341190 (*rps16*)	34.10	786	97.71	97.71	34.08	39
MH341186 (*trnTF*)	28.19	1,933	93.96	93.96	29.94	37
MH341198 (ITS)	51.73	692	89.21	89.21	53.98	50
MH341194 (ETS)	49.72	362	84.53	84.53	49.58	34

*The DNA sequences data references of genus *Lasianthus* from Genbank; *rps16*, *trnTF*, ITS and ETS regions. *L. stipularis*: *Lasianthus stipularis*

of *trnTF* was the lowest, while that of ITS was the highest. This was the first time that DNA sequences for *L. stipularis* have ever been described. The BLAST method was used to test the applicability of the four loci for species identification and similarity within the genus when compared with the sequencing data from GenBank. The *rps16* and *trnTF* showed a 97.10 and 93.96% average pairwise and maximum identity, respectively. Meanwhile, ITS and ETS showed an 89.21 and 84.53% average pairwise and maximum identity, respectively. The Bayesian and parsimony analyses of the nucleotides data set are congruent with Smedmark *et al*. (2014). Phylogenetically, the molecular data confirm the placement of *L. stipularis* within the genus *Lasianthus* and showed its monophyletic relationships in Figure 3. The DNA sequences of medicinal *L. stipularis* (Collector no. M. Poopath *et al*. 876 and 906) were submitted to the GenBank (accession number MH341186, MH341190, MH341198, and MH341194).

DISCUSSION

Phytochemically, qualitative information for specific compounds in leaf and stem bark extracts from *Lasianthus* (Rubiaceae) was investigated using color detection with different reagents on TLC plates, as well as matching Rf values, color, size, and shape of the detection zones under 365 nm UV light among samples. The results are used as evidence to support the classification of *L. stipularis*. We hypothesized that differences in profiles and chemical characteristics may affect the antibacterial activity of extracts. This study presents the first report of the chemical characteristics of *L. stipularis*, as shown by TLC and HPLC profiles. A preliminary separation of the investigated extracts was possible using TLC with a suitable mobile phase. In Lukasz and Monika (2009),[35] TLC was used as a tool for botanical material investigation, focusing on chemotaxonomy to analyze plant extracts and eventually establish the plant taxonomic classification. Moreover, HPLC analysis was used to separate and quantify bioactive compounds dissolved in solution or extracts and to determine a specific target compounds in solution.[36] Both chromatographic techniques have played a significant role in the discovery of significant bioactive compounds and natural products. The chemotaxonomical interpretation of chemical profiles may effectively predict accumulation of dominant compounds trends, which can be expected in closely related species within a genus. This facilitates an investigation of bioactive compounds as these trends have yet to be studied.[37] Many iridoid glucosides were detected in *Lasianthus* and *Ronabea* species, confirming the close taxonomic relationship between these two genera; this enabled researchers to assign them to the same tribe: Lasiantheae.[7,38] This is the first report of the chemical characteristics of *L. stipularis*, which may lead to the future discovery of chemical markers in chemotaxonomy and bioactive compounds for use in traditional plant medicines in South East Asia. A previous report on the phytochemistry of *Lasianthus* by Takeda *et al.* (2003)[8] isolated three new compounds: lasianthionoside A, B, and C from the leaves of *L. fordii*, as well as iridoid glucosides, asperuloside,[7] deacetyl asperuloside, methyl deacetyl asperuloside,[39] and citroside A.[40] Furthermore, Dallavalle *et al.* (2004)[41] were able to isolate the glucosidic compound stigmasterol 3-o-â-D-glucoside from *L. gardneri*, a species found in the mountainous forests of Sri Lanka.

This study was also the first investigation of the scopoletin in non-polar fraction of *L. stipularis* conform with Napiroon *et al*. (2018)[13] reported that coumarins and scopoletin act as antibacterial agents mostly contained in the genus *Lasianthus*. Similarly, our lipophilic extracts had promising inhibitory effects against *P. aeruginosa*, even in low concentrations, indicating that this plant could be a suitable source of

