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Antibacterial Activity of Three Medicinal *Lasianthus* (Rubiaceae) Extracts on Human Resistant Pathogenic Bacteria

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Abstract

Aim: To evaluate the activity of medicinal *Lasianthus* extracts on bacteria and to determine the chemical characters among three medicinal *Lasianthus* (Rubiaceae) species.

Materials and Methods: The phytochemical investigation of *L. cyanocarpus*, *L. hirsutus* and *L. lucidus* were performed using thin layer chromatography and high performance liquid chromatography (HPLC) techniques. Four antibacterial resistant strains including *Staphylococcus aureus* ATCC 43300 (MRSA), *Klebsiella pneumoniae* ATCC BAA 1705 (carbapenemase; KPC-producing strain) and *Pseudomonas aeruginosa* ATCC 27853 (AmpC β -lactamase producing strain) were used. The phytochemical investigation of *L. cyanocarpus*, *L. hirsutus* and *L. lucidus* were performed using thin layer chromatography and high performance liquid chromatography (HPLC) techniques. We also detected the effects of extracts on bacteria by using a scanning electron microscope.

Results: The lipophilic extracts from the three plants revealed the terpenoids, coumarin and iridoid. HPLC showed similarities in the chemical profiles of both leaf and stem bark extracts. *L. lucidus* lipophilic extracts revealed the greatest effect against *S. aureus* and *P. aeruginosa* at 400 and 100 μ g/mL respectively, whereas *L. cyanocarpus* extracts prominently affected *K. pneumoniae* at 400 μ g/mL. Cell lysis and leakage of bacteria treated with extracts were observed.

Conclusion: Our findings surprisingly showed the potential of antibacterial effect among resistant pathogenic bacteria. We also revealed the comparable signals of the chemical characters from the three *Lasianthus*. These findings

support the traditional use related infectious diseases and it might be possible to further develop the antibiotic agents.

Keywords: Resistant bacteria; *Lasianthus*; Ethnomedicine; Infectious diseases

Introduction

The World Health Organization (WHO) reports that there are globally high levels of antimicrobial resistance in common bacteria coupled with a lack of coordination and understanding of antimicrobial resistance [1,2]. A low estimate suggests that antibiotic resistance is at present causing 700,000 deaths worldwide annually and this figure is projected to reach 10 million by 2050 [3]. Pathogenic bacterial infections, especially infections from *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Acinetobacter baumannii* infect several organs including the skin and soft tissue, respiratory tract, urinary tract, bloodstream, and intra-abdomen [4]. Thus, the infectious control of resistant strains and the use of appropriate available antibacterial agents is the best way to struggle infections in the resistant era. Nowadays, scientists must encounter new antimicrobial agents from natural sources which bring new hope for treating multidrug resistant bacteria [5].

Plants in the genus *Lasianthus* (Rubiaceae) make up a large group with more than 180 species, mostly in tropical Asia where approximately 160 species are found in primary forest [6,7]. Different species of the genus *Lasianthus* (Rubiaceae) have been used as traditional medicines in tropical Asia to treat a variety of diseases, among which fevers and infectious diseases stand out [8-10].

At present, several species of *Lasianthus* are considered by authors to possess antibacterial properties [11]. It has been suggested in other reports that medicinal species of *Lasianthus* have been studied phytochemically, but there are no previous reports of isolated compounds and the bioactivity of the many species investigated here, 9 medicinal *Lasianthus* species, and in particular of their effects against pathogenic bacteria.

This study aimed to investigate antibacterial activity against seven pathogenic bacteria of 3 medicinal *Lasianthus* species related to infectious diseases. We also evaluated secondary metabolite compounds and chemical profiles from these potentially medicinal *Lasianthus* species.

Materials and Methods

Ethnomedicinal plants review

We collected all the available information on *Lasianthus* spp. Firstly, we reviewed all authoritative botanical data including Flora [12], related journals and botanical texts assembling details on plant distribution, morphological characteristics, medicinal uses and modes of preparation. We then cross-referenced these data via online databases and compared them with herbaria records in Thailand, Aarhus University Herbarium, Denmark and The Kew Herbarium, The United Kingdom.

