IDENTIFICATION OF BETA-SITOSTEROL, STIGMASTEROL AND QUANTIFICATION OF CALOPHYLLOLIDE IN *CALOPHYLLUM INOPHYLLUM* FRUIT PEEL USING PRESSURIZED LIQUID EXTRACTION FOLLOWED BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY

Ha Minh Hien[1*], Paul WS Heng[2]

(1) Institute of Drug Quality Control-Ho Chi Minh City, Vietnam

(2) Department of Pharmacy, Faculty of Science,

GEA-NUS Pharmaceutical Processing Research Laboratory

[*] National University of Singapore, Singapore

Abstract

Background: Finding an efficient tool to identify and characterize the bioactive principles from the fruit peel of *Calophyllum inophyllum* L. by determining of calophyllolide, together with two phytosterols namely β-sitosterol and stigmasterol by using pressurized liquid extraction (PLE) followed by gas chromatography–mass spectrometry (GC–MS) in avoiding the tedious and time consuming methods of extraction, isolation, purification and structural elucidation of constituents. ***Material and methods:*** The fruit peels of *Calophyllum inophyllum* L. were obtained from Ben Tre Province-Vietnam, collected in 2009, identified by the botanic specialist. The analytes were extracted by PLE using several solvents/solvent systems. The extracts were tested for antimicrobial activities to determine the solvent(s) of choice. The GC-MS method was validated according to ICH guidelines. ***Results:*** The proposed method was selective against mass spectral deconvoluting software AMDIS32. The calibration curves obtained from the base peak area, was linear over the range of 3.19–51 $\mu g.mL^{-1}$. The RSD of intra-and-inter-day precision variations were less than 2.1% and the mean recovery was (99.53 ± 2.05)% (RSD = 2.06%). The identification of calophyllolide was strictly confirmed using FDA and EU specifications whereas those of stigmasterol and β-sitosterol were done using the NIST database. The quantitative results were: 0.0255 ± 0.0005% (m/m) for PLE extract and 0.025 ± 0.0005% (m/m) for soxhlet extract. **Conclusion:** For the first time in literature**,** the finding of calophyllolide together with stigmasterol and β-sitosterol, the phytosterols in *Calophyllum inophyllum* L. fruit peel using PLE followed by GC-MS. This has proved that this waste is a promising material as studies showed that calophyllolide is the biologically active component which possesses an antimicrobial and cytotoxic activities.

Key words: Calophyllum inophyllum L. Clusiaece; calophyllolide; β-sitosterol; stigmasterol; pressurized liquid extraction; gas chromatography–mass spectrometry.

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1. INTRODUCTION

Calophyllum inophyllum L. is a large tree with broad, glossy, leathery, elliptic-oblong leaf blades, 8–16 cm by 4–8 cm, and with numerous parallel side veins. The tree bears sweetly scented white flowers in erect racemes. Fruits are globose, 2 cm across and are green in colour. In the world, it is found in Africa, tropical and temperate Asia, Australasia and the Pacific [1]. The tree is either grown wild or widely cultivated along Vietnam especially in the coastal area [2]. *Calophyllum inophyllum* L. nut is used to extract oil. In Vietnamese traditional medicine, oil is specific for rheumatism and various skin diseases (i.e., scabies, ringworm and dermatosis), wounds, leprous nephritis, bone infections and inflammations [3].

Calophyllum inophyllum L. fruit peel which is abundantly available in Vietnam is a waste product when oil is extracted to use as natural medicine. Previous study showed that this fruit peel's extracts had demonstrated antimicrobial property [4]. Thus, it is necessary to identify the active compounds which are responsible for the observed antimicrobial activity.

The GC methodologies have been used by the pharmaceutical industry for analysis of raw, intermediary and final products which are volatile or semivolatile. Detection can be obtained by FID,

- *Corresponding author: Ha Minh Hien, email: haminhhien@yahoo.com*
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ECD or MS. The successful of the GC-MS technique is due to its sensitivity, specificity, and simplicity of sample treatment in identification and quantitation in comparision with LC [5]. PLE offers the possibility of performing extractions in an inert atmosphere protected from light, which represents an advantage since phenolic compounds are very sensitive to these two factors [6].