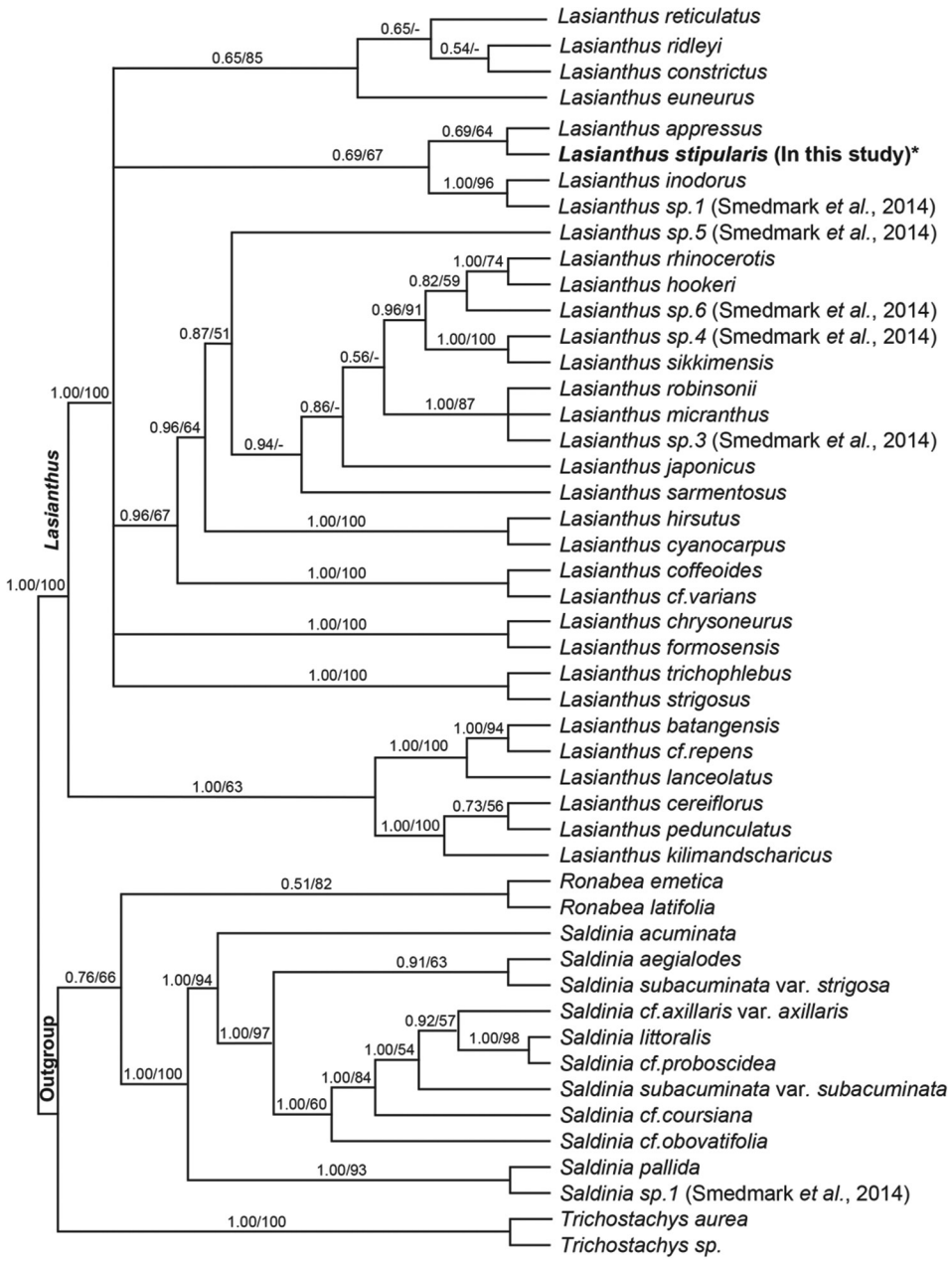

Figure 3: Phylogenetic placement of *Lasianthus stipularis* (bold font) in the genus *Lasianthus* inferred from Bayesian analysis. Bayesian posterior probabilities (PP) and bootstrap values (BP) are indicated at each node (PP/BP).

antibacterial agents for this particular genus of pathogens. Although scopoletin showed a significant antibacterial effect in MIC assays, this was indistinctive of inhibition when tested in disk diffusion assays. This discrepancy may be due to the diffusion of the compounds into the agar medium, making their antibacterial activity less competent than MIC assay.[42] Moreover, one pure compound might be less effective than the synergetic action of two or more compounds on the specific bacterial strain, as observed from differences in antibacterial effects of the crude extracts and bioactive compound in this study.

