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Chemical composition and antimicrobial activity of the extracts and essential oil of *Blumea balsamifera* from the Philippines

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Abstract
Blumea balsamifera is a registered traditional medicine in the Philippines for the diuretic treatment of kidney stones. It is commonly available in tablet, capsule, and tea formats. This study aims to investigate the antibacterial activity and chemical components of local *Blumea balsamifera* leaf methanol extract, hexane extract, and essential oil to determine their potential application in treating bacterial urinary and digestive tract infections. The identified compounds were β-carotene and predominantly flavonoids, including quercetin, blumeatin, hyperoside, padmatin, 3′, 5, 5′, 7-tetrahydroxyflavanone, 3-*O*-methylquercetin, and 7-methyleriodictyol, from high-performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) data. These compounds were found in the methanol extract. Terpenes, including eudesmol, borneol, caryophyllene oxide, guaiol, (*E*)-caryophyllene, and α-curcumene were identified as the primary components of the essential oil by gas chromatography-mass spectrometry (GC-MS) analysis. Methanol and hexane extracts exhibited antibacterial activity against methicillin-sensitive *Staphylococcus aureus* with MICs of 3 and 5 mg/mL, respectively. The essential oil inhibited methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* with respective MIC values of 2 and 3 mg/mL. However, no potential activity against other test organisms was detected. Isolation and standardization of the bioactive components from *Blumea balsamifera* leaf extracts can be further studied in an application to treat microbial infections.

Keywords: *Blumea balsamifera*, extracts, essential oil, antimicrobial activity, LC-MS/MS, GC-MS

1. Introduction
Blumea balsamifera (BB) Lin., also locally known as Sambong, is a 1–3-meter tall, half-woody, very scented shrub belonging to the genus *Blumea* and family Asteraceae. The plant is prevalent in eastern and southeastern Asia. It has been used as traditional medicine in China, Malaysia, Thailand, Vietnam, and the Philippines for thousands of years [1]. It is utilized in Thai and Chinese medicine to treat septic wounds, respiratory infections, and stomachaches [2]. Traditional healers in the Philippines advise using Sambong to treat various conditions, including cough, fever, influenza, dysentery, sore throat/eyes, malaria, boils, infected umbilical cord, and tuberculosis [3].
A review of previous *in vitro* and *in vivo* studies on Sambong revealed that it has a variety of biological activities, including antitumor, hepatoprotective, superoxide radical scavenging, antioxidant, antimicrobial, anti-inflammatory, antiplasmodial, antityrosinase, wound healing, and anti-obesity [1].
The usage of Sambong to cure kidney stones and as a diuretic has been clinically validated in the Philippines, where it is included on a list of ten medicinal plants recommended by the Philippine Department of Health. Furthermore, a group of medical researchers from the National Institutes of Health, University of the Philippines, Manila, conducted a phase III randomized placebo-controlled clinical trial to treat urinary tract stones (NIRPROMP. unpublished, 1994). Consequently, a leaf tablet is licensed to local pharmaceutical firms and placed under diuretics on the essential drugs list. In addition, a recent systematic review of its safety and efficacy as a therapy for urinary tract stones has been reported. Four clinical studies matched the inclusion criteria, two of which were double-blind and two of which were open-label. Results showed that Sambong successfully treated patients with urolithiasis, as demonstrated by radiographic evidence of stone passage and a reduction in stone size or number, with no significant adverse reactions [4].

Given that Sambong leaf is being used as a diuretic, it may be beneficial to explore its potential use for bacterial urinary and gastrointestinal infections. In the Philippines, these bacterial infections continue to be one of the major causes of death [3]. A previous study tested five common bacterial pathogens resistant to specific antibiotics with Philippine Sambong aqueous and ethanol extracts. The extracts tested had low antibacterial activity, as indicated by the MICs, ranging from 12.5 to >50 mg/mL [3]. In addition, another study on the phytochemical analysis of Sambong leaves revealed the presence of icthyothereol acetate, cyptomeridiol, lutein, and ß-carotene, wherein icthyothereol acetate showed moderate activity against *Aspergillus niger*, *Trichophyton mentagrophytes*, and *Candida albicans*. In contrast, cyptomeridiol showed low activity against the same pathogens [5]. Although some preliminary studies have been performed to analyze the antimicrobial properties and metabolites of BB leaf extract from the Philippines, further research would be necessary, including phytochemical standardization and bioactivity validation. The objective of this study was to assess the viability of using Sambong components in contemporary medicine for microbial infections. Therefore, different extracts and essential oil of Sambong leaves were studied for their chemical compositions and antibacterial properties.

2. Materials and Methods

2.1 Plant materials

BB leaves were acquired from a Palawan Center for Appropriate Rural Technology, Inc. farm in Bacungan, Puerto Princesa, Philippines. Plants were authenticated and validated by the Department of Pharmacology of the University of the Philippines, Manila. The leaves of BB were harvested just before the onset of flowering. Briefly, processing raw materials entailed the following steps: harvesting leaves was carried out by the farmers in the morning and then placed in new plastic bags. The harvests were then transported to the facility's garbling and washing sections. Air-drying was completed within two hours from harvesting or until the moisture content of dried leaves reached between 7-8%. The dried leaves were allowed to cool and then placed in food-grade polyethylene bags, sealed, and delivered to the processing facility. The dried leaves were subjected to ozonation at the processing facility, pulverized to the necessary mesh size, and dried in an industrial oven to a maximum of 5% moisture content.

