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Sanseera, Duangsuree; Niwatananun, Wirat; Liawruangrath, Boonsom; Liawruangrath, Saisunee; Baramée, Apiwat; and Pyne, Stephen G., "Chemical composition and biological activities of the essential oil from leaves of *Cleidion javanicum* Bl" (2012). *Faculty of Science, Medicine and Health - Papers: part A*. 182. <https://ro.uow.edu.au/smhpapers/182>

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Abstract

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Keywords

cleidion, bl, leaves, chemical, composition, oil, biological, essential, activities, javanicum, CMMB

Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

Publication Details

Sanseera, D., Niwatananun, W., Liawruangrath, B., Liawruangrath, S., Baramée, A. & Pyne, S. G. (2012). Chemical composition and biological activities of the essential oil from leaves of *Cleidion javanicum* Bl. *Journal of Essential Oil-Bearing Plants*, 15 (2), 186-194.

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Chemical Composition and Biological Activities of the Essential Oil from Leaves of *Cleidion javanicum* Bl.

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Received 09 March 2011; accepted in revised form 28 November 2011

Abstract: The essential oil from the leaves of *Cleidion javanicum* Bl. was isolated by hydrodistillation with the percentage yield of 0.003 % as a pale yellow liquid. The composition of the essential oil was analysed by means of GC-(FID) and GC-MS. Ten constituents accounting for 92.60% total oil were identified. The major components were ethyl linoleolate (32.12 %), hexadecanoic acid (26.77 %), *trans*-phytol (24.64 %) and *iso*-phytol (4.80 %). The antimicrobial, anticancer, antioxidant activities and cytotoxicity test of this essential oil were investigated. The oil showed non-cytotoxic effects against Vero cells (African green monkey kidney) because it inhibited more than 50 % cell growth. The anticancer activity of the essential oil was performed using the Resazurin Microplate Assay (REMA). The oil showed anticancer activity against three cancerous human cell lines; KB-Oral Cavity Cancer, MCF7-Breast Cancer and NCI-H187-Small Cell Lung Cancer with the IC₅₀ of 47.16 µg mL⁻¹, 40.23 µg mL⁻¹ and 49.95 µg mL⁻¹ respectively. The oil also showed antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* with the Minimum Inhibition Concentration (MIC) values of 25.00 mg/mL and 12.50 mg/mL respectively, using agar diffusion method. In addition, the essential oil also showed significant antioxidant activity with the IC₅₀ of 27.05 mg mL⁻¹ by means of DPPH radical scavenging assay.

Key words: GC-MS, essential oil, *Cleidion javanicum* Bl., antimicrobial, anticancer and antioxidant activities.

Introduction

Cleidion javanicum Bl. is a large shrub found in forests at low and medium altitudes from India to New Guinea²⁵. Various parts of this plant are used medicinally in Thailand and the Philippines. Its stem has been employed as analgesic, antipyretic and diaphoretic³. Decoction of its leaves is reputed to cause abortion, whereas a decoction

of the bark is used for treatment of stomachic. Its seeds are used for treatment of constipation²⁴. The chemical constituents which were isolated from the leaves of *C. javanicum* including: tilianin, diosmetin 7-O-lucopyrananoside, 24S-methyl-5 α -lanosta-9(11), 25-dien-3 β -ol, *trans*-phytol, anol glucopyranoside^{18,19}. The isolation and structure determination of polyoxygenated clerodane

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spiciflorin has also been described. Columbin, scopoletin, 3,3',4-O-trimethylellagic acid, acetylleuritic acid, acetylleuroic acid and its methyl ester, taraxerol, taraxerone, β -sitosterol, stigmasterol, 3,5-dimethoxy-4-hydroxybenzoic acid, vanillic acid, *trans*-4-propenylphenol (anol) glucoside and 5-hydroxymethylfurfural were isolated from the root by Naengchomnong, *et al.*,²¹.

There is no previous report on the composition of essential oil analysis and biological activity of *C. javanicum*. The aim of this work was to isolate and identify the chemical constituents and biological activities of essential oil obtained from *C. javanicum* leaves.