At present, the genetic molecular data for the genus *Lasianthus* comes from *rps16*, *trnTF*, ITS, and ETS,[24,43,44] while DNA barcoding assays and DNA identification for this species have not yet been reported. For the four DNA regions evaluated in this study, the ITS and ETS can be used to discriminate *L. stipularis* from other genera in the tribe Lasiantheae. From our results, it is clear that the variables %GC, DNA length, % average pairwise identity, and % average maximum identity for ITS and ETS, when examined together, could reliably identify *L. stipularis* at the species level. ITS and ETS exhibited a GC content of 51.73 and 49.72%, respectively. In addition, results from DNA analyses based on nuclear and plastid markers strongly support the recognition of *L. stipularis* as a member of *Lasianthus*. This is the first molecular report on a medicinal plant used by inhabitants of the Malay peninsula,

providing a basis for future studies on the conservation and management of this species.

Future research should aim to precisely and quantitative measured the antibacterial activity and the exact action mechanism of the plant extracts. Researchers should also investigate chemical compounds from other plant resources and their potential use in drug therapy development. The previous research suggested that plant extracts from *Lasianthus* spp. showed bioactivity against microorganisms, but our work is the first report of antibacterial effects in *L. stipularis* extracts against harmful bacterial pathogens.

CONCLUSIONS

For chromatographic techniques, our findings showed that coumarins, terpenoids, and phenolic compounds are important constituents in all of these extracts. The pure compound of stem bark lipophilic extracts found in this study was profuse with scopoletin. This is the first report of the high antibacterial activity of *L. stipularis,* particularly on human pathogenic bacteria. The differences in the chemical profiles of the leaf and stem bark extracts highlighted their differential composition and potential antibacterial effect. Such bioactive compounds found in the plant extracts and scopoletin may have useful applications in developing drugs to target antibiotic-resistant bacterial strains, or simply to use as alternative chemicals in chemotaxonomy and ethnopharmacology. Further research should be conducted to investigate the toxicity and clinical trial of these extracts, evaluating their applicability to various therapeutic uses. Furthermore, our sequencing data for *L. stipularis* revealed that the ITS and ETS were suitable loci for reliable species identification.

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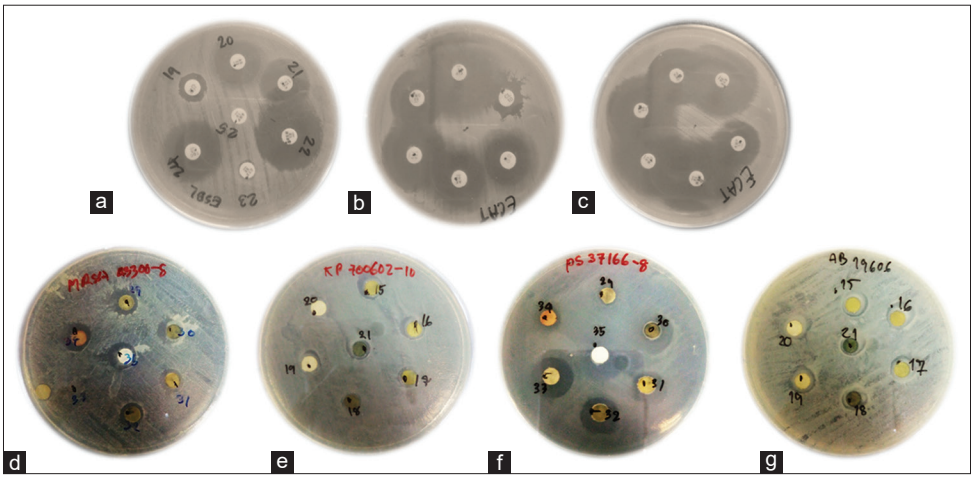
REFERENCES