2.2 Chemicals

HPLC-grade acetonitrile, methanol, hexane, and other analytical-grade reagents were obtained from Theo-Pam Trading, Philippines. The analytical-grade chemicals were purchased from Belman Laboratories, Philippines. Standard quercetin (Sigma-Aldrich) was purchased from Merck, Philippines. Standard β-carotene, cryptomeridiol, and blumeatin were purchased from ChemFaces Biochemical Co., Ltd., China.

2.3 Preparation of Standards

Stock solutions of standard compounds β-carotene, quercetin, cryptomeridiol (1 mg/mL), and blumeatin (0.5 mg/mL) were prepared separately in methanol. All the standard solutions were stored at 4°C until analysis. An appropriate volume of the individual stock standards was taken for a mixed standard solution to make the desired final concentration. Before analysis, the mixed standard solution was sonicated and filtered through a 0.20 µm polytetrafluoroethylene polymer (PTFE) membrane filter. Then, standard compounds were analyzed with the samples of BB leaves using HPLC.

2.4 Preparation of Plant Extracts

The powdered leaves of BB were extracted using 100% methanol or hexane (25 g in 200 mL solvent) using an ultrasonic bath (MRC Scientific Instruments Professional Ultrasonic, UK) operating at 40 kHz, 30°C for 60 minutes. After extraction, the mixture was filtered twice using cheesecloth followed by filter paper, concentrated, and dried using a rotary evaporator (IKA, Germany). The resulting concentrate was kept in an amber bottle at 4°C for up to one month. In each subsequent assay, an extract solution was freshly prepared.

2.5 Extraction of Essential Oil

The essential oils from BB leaves were extracted by hydrodistillation using a modified Clevenger apparatus. In a 6-liter Erlenmeyer flask, 250 g of *B. balsamifera* powder was mixed with 4 L of distilled water. The mixture was heated and kept at a low boil for approximately 4.5 hours until the amount of oil condensate in the collecting vessel no longer increased, then heating was stopped. Essential oils were extracted with diethyl ether with an approximate volume enough to dissolve the oil. The extracted oil was dehydrated with anhydrous sodium sulfate and concentrated using rotary evaporation to remove any excess solvent. The resulting volatile oil extract was stored at 4°C until further analysis.

2.6 High-Performance Liquid Chromatography (HPLC)

HPLC analysis was performed to obtain chromatographic fingerprints of BB extracts. Methanol (1mg/mL) or hexane (5mg/mL) BB extracts dissolved in methanol were passed through a 0.2 µm PTFE membrane filter for HPLC injection. Each extract and the standard mixture were subjected to HPLC analysis separately. The HPLC system used consisted of a separation module (Shimadzu Prominence) equipped with LabSolutions software (Shimadzu) with a binary pump, needle-in-flow path autosampler, and a photodiode array (SPD-M20A) detector. The analysis was carried out on a Waters Xbridge C18 (4.6×250 mm, 5.0 µm) column using 0.5% phosphoric acid (solvent A) and acetonitrile (solvent B) as the mobile phase for gradient elution. All the mobile phase solvents were passed through a 0.45 µm membrane filter before use. Mixed standard (10 µL) and samples (5 or 10 µL) were injected at a flow rate of 0.5 mL/min into the HPLC. The column oven was at 30°C, and the HPLC peaks were observed at 254 nm. The mobile phase gradient elution used was: 0-10 min, 85-60% A; 10-30 min, 60-45% A; 30-40 min, 45-25% A; 40-60 min, 25-20% A; 60-70 min, 20-5% A; 70-75min, 5-85% A.

2.7 Liquid chromatography-tandem Mass spectrometry (LC-MS/MS)

LC-MS/MS analysis of methanol and hexane BB extracts was carried out at Pascual Pharma laboratory, using an ESI-QTOF-MS/MS system comprised of a Waters ACQUITY I-Class UPLC coupled with a Waters Xevo G2-S QTOF mass spectrometers. Two µL samples were separated on reverse-phase Waters ACQUITY HSS C18 column (2.1×100 mm, 1.8 µm) at 30 °C with gradient elution at a flow rate of 0.25 mL/min. The mobile phase was 0.1% formic acid-water (solvent A) and 0.1% formic acid-acetonitrile (solvent B). Using columns calculator ver. 2.0.53.0 (Waters Corporation),

the HPLC gradient method was converted into a UPLC method to match the LC-MS system with minimal optimization. Data processing was performed using MassLynx 4.1. The acquisition parameters were: data range, 100-1500 Da; applied source temperature, 20 °C; desolvation temperature, 450 °C; cone gas (argon) flow rate, 50 L/h; desolvation gas (nitrogen) flow rate, 600 L/h; Electrospray ionization, positive mode capillary voltage, 3.0 kV and cone voltage, 80 V (source offset, 80 V). MSE mode, low and high collision energy scans; low energy scan, 6 eV, and high energy scan, 30 to 50 eV; scan time, 0.1s. The RAW files output was converted to ABF for peak alignment, peak picking, and identification processing using MS-DIAL software. The spectral databases include the following libraries: GNPS, Sumner, ReSpect, MassBank EU, Massbank NA, Faulkner Legacy, NIH Natural Products, Prestwick Phytochemicals, and Dorrestein/FDA Natural Products. Several MS-DIAL-processed data were additionally processed in GNPS for compound matching.