Plant material

Mature leaves and stem bark of the medicinal *Lasianthus* species were collected between July and December, 2015, from tropical forests in Thailand. Voucher specimens of all *Lasianthus* were deposited at the Department of Botany, Kasetsart University, Bangkok Forest Herbarium (BKF), and Aarhus University Herbarium (AAU). The plant samples were identified and compared with type specimens: *L. cyanocarpus* (syntype K! 000763940), *L. hirsutus* (syntype K!000777044) and *L. lucidus* (type K!000763966, (S.S. Larsen, T. Christian. and D. Sookchaloem 46739 (AAU)).

Preparation of plant extractions

Two-hundred gram of mature leaves and stem bark were dried under shade and powdered using an electric mill. The powder was macerated with methanol for seven days. Extracts were subsequently filtered and then concentrated using rotary evaporation at 37°C, until the crude extracts were semi-solid. The concentrated crude extracts were partitioned into a hydrophilic extract in distilled water and lipophilic extract in chloroform. The lipophilic extracts were stored at a temperature below -45°C.

Antibacterial Activity

Bacterial strains

For the antimicrobial assays, plant extracts were evaluated using pathogenic bacteria strains from the Department of Medical Science, Thailand, which included 2 Gram-positive bacteria; *Staphylococcus aureus* ATCC 25923 and *S. aureus* ATCC

43300 (MRSA) and 3 Gram-negative bacteria; *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853 (AmpC β -lactamase producing strain), *Klebsiella pneumoniae* ATCC-BAA 1705 (carbapenemase; KPC-producing strain).

Antibacterial screening

Screening of the lipophilic extracts for antibacterial effect was determined by diffusion method. The standard inoculums (0.5 McFarland) were spread evenly on Muller-Hinton agar. After drying, each disk paper containing 200 μ g of extract was placed on Muller-Hinton agar with the tested organisms. The agar plate was incubated at 37°C for 24 hours. *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 strains were used as control species with standard antibiotic disks [13].

The leaf and stem bark extracts solutions in dimethyl sulfoxide (DMSO-Sigma-Aldrich, USA) were serially diluted with 2-fold concentrations ranged from 6.25 μ g/mL to 3,200 μ g/mL. A standard inoculum of test bacteria in Mueller-Hinton broth (MHB-Oxoid, Basingstoke, UK) was poured into each well of the 96-well plate. 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 extracts that inhibited visible growth.

Chromatography techniques

The lipophilic extracts were placed and investigated on Thin Layer Chromatography pre-coated silica gel 60 F254 plates (Merck) 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 of each fluoresced spot were determined as a TLC pattern. The TLC plates were sprayed with detecting reagent to screen major secondary metabolites. We used an anisaldehyde sulfuric acid reagent for terpenoid detection. Dragendorff's reagent, vanillin- sulfuric acid reagent, 10% NaOH in ethanol and Wieffering test were used for alkaloids, higher alcohols (including steroids, phenols, essential oils), coumarin and iridoids, respectively (Merck). Samples of 10 mg/mL of lipophilic extracts in methanol (Merck) were performed using HPLC with methanol gradient of 60–100% in an aqueous buffer (0.015 M ortho-phosphoric acid pH 3 and 0.015 M tetrabutyl ammonium hydroxide). The HPLC analysis was done on an Agilent 1100 series system and detected with a UV photodiode arrays detector with wavelengths of 230 nm.

Scanning electron microscopy (SEM)

For scanning electron microscopy (SEM), *P. aeruginosa*, *S. aureus* and *K. pneumoniae* (105 CFU/mL) in lipophilic extracts at the MIC of each such strain combined with MHB were incubated at 37°C for six hours and were then filtered through a 0.22 μ m filter paper. The bacteria on the filter paper were fixed by a 2.5% glutaraldehyde in 0.2 M sodium phosphate saline buffer (pH 7.2) for 12 hours at 4°C. The fixed bacteria were washed three times with 0.2 M sodium phosphate saline buffer (pH 7.2) and were then post-fixed in 1% osmium tetroxide (OsO₄) in distilled water for one hour [14]. After that, the filter paper was cleaned up with three cycles of distilled water. The fixed samples were dehydrated in a series of acetone and then dried at the critical

point of CO₂ (Polaron Range SC7620 Sputter Coater & Carbon Accessory). Finally, the filter papers on carbon tape were coated with platinum (AUTOLAB, Spin coater) and observed under a SEM (HITACHI SEM S-2500) at Nanoimaging Center, Mahidol University.