1. Wiart C. Medicinal Plants of the Asia Pacific: Drugs for the Future? Singapore: World Scientific Publishing Co. Pte. Ltd.; 2006.
2. Quattrocchi U. CRC World Dictionary of Medicinal and Poisonous Plants. Florida: CRC Press Taylor and Francis Group; 2012.
3. Napiroon T, Chayamarit K, Dawson S, Till W, Balslev H. A synopsis of *Lasianthus* (Lasiantheae, Rubiaceae) in Thailand and two additional new species. Phytotaxa 2020;439:1-38.
4. Zhu H, Roos MC, Ridsdale CE. A taxonomic revision of the Malesian species of *Lasianthus* (Rubiaceae). Blumea 2012;57:1-102.
5. Perry LM, Metzger J. Medicinal Plants of East and Southeast Asia: Attributed Properties and Uses. Massachusetts: MIT Press; 1980.
6. Werner R. Medicines in Malay Villages. Kuala Lumpur: University of Malaya Press; 2002.
7. Takeda Y, Shimizu H, Mizuno K, Inouchi S, Masuda T, Hirata E, *et al*. An Iridoid glucoside dimer and a nonglycosideiridoid from the leaves of *Lasianthus wallichii*. Chem Pharm Bull 2002;50:1395-97.
8. Takeda Y, Shimizu H, Masuda T, Hirata E, Shinzato T, Bandoand M, *et al*. Lasianthionosides A-C megastigmane glucosides from leaves of *Lasianthus fordii*. Phytochemistry 2003;65:485-9.
9. Choudhury KD, Choudhury MD, Talukdar AD. Chemical constituents and biological activities of the genus *Lasianthus* Jack: A review. Assam Univ J Sci Tech 2010;6:129-38.
10. Bin L, Dong MZ, Yong ML, Xiao CG. Three new and antitumor anthraquinone glycosides from *Lasianthus acuminatissimus* MERR. Pharm Bull (Tokyo) 2006;54:297-300.
11. Saha K, Lajis NH, Israf DA, Hamzah AS, Khozirah S, Khamis S, *et al*. Evaluation of antioxidant and nitric oxide inhibitory activities of selected Malaysian medicinal plants. J Ethnopharmacol 2004;9:2263-7.
12. Napiroon T, Balslev H, Duangjai S, Sookchaloem D, Chayamarit K, Santimaleeworagun W, *et al*. Antibacterial property testing of two species of tropical plant *Lasianthus* (Rubiaceae). Southeast Asian J Trop Med Public Health 2017;48:117-23.
13. Napiroon T, Bacher M, Balslev H, Tawaitakham K, Santimaleeworagun W, Vajrodaya S. Scopoletin from *Lasianthus lucidus* Blume (Rubiaceae): A potential antimicrobial against multidrug-resistant *Pseudomonas aeruginosa*. J App Pharm Sci 2018a;8:1-6.
14. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, *et al*. Bad bugs, no drugs: No ESKAPE! An update from the infectious diseases society of America. Clin Infect Dis 2009;48:1-12.
15. Otter JA, French GL. Molecular epidemiology of community-associated meticillin-resistant *Staphylococcus aureus* in Europe. Lancet Infect Dis 2010;10:227-39.
16. Hotez PJ. Aboriginal populations and their neglected tropical diseases. PLoS Negl Trop Dis 2014;8:e2286.
17. Pantosti A. Methicillin-resistant *Staphylococcus aureus* associated with animals andits relevance to human health. Front Microbiol 2012;3:1-12.
18. Cuny C, Köck R, Witte W. Livestock associated MRSA (LA-MRSA) and its relevance for humans in Germany. Int J Med Microbiol 2013;303:331-7.
19. Köck R, Schaumburg F, Mellmann A, Köksal M, Jurke A, Becker K. Livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) as causes of human infection and colonization in Germany. PLoS One 2013;8:e55040.
20. Aksoy DY, Unal S. New antimicrobial agents for treatment of Gram-positive bacterial infections. Clin Microbiol Infect 2008;14:411-20.
21. Lee AS, Huttner B, Harbarth S. Control of methicillin-resistant *Staphylococcus aureus*. Infect Dis Clin North Am 2011;25:155-79.
22. Bassetti M, Merelli M, Temperoni C, Astilean A. New antibiotics for bad bugs: Where are we? Ann Clin Microbiol Antimicrob 2013;12:1-15.
23. Vajrodaya S, Bacher M, Greger H, Hofer O. Organ-specific chemical differences in *Glycosmis trichantera*. Phytochemistry 1998;48:897-902.
24. Napiroon T, Poopath M, Duangjai S, Balslev H, Vajrodaya S. *Lasianthus yalaensis* (Rubiaceae) a new species from peninsular Thailand. Phytotaxa 2018b;364:172-80.
25. Napiroon T, Tanruean K, Vajrodaya S. Comparative phytochemical evaluation and biological control properties from *Lasianthus verticillatus* (Lour.) Merr. (Rubiaceae) extracts. Res J Biotech 2019;14:41-9.
26. Clinical and Laboratory Standards Institute. Performance