Materials and methods

Plant material

Fresh leaves of *C. javanicum* Bl. were collected from Huay Kaew Arboretum, Huay Kaew Road, Chiang Mai, Thailand in June 2009. A voucher specimen (No. 2) was deposited in the herbarium of Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand (Figure 1).



Figure 1. The fresh leaves and fruits of *Cleidion javanicum* Bl.

Preparation of the essential oil

Fresh plant material (1 kg) was chopped into small pieces and subjected to hydrodistillation for 8 hours, in a modified Clevenger-type apparatus, with a water-cooled oil receiver to reduce formation of artifacts due to overheating during hydrodistillation to yield a pale yellow oil (0.003

% of the fresh weight of plant material). The oil was collected over water, separated and dried over anhydrous sodium sulphate. After that the oil was transferred to a glass vial and kept at a temperature of 4-7°C for further analysis by Gas Chromatography-Mass Spectroscopy (GC-MS).

GC-MS Analysis

The essential oil was analysed on a Hewlett Packard GC-MSD 5890 series 2 mass spectrometer fitted with a HP-5 (Hewlett Packard 19091J-433E) cross-linked fused silica capillary column (30 m, 0.25 mm i.d.), coated with 5 % phenyl Methyl Siloxane (0.25 μ m film thickness). The analytical conditions were: the oven temperature was programmed from 50°C for 2 min, isothermal, then heating by 6°C min⁻¹ to 230°C and isothermally for 15 min at 260°C. Injector temperature was 250°C. Samples were injected automatically by splitting and the split ratio was 1:100. The mass spectrometer had a delay of 3 min to avoid the solvent peak and then scanned from m/z 35 to m/z 550. Ionization energy was set at 70 eV. The carrier gas was He at a flow rate of 1.0 mL/min.

The identification of volatile components were accomplished by comparison of their GC retention indices (RI) as well as their mass spectra (NIST, WILEY7n.1) with corresponding data of authentic compounds or published spectra^{2,7,9,14,14,17,20,26}.

Antimicrobial activity assay:

The antimicrobial activity of the essential oil was evaluated by the standard agar diffusion technique¹. The microorganisms used were *Staphylococcus aureus* (ATCC25923), *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27553) *Aspergillus flavus*, *Candida albican* and *Trichophyton mentagrophyte*. All microorganisms were obtained from the Central Diagnostic Laboratory, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

The strains were maintained in agar conservation at room temperature. The strains inoculum were diluted in sterile 0.85 % Saline to obtain turbidity visually comparable to a McFarland N° 0.5 standard (10⁷⁻⁸ CFU/mL) for bacteria, but com-

parable to a McFarland N° 1.0 standard (10^{15} CFU/mL) for fungi. Every inoculum was spread over plates containing Nutrient Agar (NA) for bacteria and Potato Dextrose Agar (PDA) for fungi. In each of these plates, 4 wells (9 mm) were cut out using sterilized cork borer. One hundred μL (30 mg/mL) of the oil was carefully added into the wells by means of sterilized dropping automatic pipette. The samples were then incubated at 37°C for 24 h for bacterial pathogens and 3 days for fungal pathogens. The antimicrobial activity was evaluated in mm by measuring the diameter of inhibition zone. A positive control was also assayed to check the sensitivity of the tested organisms. Gentamicin $75 \mu\text{g mL}^{-1}$ and ketoconazole $250 \mu\text{g mL}^{-1}$ were used as positive controls for bacteria and fungi respectively. The experiments were repeated thrice.

Minimum inhibitory concentration (MIC)

The MICs of the essential oil against the test bacterial strains were determined by tube dilution method (National Committee for Clinical Laboratory Standards)²². The solutions of varying concentrations (3.13, 6.25, 12.5, 25.00 and 50.00 mg mL^{-1}) were prepared. These dilutions were taken in separate test tubes and labeled respectively. To each of the test tubes, 3 mL of Muller Hinton broth, 0.5 mL of bacterial suspension and 0.1 mL of the essential oil solution were added. A positive control tube containing the growth medium and bacteria suspension, without the essential oil solution, was also prepared. The tubes were incubated at 37°C for 24 hours. After 24 hours the turbidity was measured spectrophotometrically at 520 nm. The turbidity measurement was taken as an indicator of bacterial density. The rate of inhibition was found to be directly related to the turbidity of the medium. The lowest concentration, which did not permit any visible microbial growth when compared with that of the control, was recorded as the MIC value.