Statistical Analysis

The assay was done in triplicate to get the statistical average of the result. Mean value for the zone of inhibition in mm and MIC values in µg/mL were calculated.

Results

Medicinal plant species review

With respect to ethnomedicinal information, a total of 3 species for treating infected wounds which have been documented from tropical Asia have been listed in the present article. Fresh dosing of the 3 species, direct application and use of the leaves as poultice wounds, as well as the usage of leaves and stem bark, are the most frequently recorded ways of administration. Their uses and modes of remedy are reported in **Table 1**.

Table 1: The three medicinal Lasianthus (Rubiaceae) – information and modes of preparation and remedies.

Botanical name	Vernacular name	Distribution & Ecology	Part used	Mode of remedies	References
<i>L. cyanocarpus</i> Jack	Ched chang san yai (Thai) Loi (India)	Evergreen forest in Thailand, Malaysia, Indonesia to Papua New Guinea	Leaves	Leaf paste applied on fractured bone, rheumatoid arthritis and stopping bleeding	[10], [22]
<i>L. hirsutus</i> (Roxb.) Merr.	Pat nam ngoen khon dam (Thai) Thingchangnei (India) Ji shi shu (China)	Evergreen forest in India, south of China to South eastern Asian Archipelago	Leaves	Juice from crushed leaves applied on cuts and wounds and stopping bleeding, or haemostatic.	[10], [23]
<i>L. lucidus</i> Blume	Pat nam ngoen (Thai) Yuan bian zhong (China)	Evergreen forest in northeastern India, Southeastern Asian archipelago to New Guinea	Leaves, Stem bark and Roots	Juice from leaves to stop bleeding, on wounds, and stem bark or roots boiled as decoction together, the liquid being drunk to reduce fever.	[23]

Antibacterial activity

From the results of the zone diameters, the 3 plant extracts showed the highest antibacterial activity against *P. aeruginosa*, followed by *S. aureus* and *K. pneumoniae*, respectively (**Table 2**). With the clear zone of lipophilic extract, the leaf and stem bark extracts of *L. lucidus* showed the best potential antibacterial activity against *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC

43300(MRSA). Similarly, MIC of *L. lucidus* stem bark and leaf extracts were determined at 200 µg/ml and 100µg/ml for *P. aeruginosa* ATCC 27853 and 400 µg/ml for *S. aureus* ATCC 43300 (MRSA) respectively (**Table 3**). The leaf and stem bark extracts of *L. hirsutus* inhibited *P. aeruginosa* ATCC 27853 at 800 µg/ml concentration.

Table 2: Inhibition zone diameter (mm) of extracts of leaves and stem bark from 3 medicinal Lasianthus against bacterial strains.

Lipophilic extracts**	Diameter of inhibition zone (mm), (Mean ± SE)				
	<i>S. aureus</i> ATCC 43300	<i>S. aureus</i> * ATCC 25923	<i>E. coli</i> † ATCC 25922	<i>P. aeruginosa</i> ATCC 27853	<i>K. pneumoniae</i> ATCC-BAA 1705
<i>L. cyanocarpus</i>					
Leaves	8.33 ± 0.33	12.66 ± 0.33	6.00 ± 0.00	9.66 ± 0.33	6.00 ± 0.00
Stem bark	9.33 ± 0.33	9.66 ± 0.33	6.00 ± 0.00	6.00 ± 0.00	8.66 ± 0.33
<i>L. hirsutus</i>					
Leaves	6.33 ± 0.33	6.33 ± 0.33	6.00 ± 0.00	11.66 ± 0.33	12.66 ± 0.33
Stem bark	8.66 ± 0.33	10.33 ± 0.33	6.00 ± 0.00	8.66 ± 0.33	8.33 ± 0.33
<i>L. lucidus</i>					
Leaves	12.33 ± 0.33	10.66 ± 0.66	6.00 ± 0.00	12.66 ± 0.33	6.00 ± 0.00
Stem bark	8.67 ± 0.33	9.33 ± 0.33	6.00 ± 0.00	15.33 ± 0.33	6.00 ± 0.00
Antibiotics					
Ampicillin	-	28	20	-	-