For the first time, this is the report about determination of calophyllolide, stigmasterol and β-sitosterol in *Calophyllum inophyllum* L. fruit peel using pressurized liquid extraction followed by gas chromatography-mass spectrometry (GC-MS) in order to characterizeits bioactive principles.

2. MATERIAL AND METHODS

2.1. Chemical

n-Hexane, ethyl acetate, methanol and ethanol PA and LC grade were procured from Merck, Germany. Calophyllolide (purity > 96% by HPLC) was purchased from Institute of Drug Quality Control-Ho Chi Minh City, Vietnam.

2.2. Plant material

The fruit peels of *Calophyllum inophyllum* L. were obtained from Ben Tre Province-Vietnam, collected in 2009. The voucher specimen was identified by Prof. Truong Thi Dep, Head of Department of Botanic, Faculty of Pharmacy, University of Medicine and Pharmacy at HCMC, Vietnam where it was deposited.

2.3. Pressurized liquid extraction

Pressurized liquid extractions were performed on a Dionex ASE 100 (Dionex Corp., USA) system. The plant material was pulverized using a Fitz mill (M5A, Fitzpatrick, USA) and sieved to obtain a powder with a median particle size of 1.84 mm. Then 5 g of the milled material was mixed with diatomaceous earth (2 g) and placed into 11 mL stainless steel extraction cell, respectively. Glass wool and 10 µm frits were placed at each end of the extraction vessel to prevent fines contaminating the extract . The extraction cell was extracted under the extraction conditions. The vacuum concentrator system consisted of a rotary evaporator, a thermostat and a digital bath (EYELA, Japan) was used for obtaining the extracts. Then, extract was transferred to a 100 mL volumetric flask which was brought up to its volume with extraction solvent and filtered through a 0.45 µm filter (Satorius) prior to injection into the GC–MS system.

2.4. Soxhlet extraction

Soxhlet extraction was conducted using an automated Soxhlet extractor (B-811, Buchi, Switzerland). For this, 1 g milled material was accurately weighed and placed in a cellulose thimble (25 mm×27 mm×100 mm, Whatman, UK) and extraction was performed using methanol for 8 h. The extract was evaporated under reduced pressure and filtered through Whatman 40.

2.5. Instrumentation

GC–MS was performed with an Shimadzu GC-2010 gas chromatography instrument coupled to a GCMS-QP 2010 mass spectrometer and a LabSolutions GCMSSolution Ver. 2.5 software (Shimadzu Corporation, Japan). Compound was separated on a 25 m × 0.22 mm i.d. capillary column coated with 5 µm film of 5% phenyl methylpolysiloxane (DB-5MS) due to its polarity.

The injector and GC-MS interface were kept at 250 °C while the ion source held at 200 °C and helium was used as carrier gas at a flow rate of 1.43 mL min^{-1}. The analysis was carried out in the splitless mode. The data output was achieved using scanning (SCAN) mode for identification and selected ion monitoring (SIM) mode for quantification.

2.6. GC-MS

Method development

Base on the methodology on determination of calophyllolide using GC-MS in the previous study [7], the ramping rate as well as the programmed temperature was slightly modified to achieve the best resolution in the minimum time.

Method validation

Method validation was performed according to the ICH guidelines [10]. Thus, for identification purpose, the specificity of the method was studied. For content of calophyllolide, an active compound, the study was focused on accuracy, precision which comprises repeatability and intermediary precision, specificity, linearity and range. Excel 2003 (Microsoft Office) was used for statistical analysis. A 5% level of significance was selected.

Calibration curves of calophyllolide

Five concentration levels of calophyllolide ranging from 3.19 to 51 µg.mL^{-1} in methanol were prepared. The solutions were centrifuged at 6000 r·min^{-1} for 5 min. Each concentration was analyzed twice and the results represented the average. The peak areas of selected ion in SIM mode (*m/z* at 401) were plotted against the corresponding concentration of calophyllolide to obtain the calibration graph by linear regression.