Antioxidative Assay

DPPH Assay

The antioxidant activity of the essential oil was determined by DPPH radical scavenging assay. This modified method was described by Brand-

Williams *et al.*⁴. The DPPH 6.6 mg mL^{-1} (in ethanol) was prepared and stored in the dark before use. Various concentrations of Trolox standard solutions and oil solutions were prepared using ethanol as solvent. This experiment was carried out for sample in the concentrations of 10, 20, 30, 40, 50, 60, 70 and 100 mg mL^{-1} . To each well of 96 well microtitre plate, 180 μL of ethanolic DPPH solution and 20 μL of the test sample (the oil in ethanol) were added. The total volume for each reaction mixture in each well was 200 μL . Thereafter, the plates were incubated at 37°C for 30 min, to check for the colorimetric change (from deep violet to light yellow), when DPPH was reduced. The absorbance of each well was measured at 540 nm. The DPPH solution was used as negative control. Trolox was used as reference standard. Radical scavenging capacity was calculated by using the formula (Equation 1):

$$\% \text{ inhibition} = [(Ac - As) \times 100] / Ac \quad (1)$$

whereas 'Ac' is the absorbance of the control and 'As' is the absorbance of the test sample after incubated 30 min. The values of % inhibition were obtained from Eq.1. For 50 % inhibitory concentration (IC_{50}) evaluation of the essential oil, a graph showing the concentration of test sample (mg mL^{-1}) versus % inhibition (% DPPH reduction) was plotted (Figure 2). A linear regression ($R^2 = 0.9984$) of standard Trolox (Figure 3) was also used to calculate the radical scavenging capacity.

Anti-cancer assay

The anticancer activity of the oil was assayed by using three cancerous human-cell lines: KB cell line (epidermoid carcinoma of oral cavity, ATCC CCL-17), MCF 7 cell line (breast adenocarcinoma, ATCC HTB-22) and NCI-H 187 cell line (small cell lung carcinoma, ATCC CRL-5804). This assay was performed using the method described by Brien *et al.*⁵. In brief, cells at a logarithmic growth phase were harvested and diluted to 7×10^4 cells/mL for KB and 9×10^4 cells/mL for MCF-7 and NCI-H 187, in fresh medium. Successively, 5 μL of test sample (the oil) was diluted in 5 % DMSO, and 45 μL of cell suspension were added to 384-

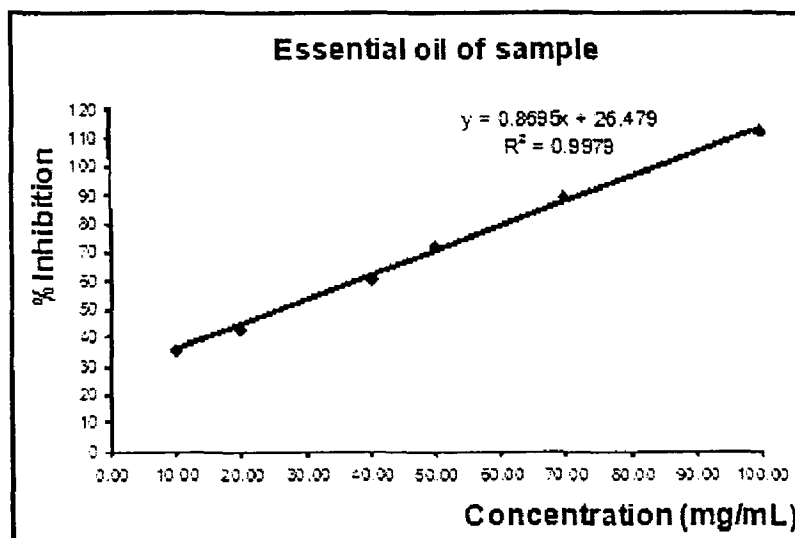


Figure 2. A linear regression ($R^2 = 0.9979$) of sample at concentrations of 10.00, 20.00, 30.00, 40.00, 50.00, 60.00, 70.00 and 100.00 mg/mL.