Piperacillin	-	28	26	-	-
Gentamicin	-	21	23	-	-
Ciprofloxacin	-	23	32	-	-

* *S. aureus* ATCC 25923 and †*E. 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 2017. (AMP (Ampicillin 10 µg), PIP (Piperacillin 100 µg), GEN (Gentamicin 10 µg), CIP (Ciprofloxacin 5 µg)).
** Average of triplicate experiments.

Table 3: Minimum inhibitory concentration (MIC) of leaves and stem bark lipophilic extracts against bacteria strains by microdilution method.

Lipophilic extracts	Minimum inhibitory concentration (MIC; µg/mL)				
	<i>S. aureus</i> ATCC 43300	<i>S. aureus</i> ATCC 25923	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> ATCC 27853	<i>K. pneumonia</i> ATCC-BAA 1705
<i>L. cyanocarpus</i>					
Leaves	1,600	1,600	>3,200	>3,200	400
Stem bark	800	1,600	>3,200	>3,200	1,600
<i>L. hirsutus</i>					
Leaves	1,600	1,600	>3,200	800	1,600
Stem bark	1,600	1,600	>3,200	400	800
<i>L. lucidus</i>					
Leaves	400	400	>3,200	200	>3,200
Stem bark	400	400	>3,200	100	>3,200

Qualitative chromatography analyses

The results on the TLC plates appeared positive to terpenoids, alkaloids, coumarin and iridoids and presented the bands after spraying with vanillin sulfuric acid reagent for detection of the higher alcohols, phenols, steroids, and essential oils. The TLC

profiles of the leaf extracts showed similarities and differences from the stem bark extracts when defined by Rf values and qualitative bands or spots from each reagent. The results of the qualitative tests performed on lipophilic extracts from leaves and stem bark are shown in **Table 4**.

Table 4: Phytochemical investigation on TLC plates and screening testing of leaf and stem bark lipophilic extracts from three medicinal *Lasianthus* species detected by specific reagents and observed under UV light.

Lipophilic extracts	Results of phytochemical investigation							
	Alkaloids		Terpenoids		Coumarin		Iridoid	
	Leaf	Stem bark	Leaf	Stem bark	Leaf	Stem bark	Leaf	Stem bark
<i>L. cyanocarpus</i>	-	-	+	+	+	+	+	-
<i>L. hirsutus</i>	-	-	+	+	+	+	+	-
<i>L. lucidus</i>	-	-	+	+	+	+	+	-

(-) Negative test, (+) Positive test

The HPLC profiles showed the three dominant peaks of the leaf extracts of the three medicinal *Lasianthus*, while only one dominant peak appeared in the stem bark extracts. The retention time and significant UV spectra of comparable signals of the lipophilic extracts of leaves and stem bark are shown in **Figure 1**. From these observations, the HPLC profiles of the leaf extracts showed similar chemical characteristics and profiles for each of the three species. The stem bark extracts of *L. cyanocarpus* and *L. hirsutus* showed similar profiles but these

differed from *L. lucidus* when diagnosed with UV λ_{max} at a signal wavelength of 230 nm.

Scanning electron microscopy (SEM)

SEM analysis showed significant morphological changes by cellular lysis in *S. aureus* ATCC 43300 (MRSA), *P. aeruginosa* ATCC 27853 (AmpC β -lactamase producing strain) and *K. pneumoniae* ATCC BAA1705 (carbapenemase; KPC-producing strain).

Moreover, the prolonged shape of *P. aeruginosa* could be observed when compared with that untreated bacteria (Figure 2).

