Regular Articles

Antimicrobial activity of *Calophyllum inophyllum* crude extracts obtained by pressurized liquid extraction

Minh Hien Ha [a*], Van Thi Nguyen [a], Khac Quynh Cu Nguyen [b], Emily LC Cheah [c], Paul WS Heng [c]

a. Research and Training Department, Institute of Drug Quality Control-Ho Chi Minh City, 200 CoBac Street, District 1, Ho Chi Minh City, Vietnam;

b. Faculty of Pharmacy, University of Medicine and Pharmacy of Ho Chi Minh City, 41 Dinh Tien Hoang, District 1, Ho Chi Minh City, Vietnam;

c. Department of Pharmacy, Faculty of Science, GEA-NUS Pharmaceutical Processing Research Laboratory, National University of Singapore, Singapore 117543, Singapore

Abstract

The fruit peel of *Calophyllum inophyllum* is considered waste material from the production of oils from its fruit and is abundantly available in Vietnam. The preparation of extracts was carried out by the pressurized liquid extraction (PLE) method. The preliminary phytochemical screening revealed the presence of phenolic compounds. The antimicrobial studies of the methanolic and *n*-hexane extracts were carried out on standard micro-organisms, *Staphylococcus aureus* (ATCC 6538 P), *Mycobacterium smegmatis* (ATCC 14468), and *Pseudomonas aeruginosa* (ATCC 9027), using the disc diffusion method. The extracts demonstrated promising antibacterial activity against *Staphylococcus aureus* and *Mycobacterium smegmatis*. The presence of phenolic compounds was likely to be responsible for these activities.

Key words: *Calophyllum inophyllum*, fruit peel, phenolic compound, antimicrobial activity

Introduction

Calophyllum inophyllum belongs to the family Clusiaceae and is a tree that can grow up to 25 m tall with a robust trunk which exudes white latex when bruised. The leaves have opposite arrangements, and are petiolate, thick and shiny with numerous parallel secondary veins. The flowers are borne in axillary cymes, of moderate size, white, and fragrant, with variable numbers of perianth parts and yellow anthers. The fruit is a purplish black globoid-to-ovoid drupe when mature with a single seed. Flowers and fruits are available throughout the year. The plant is widespread from the Indian Ocean (Africa and India) throughout Malaysia and in the Pacific islands. In Vietnam, it grows mainly in the southern warmer part of the country. Its bioactive constituents exhibit a variety of biological activities including piscicidal (phenyl coumarins), antibacterial, hypotensive, molluscicidal, antiviral, anti-retroviral effects, and phagocyte stimulation [1]. The fruit peel of *Calophyllum inophyllum* is considered a waste material from the production of oils and is available in

* Author to whom correspondence should be addressed. Address: Research and Training Department, Institute of Drug Quality Control, 200 CoBac Street, District 1, Ho Chi Minh City, Vietnam; Tel: 84-8-38374802, Fax: 84-8-38367900; Email: haminhhien@yahoo.com

Received: 2009-04-03 Accepted: 2009-07-13

abundance in Vietnam.

There is an explosion of interest in natural antimicrobials from plant sources due to the rise of multidrug resistant strains of bacteria such as MRSA, VRE and multidrug resistant tuberculosis [2]. Thus, rapid screening techniques are essential to quickly identify and isolate these compounds. Conventional solvent extraction techniques have several disadvantages in terms of high volumes of organic solvents consumed and long extraction times. Modern extraction techniques, such as pressurized liquid extraction (PLE), seek to circumvent these limitations. At the high pressures employed, extraction can be carried out above the boiling point of the solvent, which remains in the liquid phase, allowing better diffusion and improved mass transfer kinetics [3]. This drastically reduces the amount of organic solvent used as well as the extraction time, from a period of hours to only minutes. PLE also 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 [4].

Calophyllum inophyllum fruit is used to extract oil. The oil has been proven to have vulnerary and cicatrising effects [5]. The fruit peel is often discarded as waste. To our knowledge, there is no study currently available on the antimicrobial properties of the *Calophyllum inophyllum* fruit peel. Thus, this paper describes a study aimed to establish (1) PLE as a rapid extraction method for obtaining bioactive principles from botanical material, and (2) to investigate the potential of *Calophyllum inophyllum* fruit peel as a source of antimicrobial compounds.