2.8 Gas chromatography-Mass spectrometry (GC-MS)

GC-MS analysis of BB essential oil was performed at National Chemistry Instrumentation Center, Ateneo de Manila University, Philippines, using a GCMS-QP2010 Ultra Mass Spectrometer (Shimadzu) equipped with an RTX-5 column (30 m × 0.25 mm ID × 0.25 µm) and a mass spectrometry (MS) detector. The temperature of the injector was 4°C. The temperature of the oven was programmed to ramp from 40°C (3 minutes isothermal) to 100 °C (rate of 10 °C/min), then to 165 °C (rate of 1.5 °C/min), and lastly to 240 °C (10 °C/min). The interface was maintained at 280°C. The range of the mass scan was set between 40- 300 m/z. Helium was utilized as a carrier gas with a flow rate of 0.80 mL/min. Essential oil (1 µL) was injected in split mode, and the components were determined as a relative percentage of the total oil using the peak area. Using *n*-alkanes (C6–C32) as standards, the Kovats method was applied to determine the retention index of each component. Individual components were identified based on the NIST11 library with a similarity index (SI) ≥ 89%. Similarity indices below 89% were not considered.

2.9 Determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

The quality control organisms used were *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 for vancomycin and ciprofloxacin, respectively. In addition, clinical isolates of *Staphylococcus aureus* (methicillin-sensitive UL 01), *Staphylococcus aureus* (methicillin-resistant strain UL 61), *Escherichia coli* (UL 12), *Klebsiella pneumoniae* (UL 110), *Enterobacter cloacae* (UL 380), *Pseudomonas aeruginosa* (UL 10), *Acinetobacter baumannii* (UL 249), were from the clinical culture collection of Unilab, Inc., Philippines. Each strain was passaged at least twice to ensure purity and optimal viability. Working stocks were prepared on trypticase soy agar (TSA) slants and stored at 4 °C.

The MIC was determined using the broth microdilution technique described in the CLSI M07-A9, 2012, and Campbell, 2011 [6, 7]. In this assay, resazurin was added as a colorimetric indicator of viability. A bacterial suspension comparable to 0.5 McFarland standard (1.5 x 108 CFU/mL) was prepared using MHB II. The suspension was diluted to make the seed culture containing ~3 × 10^5 CFU/mL. The initial count of the seed culture was done by plating 100 µL of the ten-fold dilutions prepared from the seed culture. Next, each well was inoculated with 50 µL of the seed culture and incubated at 35°C. The final concentrations of samples were now at 10 mg/mL to 1 mg/mL. After 23 hours of incubation, 10 µL of 0.1% resazurin was added to all wells. The plates were then incubated for another hour at 35°C. Fluorescence was read using a Bio-Tek microplate reader, and percent inhibition was calculated. The method described in the NCCLS M26 document determined the MBC [8]. The color change of resazurin from blue to pink indicates bacterial growth. An aliquot of 10 µL samples from the blue-colored wells was pipetted and plated onto fresh TSA plates. Each sample was spread on the entire surface of the TSA plate that was incubated at 35°C for 24 hours. The number of bacterial colonies and the colony-forming units per milliliter (CFU/mL) was determined. The MIC and MBC were carried out in three independent tests, each with three replicate wells.

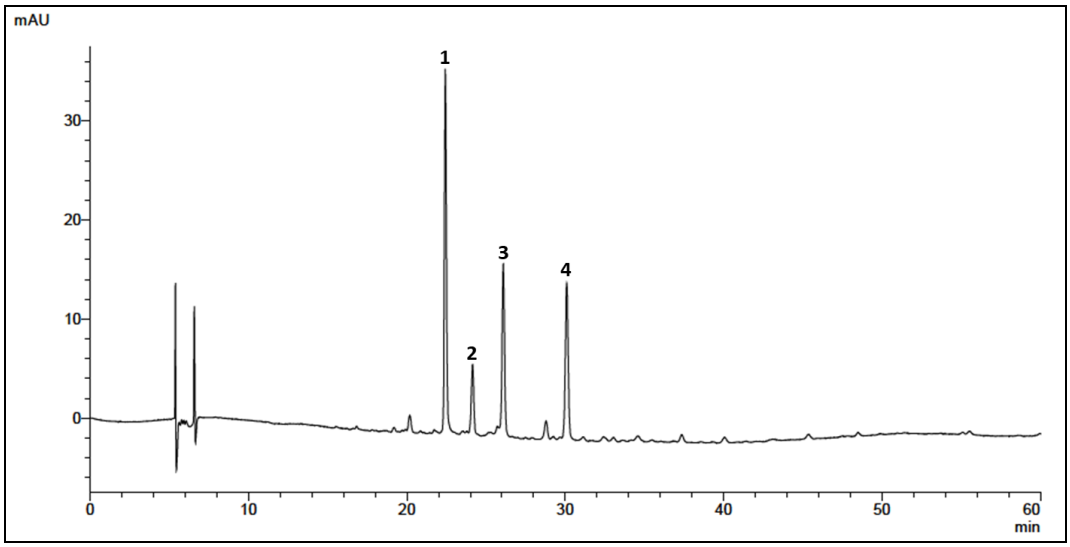
2.10 Statistical Analysis

Tests were carried out in nine replicates, and the results were calculated as the mean value.