Linearity, precision and accuracy

The method linearity, recovery and precision (repeatability and intermediary precision) were evaluated according to the ICH guidelines. The

linearity of the curves was estimated by regression using the least square method. The slope, intercept and coefficient of determination (R^2) were calculated and evaluated [10]. Thereby, samples of the solution at five different concentrations (3.19, 6.38, 12.75, 25.5 and 51 µg.mL^{-1}) were injected twice and the area recovered was calculated. In the same manner, calibration curve was built with five solutions of methanolic extract containing the compound to study the effect of matrix on the response of it using Student's *t*-test.

For the repeatability assay, six sample solutions at about 12.75 µg.mL^{-1} of compound were prepared. Each solution was injected in duplicate and the repeatability was evaluated for m/m percentage of compound through the relative standard deviation (RSD/%). The intermediary precision was calculated from data of repeatability and one additional day which actually derived from accuracy experiment. The data were expressed as relative standard deviation (RSD/%), and tested by F-test and *t*-test [11].

Specificity

The specificity of the method was tested by FDA and EU criteria for identification of known compounds [8]. Further more, the peak purity of calophyllolide was investigated by AMDIS32 software (NIST, National Institute of Standardization Technology).

3. RESULTS

3.1. Determination of PLE procedure

The PLE procedure was based on antimicrobial activity of *Calophyllum inophyllum* L. crude extracts obtained by pressurized liquid extraction. Solvents with difference polarity were studied. The thin-layer chromatography in combination with the antimicrobial activity of different extracts determined by Kirby Bauer test were used for determination of PLE procedure. Taking into account the results of TLC experiment which show the most intensity spot of calophyllolide and Kirby Bauer test which show the biggest percentage mean zone of inhibition (extract) over mean zone of inhibition (standard), the conditions of the PLE method proposed were: solvent, methanol; temperature, 120 °C; static extraction time, 15 min and 60% of the flush volume.

Table 1. Antimicrobial activity of the *Calophyllum inophyllum* fruit peel of various extract obtained by PLE (100 mg of the extract per loaded disc)

Extracting solvent	***Mean zone of inhibition (mm)***			***Mean zone of inhibition (mm)***			***Mean zone of inhibition (mm)***		
	S. aureus (ATCC 6538 P)			*M. smegmatis (ATCC 14468)*			*Ps. aeruginosa (ATCC 9027)*		
	Extract 100 µg	*Methicillin 5 µg*	ZIH_{Ext}/ZIH_{Std} (%)	Extract 100 µg	Streptomycin 10 µg	ZIH_{Ext}/ZIH_{Std} (%)	Extract 100 µg	Carbenicillin 100 µg	ZIH_{Ext}/ZIH_{Std} (%)
Water	16	31	51.6	-ve	32	Nil	-ve	23.5	Nil
Ethanol	18	31.5	57.1	14.5	32	45.3	-ve	23	Nil
Methanol	18	31	58.1	15	32	46.9	-ve	24	Nil
EtOH : water (1:1)	17	32	53.1	11.5	32	35.9	-ve	24	Nil
MeOH : water (8:2)	18.5	32.5	56.9	11.5	32	35.9	-ve	24.5	Nil
Ethyl acetate	18	32	56.25	14	32	43.8	-ve	23.5	Nil
n-Hexane	17.5	32.5	53.8	12	32	37.5	-ve	24	Nil

ZIHExt: Mean zone of inhibition (Extract)

ZIHStd: Mean zone of inhibition (Standard)

3.2. Optimization of GC-MS conditions

The GC-MS conditions were inherited from the earlier work of the authors on determination of calophyllolide in *Calophyllum inophyllum* L. [7] with modification. The initial column oven temperature was at 80 °C, then programmed at 40 °C min^{-1} to 300 °C and hold for 12 minutes. Splitless injection was conducted with and helium was used as carrier gas of 1.43 ml min^{-1} flow-rate. The spectrometer was operated in electron-impact (EI) mode, the

scan range was 40–450 amu, the ionization energy was 70 eV. The injection port, ionization source and interface temperatures were 250, 250 and 200 °C, respectively.