Materials and methods

Plant materials

The dried fruit peels of *Calophyllum inophyllum* were collected in December 2008 from Ben Tre province which is situated about 80 km from Ho Chi Minh City, Vietnam.

Pressurized liquid extraction

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 [6]. Then, 5 g of the milled material was extracted by PLE (ASE-100, Dionex, USA) and the dead space in the extraction vessel was minimized using 1-2 mm glass beads. Glass wool and 10 µm frits were placed at each end of the extraction vessel to prevent fines contaminating the extract. Extraction was carried out at a fixed pressure of 1500 psi, a temperature of 120 °C, and for a period of 15 min. A single cycle of extraction was used. Flush volume of 60 % and a nitrogen purge time of 90 s were used to rinse the equipment of any entrapped extract to enable a more accurate quantitative recovery.

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 and *n*-hexane for 8 h.

Preparation of dried extracts

The extracts obtained from both PLE and the Soxhlet extraction were dried under reduced pressure under ambient conditions using a rotary evaporator (Eyela, Japan). The yield of extract, expressed as the weight ratio over the weight of raw material used, was calculated. The extracts were stored in airtight vials and kept refrigerated (2-8 °C) until further analysis.

Screening of phenolic compounds

Thin-layer chromatography was carried out using thin-layer chromatography (TLC) plates of pre-coated silica-gel 60 F_{254} on aluminum sheets (Merck, Germany). Development of the TLC plates was

carried out vertically in a 20 cm×20 cm twin trough chromatographic tank, pre-saturated for 10 min (with filter paper) with an appropriate developing solvent. The separation was allowed to run a distance of 100 mm from the lower edge of the plate. After developing, the plates were dried at ambient temperature. Mobile phase systems used were: (A) *n*-hexane: ethyl acetate (7:3), (B) toluene: ethyl acetate (8:2), and (C) chloroform: methanol (9:1). Detection of the compounds on the TLC plates was made under UV light at 254 and 366 nm and they were subsequently sprayed with 5 % ferric chloride solution.

Antimicrobial assay

Standard culture

Standard strains used were *Staphylococcus aureus* (ATCC 6538 P), *Pseudomonas aeruginosa* (ATCC 9027), and *Mycobacterium smegmatis* (ATCC 14468) purchased in the form of inoculation loops from Oxoid (England). These micro-organisms were cultivated on nutrient agar until the 6th generation and then used for the study.

Preparation of impregnated discs

Sterile paper discs (Whatman No.54) were individually impregnated with diluted plant extracts using the respective solvent (methanol or *n*-hexane) to obtain 100 µg of dried extract per disc. These discs were then dried at 40 ºC overnight before use.

Then, 20 ml sterilized Mueller Hinton agar (3.8 % *w*/*v*) was transferred to 90 mm Petri dishes and allowed solidify. Sterile water was used to wash the surface of the colonies from fresh subcultures of the test microorganisms. The turbidity of the microbial suspension was standardized against McFarland tube 0.5 (equivalent to 1.5×10^8 CFU/ml). The standardized suspension was diluted 10 times and 200 µl (3×10^6 CFU) was inoculated at the centre of the solid medium. The suspension was evenly spread using a glass spreader on the surface of the agar and allowed to dry for 15 min. The extract-impregnated discs and a standard antibiotic disc (Table 1) were placed equidistant from each other on the surface of the inoculated agar and a period of 30 min was allowed for diffusion of the compounds. The plates were then incubated at 37 °C for 18 h (*S. aureus, Ps. aeruginosa*) and 48 h (*M. smegmatis*). The diameters of the zones of inhibition around the discs were measured to the nearest mm and recorded at the end of the incubation period. Two diameter readings, perpendicular to each other, were recorded for each zone (d_1 and d_2), and the average readings were calculated. The ratio of the average ($d_{extract}$)/average ($d_{standard}$) was calculated for each extract, and for each particular microorganism.

Table 1. Microorganisms tested and the corresponding positive controls

Microorganism	Standard antibiotic disc
Staphylococcus aureus	Methicillin 5 µg
Pseudomonas aeruginosa	Carbenicillin 100 µg
Mycobacterium smegmatis	Streptomycin 10 µg

Results

Extraction method and yield

The yields of respective extracts using methanol and *n*-hexane are shown in Table 2.

Screening of phenolic compounds

Extracted compounds were separated by TLC and the results are shown in Table 3. These spots produced a violet coloration with 5 % ferric chloride solution indicating the presence of phenolic compounds.