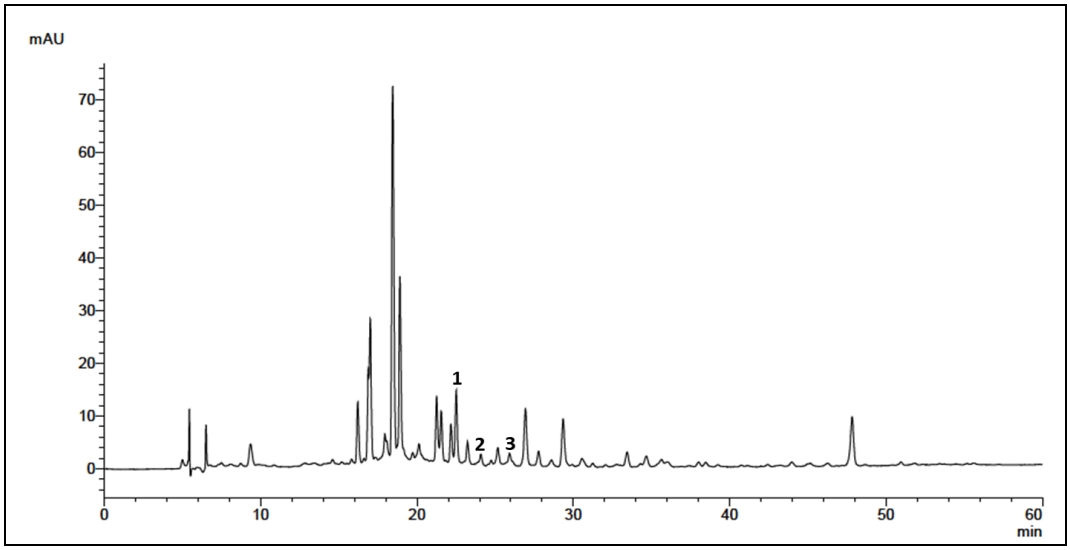
3. Results & Discussion

3.1 Chemical Composition of BB Extracts

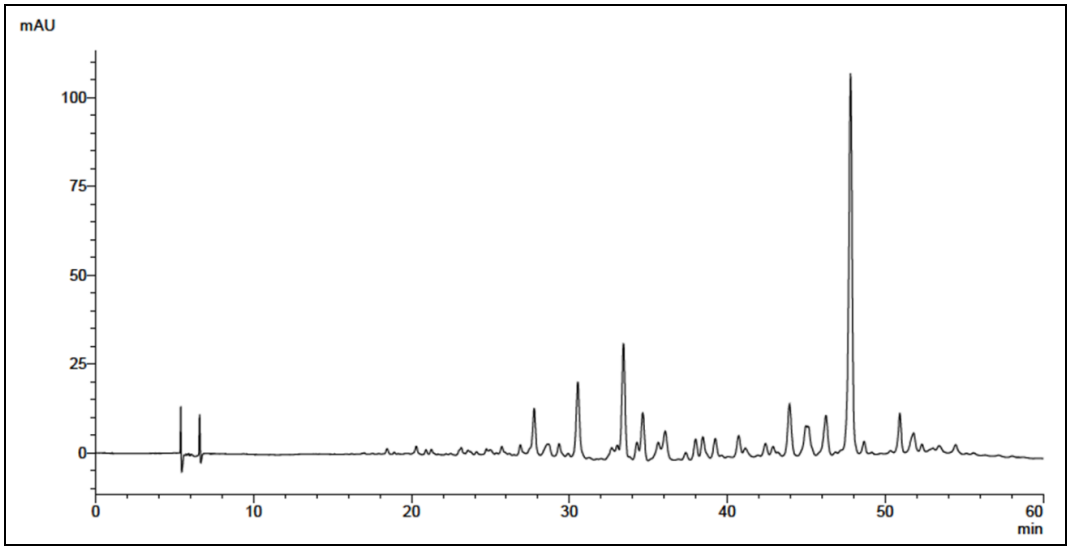
High-pressure liquid chromatography (HPLC) examined the chemical constituents in *Blumea balsamifera* (BB) extracts. Multi-component plant extracts are separated and relatively identified using this approach. The analysis utilized reversed-phase chromatography with a Photodiode Array (PDA) detector, a C18 column, and UV detection at 254 nm. Figure 1A displays the chromatogram of the available standard reference compounds for the BB sample. Figures 1B and 1C showed the chromatograms for methanol and hexane BB extracts, wherein resolved peaks could be observed. Most compounds eluted in methanol extract (Fig. 1B) were detected in the 8-34 min range indicating polar compounds. On the other hand, the eluted chemicals in hexane extract (Fig. 1C) were observed between 28 and 54 mins, indicating less polar to nonpolar compounds relative to the methanol extract. Comparison of peak retention times (RT) of these reference compounds under the same HPLC system conditions indicated that three peaks at 22.4, 24.10, and 26.1 mins could be quercetin (1), β-carotene (2), and blumeatin (3), respectively in methanol extract of BB (Figure 1B). The UV spectra of these peaks from methanol extract were comparable to the quercetin, β-carotene, and blumeatin standard spectra.



A



B



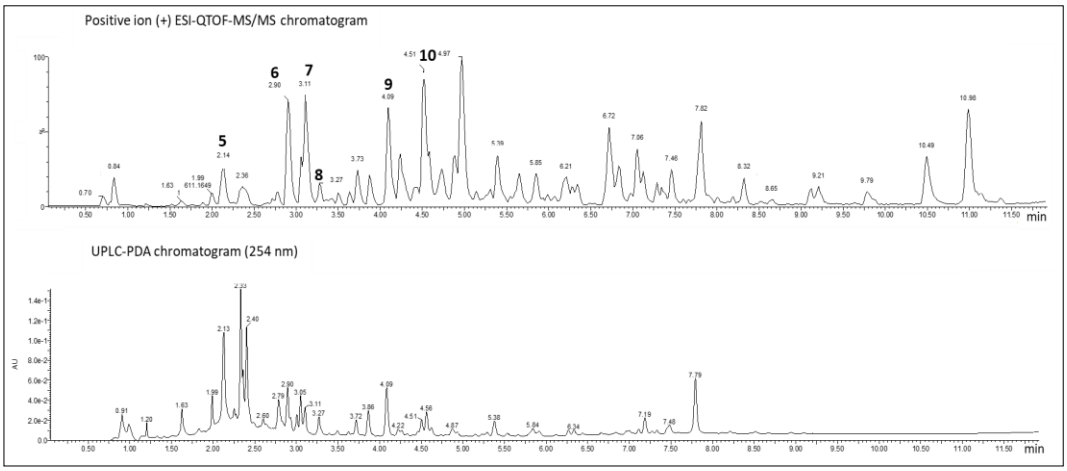
C

Fig 1: HPLC Chromatograms of A. Standard compounds: quercetin (1), β-carotene (2), blumeatin (3), cryptomeridiol (4), B. methanol, and C. hexane extracts of *Blumea balsamifera*.