Standards for Antimicrobial Susceptibility Testing, Twenty-First Informational Supplement M100-S21. Available from: https://clsi.org/media/2663/m100ed29_sample.pdf [Last accessed on 2019 Dec 15].
27. Doyle JJ, Doyle JL. Isolation of plant DNA from fresh tissue. Focus 1990;12:13-15.
28. Oxelman B, Liden M, Berglund D. Chloroplast rps16 intron phylogeny of the tribe Sileneae (Caryophyllaceae). Pl Syst Evol 1997;184:393-410.
29. Razafimandimbison SG, Bremer B. Phylogeny and classification of Nucleases (Rubiaceae) inferred from molecular (ITS, rbcL, and trnT-F) and morphological data. Am J Bot 2002;89:1027-41.
30. Popp M, Oxelman B. Inferring the history of the polyploid *Silene aegaea* (Caryophyllaceae) using plastid and homoeologous nuclear DNA sequences. Mol Phylogenet Evol 2001;20:474-81.
31. Oxelman B. RAPD patterns, nrDNA ITS sequences and morphological patterns in *Silene* section Sedoideae (Caryophyllaceae). Pl Syst Evol 1996;201:93-116.
32. NegrÓn-Ortiz V, Watson LE. Molecular phylogeny and biogeography of *Erithalis* (Rubiaceae), an endemic of the Caribbean Basin. Pl Syst Evol 2002;234:71-83.
33. Baldwin BG, Markos S. Phylogenetic utility of the external transcribed spacer (ETS) of 18S-26S rDNA: Congruence of ETS and ITS trees in *Calycadenia* (Compositeae). Mol Phylogenet Evol 1998;10:449-63.
34. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997;25:4876-82.
35. Lukasz C, Monika WH. Two-dimensional thin-layer chromatography in the analysis of secondary plant metabolites. J Chromatogr 2009;1216:1035-52.
36. Kupiec T. Quality-control analytical method: High-performance liquid chromatography. Int J Pharm Compd 2004;8:223-7.
37. Andreas H, Fasshuber J, Schinnerl WR, Brecker L, Vetschera KV. Iridoids as chemical markers of false ipecac (*Ronabeaemetica*), a previously confused medicinal plant. J Ethnopharmacol 2011;138:756-61.
38. Li B, Zhang DM, Luo YM, Chen XG. Three new and antitumor anthraquinone glycosides from *Lasianthus acuminatissimus* Merr. Chem Pharm Bull 2006;54:297-300.
39. Briggs LH, Cain BF, Le QP, Shoolery JN. The structure of asperuloside. Tetrahedron Lett 1963;2:69-74.
40. Inouye H, Okigawa M, Shimokawa N. Studies on monoterpene glucosides. VIII. Artefacts formed during extraction of asperuloside and paederoside. Chem Pharm Bull 1969;17:1949-54.
41. Dallavalle S, Jayasinghe L, Kumarihamy BM, Merlini L, Musso L, Scaglioni L. A new 3,4-seco-lupane derivative from *Lasianthus gardneri*. J Nat Prod 2004;67:911-3.
42. Mani MM, Claira AA, Uma MS, Suriyati M, Surash R, Sharif MM, *et al*. Antimicrobial activity and phytochemical screening of various parts of *Ixora coccinea*. J Med Plant Res 2014;8:423-9.
43. Xiao L, Zhu H. Paraphyly and phylogenetic relationships in *Lasianthus* (Rubiaceae) inferred from chloroplast rps16 data. Bot Stud 2007;48:227-32.
44. Smedmark JE, Razafimandimbison SG, Wikström N, Bremer B. Inferring geographic range evolution of a pantropical tribe in the coffee family (Lasiantheae, Rubiaceae) in the face of topological uncertainty. Mol Phylogenet Evol 2014;70:182-94.