The selected ion monitoring (SIM) method was used for the quantification of calophyllolide. A fragment ion *m/z* 401 of base peak was used as target ion together with two reference ions at m/z 83 and 55.

3.3. Method validation

Specificity

With the scanning (SCAN) mode, the mass spectrum of calophyllolide in the PLE extract showed a molecular ion peak at *m/z* 416; the base peak at *m/z* = 401 was due to methyl elimination. The fragment ions at *m/z* 83, 77 and 55 were also observed. The fragmentation pattern was reasonable for the compound as shown in fig. 1. The mass spectrum of calophyllolide was supported by data in literature (MS (70 eV) *m/z* 416 [M]+, 401, 83, 55)[12]. The peak purity of calophyllolide was checked by mass spectral deconvoluting software AMDIS32. In the sample, the extracted spectrum, retention time of calophyllolide matched with those obtained from the standard compound (95%).

Linearity and interferences

The calibration curves obtained from the base peak area, was linear over the range of 3.19–51 µg.mL^{-1}. The linear model was $Y = 2510.9X - 7671.4$ and the coefficient of determination

(R^2) was 0.9988. The *t*-test was used to compare the calibration curves of calophyllolide only and in the presence of the matrix of extract. The *t* value of 1.053 was less than $t_{0.05}$ of 2.776 which indicates that the two curves are not statistically different ($P = 0.05$) or there are no effect of matrix on the response of calophyllolide.

Precision and accuracy

According to ICH, the repeatability of an analytical method is described as the relative standard deviation, calculated from six times of repeated determination from concentration of 100% from the standard [6]. For calophyllolide, the concentration of about 12.75 µg.mL^{-1} was chosen as central point from the calibration curve. The result was (0.0255 ± 0.0005) % (m/m). The method was found to be precise as the repeatability had lower value of 1.89%. The intermediary precision was performed to evaluate the accumulation of the random errors. In this work the variations between different day was studied and the inter-day RSD's was 2.06%. The F-test and *t*-test indicate that the average percentage (m/m) of calophyllolide was not statistically different between days ($P = 0.05$).

The accuracy was verified by calculation the percent recoveries of the amount of calophyllolide added at 3 levels (80%, 100% and 120%) of the known amount of calophyllolide. Mean recovery was (99.53 ± 2.05)% (RSD = 2.06%).

3.4. Identification of calophyllolide, stigmasterol and β-sitosterol in *Calophyllum inophyllum L.* fruit peel

The SCAN method was use for identification calophyllolide, stigmasterol and β-sitosterol. All the components of interest were completely separated, and calophyllolide was identified according to criteria for identification of known compound including FDA and EU criteria for mass spectral matching [8]. Besides, stigmasterol and β-sitosterol were also discovered by comparing the mass spectra of the sample with database of NIST 147. LIB in the library.

Table 2. FDA criteria for mass spectral matching of calophyllolide

FDA specification	Acceptance criteria	Calophyllolide (Reference Standard)	Specimen	Results
Retention Time (min)	≤ ± 2%	12.818 ± 0.002 (0.01)	12.822 ± 0.007 (0.05)	Conforms (0.03%)
Minimum structurally specific ions (m/z)	≥ 3	416, 401, 83 and 55	416, 401, 83 and 55	Conforms (4)
General correspondence between relative abundance of sample and standard ions (%)	± 20%	m/z 55: 15.08% m/z 83: 3.28% m/z 416: 14.77%	m/z 55: 17.14% m/z 83: 3.71% m/z 416: 15.08%	Conforms (m/z 55: 13.66%; m/z 83: 13.11%; m/z 416: 2.1%
Prominent ions, not from analyte	can be explained	m/z 73	m/z 73	Conforms (Ion fragment of septum pieces that have fallen into glass insert)