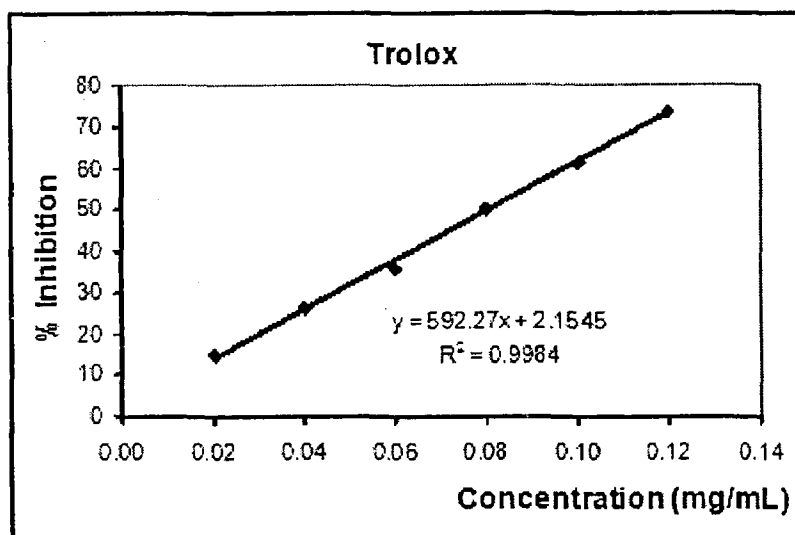


Figure 3. A linear regression ($R^2 = 0.9984$) of standard Trolox at concentrations of 0.02, 0.04, 0.06, 0.08, 0.10 and 0.12 mg/mL.

well plates, incubated at 37°C in 5 % CO₂ incubator. After the incubation period (3 days for KB and MCF-7, and 5 days for NCI-H187), 12.5 µL of 62.5 µg mL⁻¹ Resazurin solution was added to each well, and the plates were then incubated at 37°C for 4 hours. Fluorescence signal was measured using SpectraMax M5 multi-detection microplate reader (Molecular Devices, USA) at the excitation and emission wavelengths of 530 nm and 590 nm. The Percentage inhibition of the cell growth was calculated by the following equation:

$$\% \text{ Inhibition} = [1 - (FU_T / FU_C)] \times 100.$$

Whereas FU_T and FU_C are the mean fluorescent unit from treated and untreated conditions, respectively. Dose response curves were plotted from 6 concentrations of 2-fold serially diluted test compounds and the sample concentrations that inhibit the cell growth by 50 % (IC_{50}) can be evaluated by using the SOFTMax Pro software (Molecular Devices, USA).

Cytotoxicity assay

The cytotoxicity against primate cell line (Vero) of the oil was assayed by using Green fluorescent protein (GFP) detection described by Hunt *et al.*

¹³. In brief, The GFP-expressing Vero cell line was generated in-house by stably transfecting the African green monkey kidney cell line (Vero, ATCC CCL-81), with pEGFP-N-1 plasmid (Clontech). The cell line was maintained in minimal essential medium supplemented with 10 % heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g L⁻¹ sodium bicarbonate and 0.8 mg mL⁻¹ geneticin, at 37°C in a humidified incubator with 5 % CO₂.

The assay was carried out by adding 45 µL of cell suspension at 3.3 x 10⁴ cells/mL to each well of 384-well plates containing 5 µL of test compounds previously diluted in 0.5 % DMSO, and then incubating for 4 days in 37°C incubator with 5 % CO₂. Fluorescence signals were measured by using SpectralMax M5 microplate reader (Molecular Devices, USA) in the bottom reading mode with excitation and emission wavelengths of 485 and 535 nm. Fluorescence signal at day 4 was subtracted with background fluorescence at day 0. The percentage of cytotoxicity was calculated by the following equation (Equation 2), where FU_T and FU_C represent the fluorescence units of cells treated with test compound and untreated cell, respectively.

$$\% \text{ cytotoxicity} = [1 - (FU_T / FU_C)] \times 100 \quad (2)$$

IC₅₀ values were derived from dose-response curves, using 6 concentrations of 2-fold serially diluted samples, by the SOFTMax Pro software (Molecular device). Ellipticine and 0.5 % DMSO were used as a positive and negative control, respectively.