LC-MS/MS untargeted analysis was performed to obtain information about the molecular mass and further identify the BB extract's chemical composition. The components of BB leaves extracts were analyzed from crude extracts. Figure 2

represents the chromatograms with MS and UV detection of methanol (A) and hexane (B) BB extracts, and Table 1 shows the chromatographic and mass spectral properties of the identified peaks by manually comparing MS data from prior research and data matching using MS-DIAL software. As shown in Table 1, hyperoside (5), padmatin (6), 3′, 5, 5′, 7-tetrahydroxyflavanone (7), 3-*O*-methylquercetin (8), quercetin 3,7-dimethyl ether (9) and 7-methyleriodictyol (10) were putatively annotated from methanol BB extracts. In addition, the identification of quercetin was verified in MS analysis; however, it appeared to be a derivative, quercetin 3, 7-dimethyl ether.

The majority of the identified compounds in BB methanol extracts were flavonoids. Previous phytochemical studies have identified several flavonoids, such as blumeatin, velutin, tamarixetin, dihydroquercetin-7,4′-dimethyl ether, ombuine, rhamnetin, luteolin-7-methyl ether, luteolin, quercetin, 5, 7, 3′, 5′-tetrahydroxyflavanone, and dihydroquercetin-4′-methyl ether [1]. In addition, certain flavonoids and sesquiterpenoids have antibacterial properties [9, 10]; therefore, these identified compounds may affect the plant's biochemical properties. The chromatographic methods of this study helped determine the contents of flavonoids in BB. Flavonoids have been recognized for their medicinal properties, and the validation and standardization of the local plant have been developed as herbal medicine in the Philippines. Six new sesquiterpenes were discovered from earlier chemical analyses of the leaves of the Philippine medicinal plant Sambong. The study used silica gel column chromatography to isolate the chemical constituents of the plant, the structures of which were determined by spectral analysis [11]. However, in the study, multiple peaks not matched by MS-DIAL and GNPS analyses imply further interpretation and characterization. Employing more standard chemicals, mass spectrum libraries, and other spectroscopic techniques on peaks that haven't yet been recognized will be helpful. Additionally, hexane extracts can be analyzed using GCMS to identify the volatile compounds that may contribute to the antibacterial properties.



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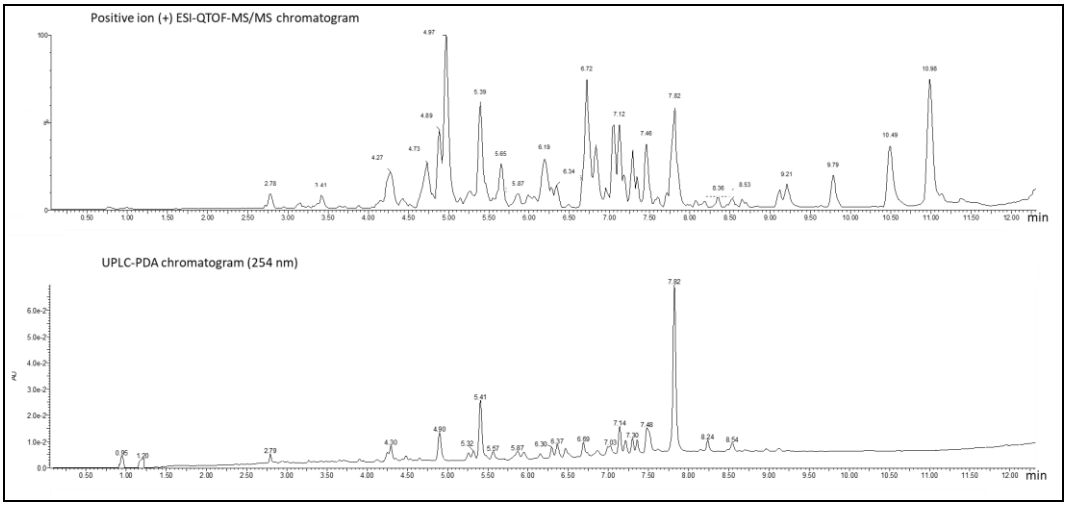

B

Fig. 2. LC/MS analysis of A. methanol and B. hexane extracts of *Blumea balsamifera* shows positive ion (+) ESI-QTOF-MS/MS and UPLC-PDA chromatograms.

Table 1. Chromatographic and mass spectral properties of peaks identified from *Blumea balsamifera* using LC/MS analysis.

No.	RT (min)	Precursor Ion	Experimental Mass	Fragments	Identification
5	2.14	[M+H]	465.1037	435.13, 303.05, 163.04	hyperoside
6	2.90	[M+H]	319.0782	301.07, 273.07, 245.08,	padmatin
				195.03, 153.02	

7	3.11	[M+H]	289.0666	287.05, 245.08, 213.05, 153.02, 149.02, 137.06	3’,5,5’,7-Tetrahydroxyflavanone
8	3.27	[M+H]	317.0621	302.04, 280.07, 229.14	3-*O*-Methylquercetin
9	4.09	[M+H]	331.0782	316.05, 285.22, 247.20	quercetin 3,7-dimethyl ether
10	4.51	[M+H]	303.0828	285.22, 247.20, 167.03	7-Methyleriodictyol

3.2 Chemical Composition of BB Essential Oil

The chemical composition of *Blumea balsamifera* essential oil (EO) was analyzed by gas chromatography-mass spectrometry (GC-MS) followed by mass spectral data matching using the NIST11 database. Figure 3 represents the chromatogram of the essential oil, and Table 2 shows the chromatographic and mass spectral properties of the identified peaks. GC-MS analysis resulted in the identification of 17 compounds in the essential oil based on the NIST11 library with a similarity index (SI) higher than 89%. The main constituents of the oil were γ−eudesmol (18.06 %), borneol (11.98 %), caryophyllene oxide (11.48 %), β-eudesmol (8.96 %), α-eudesmol (6.34 %), guaiol (2.28 %), (*E*)-caryophyllene (2.26 %), and α-curcumene (2.08 %).