Table 3. EU criteria for mass spectral matching of calophyllolide

EU specification	Acceptance criteria	Calophyllolide	Specimen	Results
Retention Time (min)	≤ ± 0.05%	12.818 ± 0,002 (0.01)	12.822 ± 0,007 (0.05)	Conforms (0.03%)
Minimum diagnostic ions (molecular ion, adducts, fragments, and isotope ions) with an intensity >10% in the standard must be observed in the sample (m/z)	≥ 4	416, 401, 83 and 55	416, 401, 83 and 55	Conforms
The inclusion of molecular ion if the relative intensity is ≥ 10% of the base peak 10% of the base peak 10% of the base peak.	Must be included	m/z 416: 14.77%	m/z 416: 15.08%	Conforms
The relative intensities of the sample diagnostic ions are required to match those of the standard, within specified tolerances	> 50 ± 10% > 20-50 ± 15% > 10-20 ± 20% ≤ 10 ± 50%	m/z 55: 15.08% m/z 83: 2.98% m/z 416: 14.77%	m/z 55: 17.14% m/z 83: 3.71% m/z 416: 15.08%	Conforms (m/z 55: 13.66%; m/z 83: 13.11%; m/z 416: 2.1%)

Table 4. Mass spectral matching of stigmasterol and β-sitosterol against NIST 147. LIB

Compound	Retention time (min)	Mass data	Database
Calophyllolide	12.67	416, 401, 83, 55	
Stigmasterol	12.97	412, 397, 83, 69, 55	NIST 147. LIB
β-sitosterol	13.87	414, 399, 69, 55, 43	NIST 147. LIB

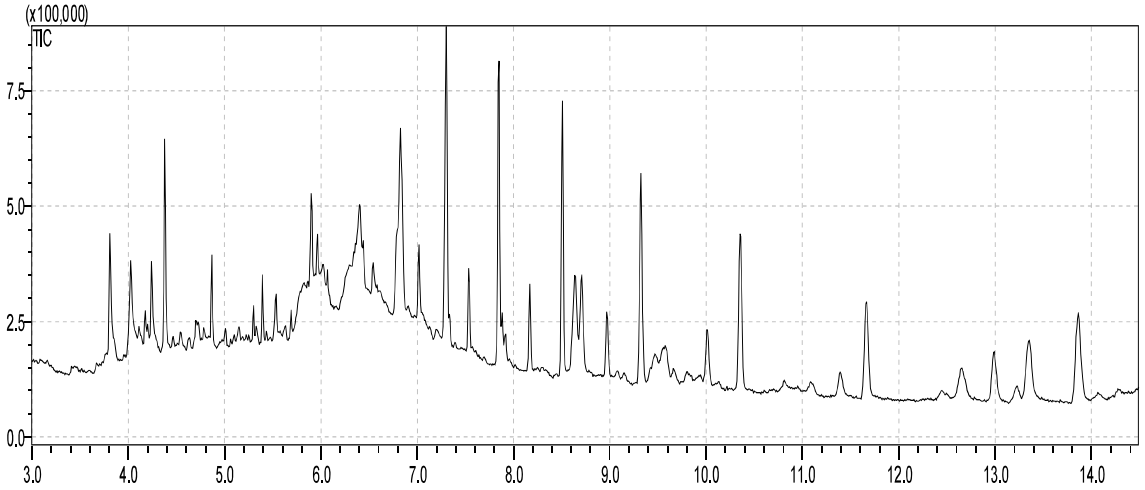
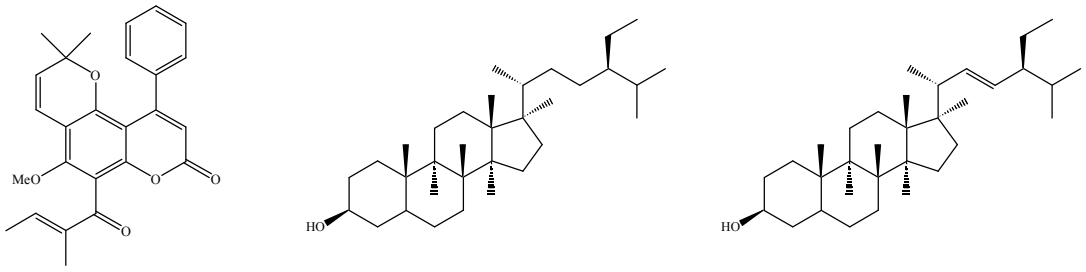


Fig. 1. GC-MS total ion chromatogram of the methanolic extract obtained from fruit peel of *Calophyllum inophyllum* L.