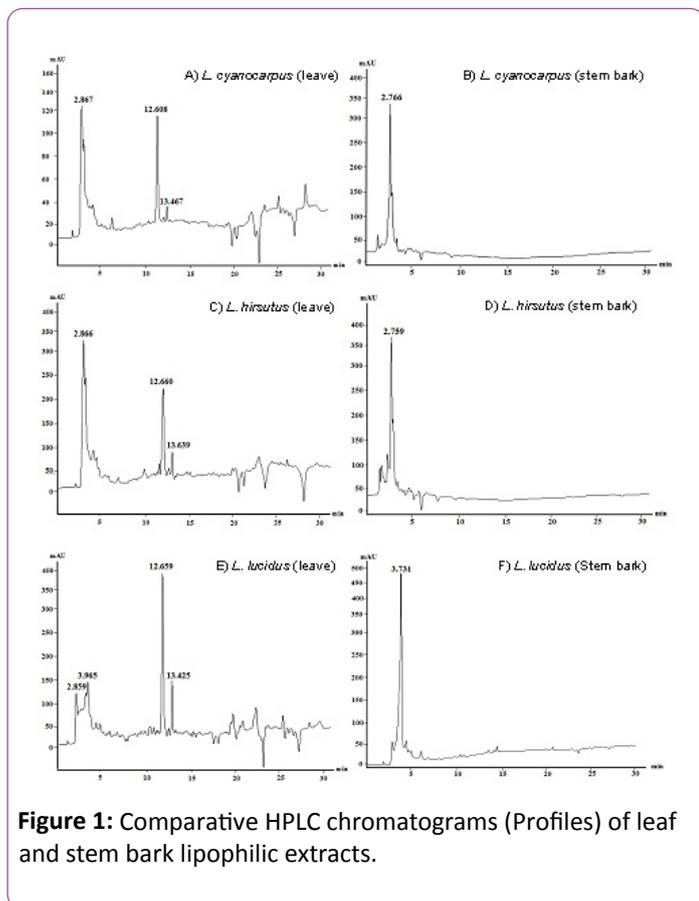


Figure 1: Comparative HPLC chromatograms (Profiles) of leaf and stem bark lipophilic extracts.

Discussion

This study was the first to investigate the effects of leaves and stem bark from *L. cyanocarpus*, *L. hirsutus* and *L. lucidus* lipophilic extracts on *S. aureus*, *P. aeruginosa*, and *K. pneumoniae*. Similarly, Dinda et al. [15] reported iridoids from Rubiaceae plants demonstrated antimicrobial activity. However, our lipophilic extracts had promising inhibitory effects against *P. aeruginosa* even at concentrations of 100 µg/ml, and thus can be a nice source for antibacterial agents. Moreover, we first revealed the cellular morphological changes via an electron microscope. The prolonged cells and the leakage of content were originally thought to be the results of by cell wall detriment like β-lactam [16].

Qualitative information for specific constituents in leaf and stem bark extracts from *Lasianthus* were investigated using color detection with different reagents was evidence to support the classification of the three medicinal *Lasianthus* species. The HPLC analysis determined the retention time and UV spectra signals. The results from this study present the first report of the chemical characteristics of these plants demonstrated with chromatographic techniques. In the report of Lukasz and Monika [17] the authors used TLC as a tool for botanical material investigation, then focused on chemotaxonomy for analysis of plant extracts and plant classification.