Volatile compounds comprise most of BB's constituents; they are the principal active constituents, containing terpenoids, fatty acids, phenols, alcohols, aldehydes, ethers, ketones, pyridines, furans, and alkanes [1]. There have been different studies about essential oil components from *Blumea balsamifera*. The components of the essential oils of BB grown in several locations in Asia, including Vietnam; Luodian, Hainan, Nanning, Yunnan, China; Bangladesh; and Thailand, are given in Table 3 based on previous literature [12-21]. The majority of the analyzed BB essential oil was harvested in China. One of the most prevalent components of Sambong discovered in this experiment is γ−eudesmol, borneol, and caryophyllene oxide, also known as essential oil components from other BB. In Yunnan and Luodian, China, EOs have a comparatively high borneol concentration. Yunnan's borneol, extracted using steam distillation, had a 52.42 % yield [19], while Luodian's was 43.55 % using hydrodistillation [15]. Borneol was also found in the BB of Bangladesh (33.22 %) [20] and Thailand [21]. The chemical compositions of six EOs (EO1-EO6) harvested at different times from Luodian, China, are determined by Wang et al. [14]. Six EOs contained primarily caryophyllene (22.9-36.4 %), xanthoxylin (11.3-15.5 %), γ-eudesmol (5.4-15.1 %), and α-cubenene (8.6-12.1 %). Only this EO produced a comparable amount of γ-eudesmol to that obtained in this study (18.06%). Moreover, the high xanthoxylin content in EO was a distinguishing feature, which could be regarded as a characteristic of a volatile constituent from Luodian *B. balsamifera*. In this experiment, the BB contains 11.48 % caryophyllene oxide, which is comparable to the level (11.20 %) found in Hainan, China [16]. Vietnam and Nanning, China, also produced this chemical at a lesser percentage of 5.98 % [12] and 5.35 % [18], respectively. Some locally specific compounds, such as thujopsene-I3 (14.45 %), were produced in the EO of Hainan, China, by headspace-solid microextraction [17], and 1, 8-cineole (20.98 %) from Nanning, China, by hydrodistillation [18]. Camphor is also a primary EO of BB in Vietnam [12], China [15, 19], and Thailand [21], with the highest concentration (43.69 %) in Lamdong, Vietnam. Except for Thailand, the BB in this study contains caryophyllene and the other areas described in Table 3. The selection of extraction techniques may have an impact on the chemical composition of the essential oils. The composition of different plant species' essential oils is also impacted by geographical variance [22].

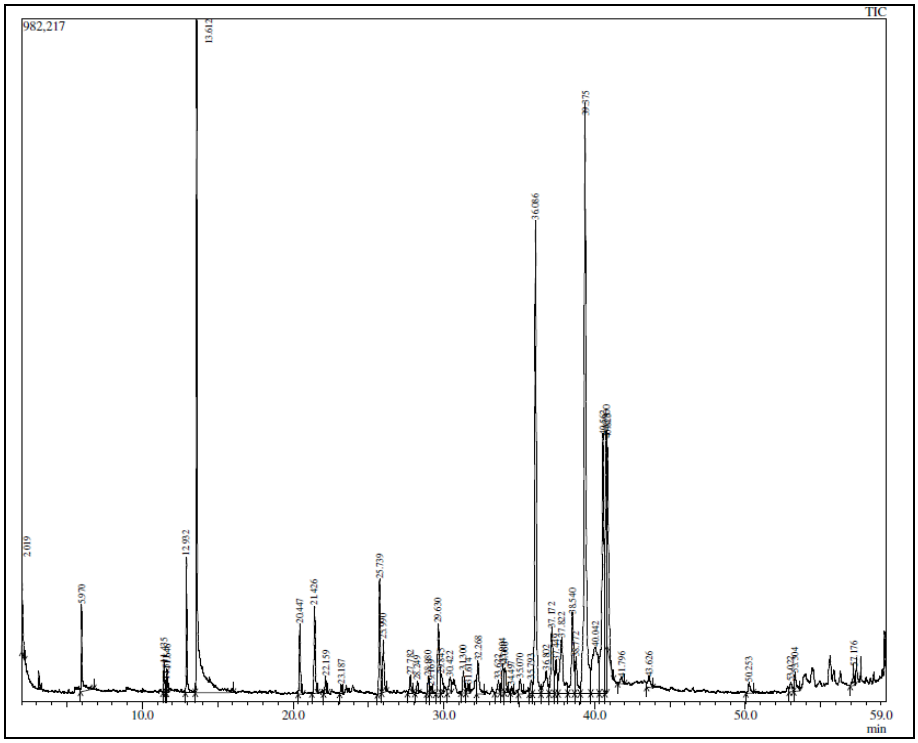

Fig 3: GC/MS total ion chromatogram of the essential oil of *Blumea balsamifera*.

Table 2: Chemical constituents of the essential oil of *Blumea balsamifera*.