Results and discussion

The essential oil obtained from the leaves of *C. javanicum* Bl. was analysed by means of GC (FID) and GC-MS. Identification of the oil constituents was performed by a comparison of their mass spectra with literature data (NIST and NISTREP) and their programmed-temperature kovats retention indices (RI) with those in the literature. Ten components, represent in 92.60 % of the total essential oil, were identified and are listed in the order of their elution on the HP-5 capillary column used for GC-MS analysis (Table 1).

The major components of the essential oil were identified as ethyl linoleolate (32.12 %), hexadecanoic acid (26.77 %), *trans*-phytol (24.64 %) and *iso*-phytol (4.80 %). The minor components were hentriacontane (1.51 %), pentadecanone (1.00 %),

Table 1. Chemical composition of the essential oil of *Cleidion javanicum* Bl.

Peak no.	Compounds	RA (%)	RI	RT	Identification	References
1	Tetradecanoic acid	0.56	1758.2	25.2	RI, MS	14
2	Pentadecanone	1.00	1845.0	26.6	RI, MS	15
3	Isophytol	4.80	1944.0	28.4	RI, MS	2
4	Hexadecanoic acid	26.77	1972.0	28.9	RI, MS	9
5	Methyl linoleate	0.57	2092.0	30.7	RI, MS	17
6	<i>trans</i> -Phytol	24.64	2111.0	31.0	RI, MS	2
7	Ethyl linoleolate	32.12	-	31.6	MS	-
8	Neophytadiene	0.28	2114.0	32.5	RI, MS	26
9	Dodecanoic acid	0.45	2489.0	34.2	RI, MS	20
10	Hentriacontane	1.51	3100.0	48.0	RI, MS	7
	Total	92.60				

RA is the relative area (raw peak area relative to total peak area)

RI is the programmed temperature retention indices as determined on HP-5 column using a homologous series of n-alkanes

RT is retention time

Identification is the methods of identification

methyl linoleate (0.57%), tetradecanoic acid (0.56%), dodecanoic acid (0.45%) and neophytadiene (0.28%).

Most of the components of the essential oil are lipidic derivatives eg. Ethyl linoleolate, hexadecanoic acid, methyl linoleate and dodecanoic acid which are used as flavour and fragrance agents, essential ingredient in making soaps and shampoos. But *trans*-phytol and *iso*-phytol are acyclic terpenoids. Their chemical structures are presented in Figure 4 and Figure 5 respectively. *Trans*-phytol is a diterpene. It can be used as anticancer, antiviral and antipyretic agents^{11,29}. It is also used as a fixer in perfumery. *Iso*-phytol is used in manufacturing synthesis of vitamins E²⁸ and K⁸. Vitamin E is the collective name for a group of fat-soluble compounds with distinctive antioxidant activities¹⁰. Vitamin K cycle could act as a potent antioxidant activity¹⁶.

From biological activity studies, the essential oil from the leaves of *C. javanicum* Bl. showed significant anticancer activity against KB-Oral Cavity Cancer, MCF7-Breast Cancer and NCI-H187-Small Cell Lung Cancer with the IC₅₀ of 47.16 µg mL⁻¹, 40.23 µg mL⁻¹ and 49.95 µg mL⁻¹ respectively (Table 2). The oil showed non-cytotoxic effects against Vero cells. The biological activity of some compounds present in this essential oil have been reported to inhibit anticancer activity; in particular, hexadecanoic acid in *Solanum spirale* oil, showed cytotoxicity to human leukemic cells MOLT-4 and it also showed *in vivo* antitumor activity in mice^{12,27}. (E)-Phytol possessed anticancer activity against HT-29 human colon cancer cells, MG-63 osteosarcoma cells and AZ-521 gastric cancer cells³⁰. The oil also possessed antibacterial activity against *S. aureus* and *P. aeruginosa* with the inhibition zone of 11

mm and 15 mm respectively. But it did not show antifungal activity (Table 3). The minimum inhibitory concentrations (MIC values) of the oil were also evaluated. The oil showed antibacterial activity against *P. aeruginosa* and *S. aureus* with the MIC values of 12.50 mg mL⁻¹ and 25.00 mg mL⁻¹ respectively (Table 4). Some fatty acids present in this essential oil have been reported to possess antibacterial activity against *S. aureus* such as tetradecanoic acid and hexadecanoic acid⁶. (E)-Phytol also possessed antibacterial activity against *S. aureus*²³. The results indicated that this essential oil may be used for treatment of infectious diseases. The antioxidant activity of the oil was also carried out using the DPPH assay. The oil showed moderate antioxidant activity with the IC₅₀ of 27.05 mg mL⁻¹ (Table 5).