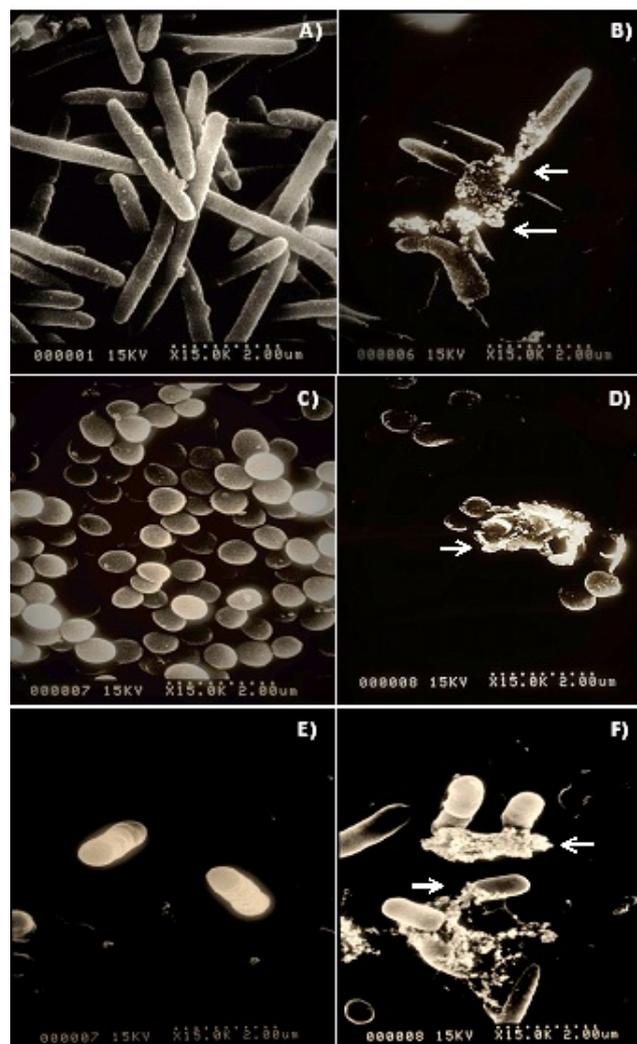


Figure 2: Scanning electron microscopy images of the antibacterial effects of lipophilic extracts of leaves and bark of three medicinal *Lasianthus* on the bacterial strains *P. aeruginosa* ATCC 27853 (AmpC β-lactamase producing strain) (A, B), *S. aureus* ATCC 43300 (MRSA) (C, D) and *K. pneumoniae* ATCC BAA1705 (carbapenemase; KPC-producing strain) (E, F). A, C and E are controls without treatment; B, D and F are with treatment. Note morphological alternations in B and F with clearly broken cells and leakage from cells.

Moreover, our HPLC profile among three species also supported the TLC findings showing the same number of compounds which could be detected under UV wavelength. Thus, HPLC is used to separate and quantify chemical compounds that have been dissolved in solution and to determine a specific compound in solution [18]. From our comparative chemical profiles of the three medicinal *Lasianthus* from each part of the plant, present similar profiles and characteristics of the extracts when detected with UV irradiation signals are shown. When determining their morphological characteristics together with their chemical profiles and medicinal usage. Iridoids are generally distributed in the family Rubiaceae, but the lack of alkaloids in *Ronabea* and the tribe

Lasiantheae which differs from the alkaloid-rich genus, *Psychotria*, is a significant chemical characteristic. The genus *Ronabea* has been placed in the same tribe as *Psychotria* (tribe *Psychotrieae*), but molecular evidence supports the separation of these two genera into different tribes as well [19]. A previous report on the phytochemistry of *Lasianthus* by Takeda et al. [20] isolated the three new compounds lasianthionoside A, B and C from leaves of *L. fordii* together with iridoid glucosides and asperuloside, deacetyl asperuloside, methyl deacetyl asperulosidate and citroside A. Furthermore, Dallavalle et al. in 2004 [21] were able to isolate the glucosidic compound (stigmasterol 3-o- β -D-glucoside) from *L. gardneri*, a species growing in the mountain forests of Sri Lanka.

This is the first report of the chemical profiles of these three medicinal *Lasianthus* which may lead to the future discovery of chemical markers in chemotaxonomy and bioactive compounds for uses of medicinal *Lasianthus* species in South East Asia. The three medicinal *Lasianthus* in this study have been reported as traditional remedies for treating infected wounds and stopping the bleeding as well as reducing fever possibly caused by infection. The results of the antibacterial activity and chromatographic trends of the extracts conform to the available information on traditional uses [22,23].

In the future, there is a need to clarify the antibacterial activity and the exact mechanism of action of the extracts as well as to undertake research to discover the chemical compounds from the plant resources, which is expected to lead to a novel discovery of drugs.

Conclusion

Our work is the first report on three medicinal *Lasianthus* extracts which supports the traditional uses related to treating infectious disease and showed antibacterial effects against bacterial pathogens which are serious health problems. Qualitative analyses of lipophilic leaf and stem bark extracts from the three *Lasianthus* were undertaken using chromatographic techniques (TLC and HPLC). Effective pure compounds should be isolated from these plants to obtain alternative chemicals for use in chemotaxonomy and medicinal plant work.

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