RT (min)	SI	Peak Area (%)	Constituent	RI (NIST11)
11.44	96	0.41	Linalool	1082
12.93	97	1.77	2-Bornanone	1121
13.61	97	11.98	Borneol	1138
25.74	96	2.26	(*E*)-Caryophyllene	1494
27.78	91	0.44	Humulene	1579
28.25	94	0.26	Alloaromadendrene	1386
29.63	97	2.08	α-curcumene	1524
35.07	90	0.44	Palustrol	1530
36.09	89	11.48	Caryophyllene oxide	1507
36.80	92	1.20	(5*Z*)-Nonadecene	1918
37.17	91	2.28	Guaiol	1614
39.38	94	18.06	γ-eudesmol	1626
40.56	94	8.96	β-eudesmol	1593
40.78	91	6.34	α-eudesmol	1598
50.25	94	0.35	1-Nonadecene	1900
53.31	93	0.61	Hexahydrofarnesyl acetone	1754
57.17	90	0.43	1-Nonadecanol	2153

RT: Retention time, SI: Similarity index, RI: Retention index.

Table 3: Major components (> 5%) of the essential oil of *Blumea balsamifera* from previous studies.

Place of Origin	Method	Main Components (>5%)	Ref
Lamdong, Vietnam	hydrodistillation	Camphor (43.69%), caryophyllene (12.71%), caryophyllene oxide (5.98%)	[12]
Luodian, Guizhou, China	vacuum-drying	Caryophyllene (18.54%), borneol (18.33%), (þ)-2-bornanone (11.28%), a-gurjunene (6.73%)	[13]
Luodian, Guizhou, China	Modified Hydrodistillation	Caryophyllene (22.9-36.4%), xanthoxylin (11.3-15.5%), γ-eudesmol (5.4-15.1%), α-cubenene (8.6-12.1%)	[14]
Luodian, Guizhou, China	Hydrodistillation	L-Borneol (43.55%), D-Camphor (9.54%), β-pinene (5.20%), (E)-β-caryophyllene (6.51%)	[15]
Sanya, Hainan, China	volatile oil extractor (XD extracting standards)	Caryophyllene (19.28%), 1,7,7 trimethyl (1S endo) bicyclo[2.2.1] Heptan 2 ol (15.54%), caryophyllene oxide (11.20%), thujopsene (10.36%), 3 t butyl 4 methoxyphenol methyl derivative (6.04%), guaiol (5.03%)	[16]
Sanya, Hainan, China	Headspace-solid Phase Microextraction	Caryophyllene (26.47 %), thujopsene-I3 (14.45%), 1,7,7-trimethyl-(1S-endo)-bicyclo[2.2.1]heptan-2-ol (9.07 %), 4: 3-t-butyl-4-methoxyphenol methyl derivative (6.91 %)	[17]
Nanning, Guangxi Zhuang, China	Hydrodistillation	1,8-cineole (20.98%), borneol (11.99%), β-caryophyllene (10.38%), camphor (8.06%), 4-terpineol (6.49%), α-terpineol (5.91%), caryophyllene oxide (5.35%)	[18]
Yunnan, China	Steam distillation	Borneol (52.42%) Camphor (17.76%)	[19]
Chittagong, Bangladesh	Hydrodistillation	Borneol (33.22%), Caryophyllene (8.24%) Ledol (7.12%), tetracyclo [6, 3, 2, 0, (2.5).0(1, 8) tridecan-9-ol, 4, 4-dimethyl (5.18%)	[20]
Thailand		L-borneol, D-camphor and cineole	[21]

3.3 Antibacterial Activity of BB Extracts and Essential Oil

A test was also performed to evaluate the antibacterial properties of BB from the Philippines. Methanol extract, hexane extract, and the essential oil of the leaves were analyzed for antimicrobial activity as determined by the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). For MIC measurements, a broth microdilution technique was used with resazurin as a colorimetric indicator. Table 4 presents the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts and essential oil against two Gram-positive bacteria: methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*, and five Gram-negative bacteria: *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*.

The MIC and MBC were tested between 1 and 10 mg/mL. Methanol and hexane extracts were mildly inhibitory against methicillin-sensitive *Staphylococcus aureus* among the bacterial strains, with MICs of 3 and 5 mg/mL, respectively. MBC data indicates that methanol and hexane extracts have bactericidal activity against methicillin-sensitive *Staphylococcus aureus* at greater than 10 mg/mL. The essential oil in this work was found to have an inhibitory effect against methicillin-sensitive *Staphylococcus aureus* with MIC of 2 mg/mL and MBC of 4 mg/mL. It also has activity against methicillin-resistant *Staphylococcus aureus* with MIC of 3 mg/mL and MBC of 4 mg/mL. The study is the first report on the antibacterial properties of the essential oil of Sambong in the Philippines. The results suggested that Sambong may have antibacterial properties, which explains its traditional medicinal use.

The antibacterial activity against *S. aureus* (25 mg/mL) and *S. pneumoniae* (12.5 mg/mL) at higher MIC values have been reported in a prior investigation of ethanol extract from the Sambong Philippine variety [3]. There appeared to be no antibacterial property in BB crude aqueous and ethanolic extracts against isolates of *Staphylococcus aureus* and *Escherichia coli* reported by Ongsakul [23]. Moreover, the essential oil revealed the highest activity level against *S. Aureus* among the tested plant extracts. According to Sakee et al., the essential oil of BB from Roi-Et, Thailand, was found to be the most effective of their extracts, having MICs of 150 µg/mL against *B. cereus* and 1.2 mg/mL against *S. aureus* and *C. albicans* [2]. The BB essential oil purchased from Guizhou Ainaxiang Technology Development Company; China, has a potent bacteriostatic effect against *H. parasuis*. The MIC was determined to be 0.625 µg/mL, and the MBC was 1.25 µg/mL [24].