SUPPLEMENTARY



Supplementary Figure 1: Samples of inhibition zones of standard antibiotics, leaf, stem bark, non-polar fractions, and scopoletin on agar plates which inoculated with bacterial strains: (a) *Staphylococcus aureus* ATCC 25923, (b and c) *Escherichia coli* ATCC 25922, (d) *S. aureus* (methicillin-resistant), (e) *Klebsiella pneumoniae* (BAA 1706), (f) *Pseudomonas aeruginosa* (ATCC 37166), and (g) *Acinetobacter baumannii* (ATCC 19606)

Supplementary Table 1: List of samples in the study (*Lasianthus*), their voucher specimens and the GenBank accession numbers of DNA sequences

Species	Origin	Vouchers	*trnTLF*	*rps16*	ITS	ETS
Lasianthus appressus	Thailand	Larsen 46093 (AAU)	KF704918	KF704875 AY538439	KF704969	KF704821
Lasianthus batangensis	Gabon, Liberia	Andersson & Nilsson 2284 (GB), Adam 20063 (UPS)	KF704919		KF704971	KF704823
Lasianthus cereiflorus	Tanzania	B. Bremer 3092 (UPS), Manktelov *et al*. 89214 (UPS)	KF704920	KF704877	KF704973	KF704824
Lasianthus cf. *repens*	Gabon	Andersson & Nilsson 2307 (GB)	KF704938	KF704890	KF704987	KF704840
Lasianthus cf. *varians*	Sri Lanka	Klackenberg 189 (S)	KF704951	KF704901 DQ282642	KF705000	KF704852
Lasianthus chrysoneurus	China, Thailand	Zhu 03159 (?), Larsen *et al*. 43311 (AAU)	KF704921		KF704974	KF704825
Lasianthus coffeoides	India	Klackenberg & Lundin 80 (S)	KF704922	AF004061	KF704975	KF704826
Lasianthus congesticymus	Thailand	Larsen *et al*. 45522 (AAU)	KF704923	KF704878	KF704976	KF704827
Lasianthus constrictus	Thailand	Larsen *et al*. 45643 (AAU)	KF704924	KF704879	-	-
Lasianthus cyanocarpus	-	Ridsdale 11/8 XVII 11.8.17.136 (?)	KF704925	KF704880	KF704977	KF704828
Lasianthus ellipticus	Thailand	Larsen *et al*. 45620 (AAU)	KF704926	KF704881	KF704978	KF704829
Lasianthus euneurus	Borneo	Beaman 9088 (S)	KF704927	KF704882	KF704979	KF704830
Lasianthus formosensis	Vietnam	Averyanov and Binh VH3772 (AAU)	KF704928	KF704884	KF704981	KF704831
Lasianthus hirsutus	Vietnam, Thailand	Gong 04298 (?), Larsen *et al*. 45047 (AAU)	KF704929	DQ282637	KF704982	-
Lasianthus hookeri	China, Thailand	Zhu 03157 (?), Larsen *et al*. 46728 (AAU)	KF704930	DQ282643	KF704983	KF704832
Lasianthus inodorus	Borneo	Beaman 7194 (S)	KF704931	KF704885	-	KF704833
Lasianthus japonicus	Japan	Chevi 139 (GB)	KF704932	KF704886	KF704984	KF704834

(Contd...)