1 **2** **3**

Fig. 2. Chemical structures of calophyllolide (1), β-sitosterol (2) and stigmasterol (3)

3.5. Quantification of calophyllolide in *Calophyllum inophyllum L.* fruit peel

The yields of respective extracts using methanol are presented in Table 5.

Table 5. Extraction of Calophylluminophyllum fruit peel with methanol by PLE and Soxhlet extraction

	Crude extract yield (% w/w)			Crude extract mass (mg)		Extraction time	Extract descriptio
	1st	2nd	Average	Vial 1	Vial 2		
PLE extraction	11.3	9.9	10.6	514	494	25-30 min	Dark brown sticky oil
Soxhlet extraction	3.85	4.09	3.97	81	85	8 h	Oily dark yellow

The selected ion monitoring (SIM) method was used for quantification of calophyllolide. A fragment ion *m/z* 401 of calophyllolide's base peak was used as target ion together with two reference ions at m/z 83 and 55 for quantification. The results were: 0.0255 ± 0,0005% (m/m) for PLE extract and 0.025 ± 0.0005% (m/m) for soxhlet extract.

4. DISCUSSION

Calophyllolide as chemical reference substance has been established using WHO guidelines's procedure and distributed by the institute of drug quality control-Ho Chi Minh City, Vietnam which is an assigned laboratory by the country for the job. The method used for the determination of purity was a validated HPLC-DAD. Because of the non-availability of calophyllolide as primary or secondary reference standard for traceability in the market, therefore area percent for determination of its purity was the method of choice. Information on method validation such as specificity, linearity and precision was available. Statistical treatment of data such as assigned value, standard deviation or standard uncertainty measurement was strictly followed ISO 13528:2005. Earlier study has suggested the presence of β-sitosterol and stigmasterol in the fruit peel of *Calophyllum inophyllum* L. grown in Vietnam using VLC and spectroscopic methods: IR, NMR [9]. However, the problem is that due to the similarity in structure of β-sitosterol and stigmasterol, it is difficult to purify β-sitosterol contaminated with stigmasterol. GC-MS proved to be an effective measure in confirmation of those two compounds as the method is suitability, simplicity, rapidity and precision.

The results showed that PLE employed to extract calophyllolide was comparable with and even better than the exhausted extraction using soxhlet in term of calophyllolide's concentration, time and solvent consuming. Moreover, the crude extracts obtained by PLE using solvents with different polarity demonstrated promising antimicrobial activities against Staphylococcus aureus, and Mycobacterium smegmatis (with the exception of water extract). This result is promising as M. smegmatis is closely related to Mycobacterium tuberculosis, which is the pathogen involved in tuberculosis.

5. CONCLUSION

For the first time in literature, the finding of calophyllolide together with stigmasterol and β-sitosterol, the phytosterols in *Calophyllum inophyllum* L. fruit peel using PLE followed by GC-MS. Various PLE extractsusing solvent/ solvent mixture of different polarityshowed activities against Staphylococcus aureus and Mycobacterium smegmatis. The PLE methanolic

extract demonstrated the strongest antimicrobial property. It was then selected forPLE procedure to characterize the bioactive principles from thefruit peel of Calophylluminophyllum L. This has proved that this waste is a promising material as studies showed that calophyllolide is the biologically active component which possesses an antimicrobial and cytotoxic activities [12]. Further investigation including standardization for quality control and examination the effects resulted from collecting location, harvesting time and storage time on the concentration of calophyllolide as well as the antimicrobial property of fruit peel should be considered.

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