B. balsamifera methanol and chloroform extracts inhibited the growth of gram-positive bacteria (*B. cereus, S. aureus, and S. pneumoniae*) with inhibition zones ranging from 7.8 mm ± 0.41 to 10.5 mm±0.71. Inhibitory action was also seen against gram-negative *P. aeruginosa*, with zones of inhibition of 7.5 mm±0.58 and 8.0 mm±0.82, respectively. Alkaloids, flavonoids, steroids, and cardiac glycosides were the

discovered phytochemical constituents in the extracts [25]. Ali et al., reported efficient isolation of flavonoids, namely, 3,4,5-trihydroxy-3,7-dimethoxyflavanone, 3,4,5-trihydroxy-7-ethoxyflavanone, and biflavonoid 3-*O*-7-biluteolin from BB leaves using soxhlet extraction method [26]. The bacterial inhibitory activity is supported by the fact that the identified components of BB methanol extract in this study could be flavonoids. Using GC-MS, the chemical components of *B. balsamifera* leaf extracts in n-hexane from South Aceh, Indonesia, were identified. There were 27 chemical components found, with jasmoline (14.32%) being the most prevalent, followed by borneol (13.2%) and caryophyllene (10.03%) [27]. As demonstrated in the Sakee investigation, the hexane extract inhibits gram-positive *S. aureus* and gram-negative *E. cloacae*, with MICs of 9.6 and 4.8 mg/mL, respectively [2]. It is possible that the chemicals identified in the latest report contribute to this plant's action. The antibacterial properties of BB essential oil against *Haemophilus parasuis* were discovered by He in 2020. In their study, borneol, (*E*)-caryophyllene (β-caryophyllene), and camphor were the top three constituents of the oil [24]. An analysis was performed on a green, efficient, and solvent-free improved hydrodistillation (IHD) technique for the production of high-purity natural (–)-borneol from B. balsamifera leaves; the purity of the isolated (–)-borneol was 92%, and the recovery rate was 96%. Standard (–)-borneol, isolated (–)-borneol, and commercial (–)-borneol, expressed as minimum inhibitory concentration (MIC), exhibited consistent antibacterial and antifungal activity against the tested microorganisms [28]. Camphor, caryophyllene, caryophyllene oxide, β-eudesmol, thymol hydroquinone dimethyl ether, and τ-eudesmol comprised most of the essential oil extracted from fresh *Blumea balsamifera* leaves in Lamdong, Vietnam. Different quantities of this species' essential oil exhibited significant antibacterial activity against *E. coli* and *S. aureus*, as measured by the diameter of the inhibition zone [12]. *Blumea balsamifera* (L.) DC. Leaves growing in China's Luodian county were subjected to a modified hydrodistillation to extract the essential oil. Analyses revealed that xanthoxylin, γ-eudesmol, and α-cubenene were the predominant components. In addition, the EO displayed antifungal action with MIC values ranging from 62.5 to 250 µg/mL against different fungi and antibacterial activity against *Staphylococcus aureus* with a MIC value of 2,000 µg/mL [29]. Our research suggested that the essential oil contained comparable components with antibacterial action based on the above findings.

Table 4: Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of methanol and hexane extracts and essential oil of *Blumea balsamifera* against the tested organisms.

Test Organism	BB Methanol		BB Hexane		BB Essential Oil	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
Staphylococcus aureus (Methicillin-sensitive)	3	>10	5	>10	2	4
Staphylococcus aureus (Methicillin-resistant)	>10	ND	>10	ND	3	4
Escherichia coli	>10	ND	>10	ND	>10	ND
Klebsiella pneumoniae	>10	ND	>10	ND	>10	ND
Acinetobacter baumannii	>10	ND	>10	ND	>10	ND
Pseudomonas aeruginosa	>10	ND	>10	ND	>10	ND
Enterobacter cloacae	>10	ND	>10	ND	>10	ND

BB: *Blumea balsamifera*, NT: not tested due to insufficient activity in MIC assay, ND: not determined as the succeeding higher concentrations still showed growth Data represented the mean and was performed in three independent experiments; each experiment was performed in triplicates (n=9). MIC assay was done by the Resazurin-aided broth microdilution method.

4. Conclusions

The HPLC and LC-MS/MS analysis determined some chemical constituents in *Blumea balsamifera* (BB) leaves extract: quercetin, beta-carotene, blumeatin, hyperoside, padmatin, 3′, 5, 5′, 7-tetrahydroxyflavanone, 3-*O*-methylquercetin, and 7-methyleriodictyol. GC-MS analysis of the essential oil compounds showed that the oil's main constituents were γ−eudesmol, borneol, caryophyllene oxide, β-eudesmol, α-eudesmol, guaiol, (*E*)-Caryophyllene, and α-Curcumene. BB methanol extract, hexane extract, and essential oil displayed potential antibacterial efficacy against *Staphylococcus aureus* in the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. However, the essential oil exhibited better antibacterial activity among the different plant extracts. The study presented that *B. balsamifera* from the Philippines has antimicrobial activity, a finding which can be explored for microbial infection applications, especially of *S. aureus*. Isolation and identification of the active components can be further studied.

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6. Competing Interest

The authors have stated that there are no competing interests.

7. Authors Contributions

ZPR conceptualized the study plan, assisted and supervised the laboratory work, conducted data analysis and interpretation, wrote and edited the manuscript, and made an application for financial approval. JDC and CGT performed experiments, gave discussion and insights, and reviewed the paper. FA assisted in some experimental work. The final manuscript was read and approved by the authors.

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