Supplementary Table 1: *(Continued)*

Species	Origin	Vouchers	*trnTLF*	*rps16*	ITS	ETS
Lasianthus kilimandscharicus	Malawi	Lantz 119 (UPS)	KF704933	AM117327	EU145366	-
Lasianthus lanceolatus	Puerto Rico	Taylor CM 11719 (MO)	EU145554	-	EU145367	KF704835
Lasianthus maingayi	Thailand	Larsen *et al*. 43312 (AAU)	KF704934	KF704887	KF704985	KF704836
Lasianthus micranthus	Vietnam	Tirvengadum *et al*. 3287 (AAU)	KF704935	KF704888	-	KF704837
Lasianthus pedunculatus	Tanzania	Andreasen 71 (UPS)	KF704936	EU145504	EU145368	KF704838
Lasianthus pilosus	Thailand	Larsen *et al*. 43154 (AAU)	KF704937	KF704889	KF704986	KF704839
Lasianthus pilosus var. *angustifolius*	Thailand	Napiroon and Vajrodaya (BKF)	MH341185	MH341189	MH341197	MH341193
Lasianthus rhinocerotis	Malaysia, Thailand	Zhu 03123 (?), Larsen *et al*. 2013 (AAU)	KF704939	DQ282639	KF704988	KF704841
Lasianthus ridleyi	Thailand	Larsen 46088 (AAU)	KF704940	KF704891	KF704989	KF704842
Lasianthus robinsonii	Brunei	Bygrave 35 (K)	KF704941	KF704892	KF704990	-
Lasianthus sarmentosus	Thailand	Niyomdham 4488 (AAU)	KF704942	KF704893	KF704991	-
Lasianthus scalaris	Thailand	Larsen *et al*. 44161 (AAU)	KF704943	KF704894	KF704992	KF704843
Lasianthus sikkimensis	China, Thailand	Zhu 03155 (?), Larsen *et al*. 46316 (AAU)	KF704944	DQ282644	KF704993	KF704844
Lasianthus sp. 5JS2013	Vietnam	Krüger *et al*. 31 (S)	KF704948	KF704898	KF704997	KF704848
Lasianthus sp. 1JS2013	Borneo	Nielsen & Balslev 1086 (AAU)	KF704945	KF704895	KF704994	KF704845
Lasianthus sp. 3JS2013	Vietnam	Kainulainen *et al*. 17 (S)	KF704946	KF704896	KF704995	KF704846
Lasianthus sp. 4JS2013	Vietnam	Kainulainen *et al*. 57 (S)	KF704947	KF704897	KF704996	KF704847
Lasianthus sp. 6JS2013	Vietnam	Krüger *et al*. 4 (S)	KF704949	KF704899	KF704998	KF704849
L. stipularis	Thailand	Napiroon & Vajrodaya (BKF)	MH341186	MH341190	MH341198	MH341194
Lasianthus strigosus	Australia	B & K Bremer 3902 (UPS)	EU145556	EU145505	EU145369	KF704850

L. stipularis: *Lasianthus stipularis*

Species	Origin	Vouchers	*trnTLF*	*rps16*	ITS	ETS
Lasianthus trichophlebus	Thailand	Larsen 45393 (AAU)	KF704950	KF704900	KF704999	KF704851
Lasianthus yalaensis	Thailand	M. Poopath *et al*. 876 (BKF)	MH341183	MH341183	MH341195	MH341191
Lasianthus yalaensis	Thailand	M. Poopath *et al*. 906 (BKF)	MH341184	MH341188	MH341196	MH341192
Ronabea emetica	Bolivia	Steinbach 434 (S)	KF704955	KF704903	KF705003	KF704857
Ronabea latifolia	Guatemala	Contreras 9152 (S)	KF704956	KF704904	KF705004	-
Saldinia acuminata	Madagascar	Razafimandimbison *et al*. 605 (S)	KF704957	KF704905	KF705005	KF704858
Saldinia aegialodes	Madagascar	Razafimandimbison 506 (UPS)	KF704958	KF704906	KF705006	KF704859
Saldinia cf. *obovatifolia*	Madagascar	Razafimandimbison *et al*. 981 (S)	KF704964	KF704913	KF705014	KF704867
Saldinia cf. *axillaris* var. *axillaris* JS2013	Madagascar	Razafimandimbison *et al*. 1004 (S)	KF704959	KF704907	KF705007	KF704860
Saldinia cf. *coursiana* JS2013	Madagascar	Kårehed *et al*. 286 (UPS)				
Saldinia cf. *proboscidea* JS2013	Madagascar	Kainulainen *et al*. 64 (S)	KF704961	KF704909	KF705010	KF704863
Saldinia littoralis	Madagascar	Schatz & Lowry 1307 (K)	KF704966	KF704915	KF705016	KF704869
			KF704962	KF704910	KF705011	KF704864
Saldinia pallida	Madagascar	Bremer *et al*. 4038-B38 (UPS)	KF704965	KF704914	KF705015	KF704868
Saldinia sp. 1 4038BB38	Madagascar	Razafimandimbison 506 (UPS)	KF704958	KF704906	KF705006	KF704859
Saldinia subacuminata var. *strigosa*	Madagascar	Kårehed *et al*. 202 (UPS)	KF704967	KF704916	KF705017	KF704870
Saldinia subacuminata var. *subacuminata*	Madagascar	Razafimandimbison *et al*. 606 (UPS)	KF704968	KF704917	-	KF704871
Trichostachys aurea	Gabon	Andersson & Nilsson 2304 (GB)	EU145559	EU145507	EU145372	KF704872
*Trichostachys sp.*Sonke1725	Cameroon	Sonké 1725 (UPS)	EU145560	AM900595	EU145373	-