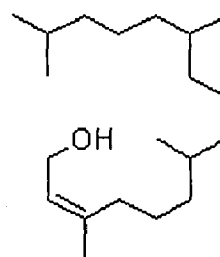


Figure 4. Chemical structure of *trans*-phytol ((E,7R,11R)-3,7,11,15-tetramethylhexadec-2-en-1-ol; C₂₀H₄₀O; MW 296.531 g/mol).

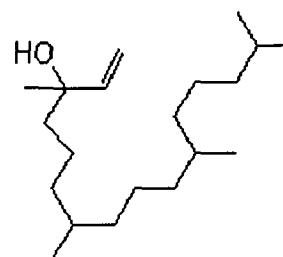


Figure 5. Chemical structure of *iso*-phytol (3,7,11,15-tetramethylhexadec-1-en-3-ol; C₂₀H₄₀O; MW 296.531 g/mol).

Table 2. Anticancer activities of the essential oil of *Cleidion javanicum* Bl.

Cancer Cell Lines	IC ₅₀ (µg/mL)
KB-Oral Cavity Cancer	47.16
MCF7-Breast Cancer	40.23
NCI-H187-Small Cell Lung Cancer	49.95

IC₅₀ is inhibition cell growth by 50 %

Table 3. The zone of inhibition of the essential oil of *Cleidion javanicum*, Gentamicin and Ketoconazole

Species of Microorganisms	Zone of Inhibition (mm) ¹		
	Essential oils (30 mg/mL)	Gentamicin (75 µg/mL)	Possitive control ² Ketoconazole (250 µg/mL)
Bacterial strains	11	35	-
<i>S. aureus</i>	-	27	-
<i>E. coli</i>	15	27	-
<i>P. aeruginosa</i>			
Fungal strains			
<i>A. flavus</i>	-	-	25
<i>C. albican</i>	-	-	37
<i>T. mentagrophyte</i>	-	-	16

¹ Represents that inhibition zones are the mean cork borer (9 mm) diameter

² Represents that possitive control, Gentamicin (75 µg/mL) and Ketoconazole (250 µg/mL)

(-) Represents that no inhibition zone

Table 4. Minimum inhibitory concentration of the essential oil of *Cleidion javanicum* Bl.

Test organism	Concentrations (mg/mL)(Zone of inhibition in mm)				
	3.13	6.25	12.50	25.00	50.00
<i>S. aureus</i>	-	-	-	11	15
<i>P. aeruginosa</i>	-	-	11	15	19

(-) Represents that no inhibition zone

Table 5. Antioxidant activity of the essential oil of *Cleidion javanicum* Bl.

	IC ₅₀ (mg/mL)
Sample	27.05
Trolox	0.08

IC₅₀ is inhibition cell growth by 50 %

Conclusion

In conclusion, this is the first report that describe the chemical composition and biological activities of the essential oil from leaves of *Cleidion javanicum* Bl. The essential oil from leaves of *C. javanicum* showed significant antimicrobial, antioxidant and anticancer activities. The essential oil showed non-cytotoxic to primate cell line. Therefore, this essential oil may play an impor-

tant role for the production of health supplement. Some bioassay results, antimicrobial and anticancer activities, support its folklore use and there is need to do more studies for isolation of compounds needed for drug development.

Acknowledgements

We would like to express our sincere thanks to National Research Council, Thailand, Faculty of

Pharmacy and the Graduate School, Chiang Mai University and also Rajamangala University of Technology Krungthep for financial support. Additionally Saisunee Liawruangrath would like to

express her sincere thank to Center for Innovation in Chemistry, PERCH-(CIC), Chemistry Department, Faculty of Science, Chiang Mai University.

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