Antimicrobial activity

The methanolic and *n*-hexane extracts were tested for their activity against *Staphylococcus aureus, Mycobacterium smegmatis,* and *Pseudomonas aeruginosa* and the results of their anti-microbial properties are shown in Table 4.

Table 2. Extraction of *Calophyllum inophyllum* fruit peel with methanol or *n*-hexane by PLE and Soxhlet extraction

No.	Solvent	Crude extract yield (% *w*/*w*)			Crude extract mass (mg)		Extraction time	Solvent : feed ratio	Extract description
		1st	2nd	Average	Vial 1	Vial 2			
Pressurized liquid extraction									
1	Methanol	10.19	9.78	9.98	510	490	25-30 min	80:10:00	Dark brown sticky oil
2	*n*-Hexane	1.12	0.79	0.96	57	40			Oily yellow
Soxhlet extraction									
1	Methanol	3.91	4.18	4.05	80	86	8 h	100:01:00	Oily dark yellow
2	*n*-Hexane	0.9	0.99	0.95	18	20	6 h		Yellow amorphous solid

Table 3. TLC-monitoring of methanolic and *n*-hexane crude extract of *Calophyllum inophyllum* fruit peel.

No.	Crude extract	Mobile phase A			B			C		
		Detection								
		UV 254	UV 366	$FeCl_3$ 5 %	UV 254	UV 366	$FeCl_3$ 5 %	UV 254	UV 366	$FeCl_3$ 5 %
		R_f value								
1	Methanol	0.56	-	-	0.62	-	-	0.85	0.85	-
		0.53	0.53	0.53	0.58	0.58	-	0.70	0.70	0.70
		0.47	-	-	0.52	-	-	0.64	0.64	0.64
		0.41	0.40	0.40	0.40	0.40	0.40	0.47	0.47	0.47
		0.34	0.32	0.32	-	0.33	0.33	0.41	0.41	0.41
		0.27	-	-	-	0.26	0.26	0.35	0.35	0.35
		0.22	0.24	0.24	0.22	-	-	0.30	0.30	0.30
		0.18	-	-	0.14	-	-	0.20	0.20	0.20
		0.11	0.13	0.13	-	0.18	0.18			
		0.06	0.05	0.05	-	0.05	0.05			
Number of spots		10	6	6	6	6	5	8	8	7
2	*n*-Hexane	0.79	0.79	0.79	0.82	0.82	-	0.85	0.85	-
		0.53	0.65	0.65	-	0.64	0.64	0.78	0.78	0.78
		0.47	0.47	0.47	0.59	0.59	0.59	0.70	0.70	0.70
		0.39	0.39	0.39	0.48	0.44	0.44	0.64	0.64	0.64
		0.32	-	-	-	0.35	0.35	0.51	0.51	0.51
		0.26	0.24	0.24	0.28	0.26	0.26	0.47	0.47	0.47
		0.20	-	-	0.22	-	-	0.35	0.35	0.35
		0.13	0.13	0.13	0.15	-	-	0.30	0.30	0.30
		0.07	-	-						
		0.03	-	-						
Number of spots		10	6	6	6	6	5	8	8	7

Table 4. Antimicrobial activity of the *Calophyllum inophyllum* fruit peel methanolic and *n*-hexane extracts obtained by PLE (100 µg of the extract per loaded disc).

Extraction solvent employed	Mean zone of inhibition (extract) / Mean zone of inhibition (stabdard) % (n = 2)		
	S. aureus	*Ps. aeruginosa*	*M. smegmatis*
Methanol	58.1	Nil	46.9
n-Hexane	53.8	Nil	37.5

Discussion

This results indicate that PLE can be employed to extract phenolic and antimicrobial compounds from the fruit peel of *Calophyllum inophyllum*. While extraction using *n*-hexane as the solvent resulted in similar extraction yield as Soxhlet extraction, methanolic PLE extracts resulted in a 2.5-fold greater yield. *n*-hexane usually extracts lipophilic material, such as plant waxes and essential oils, which normally account for a small fraction of the total dry weight of the plant. Methanolic extracts, on the other hand, mainly extract moderately to highly polar components, such as tannins, plant phenolic compounds, as well as high molecular weight compounds, such as sugars, starches, thus accounting for their much higher yields [4]. Soxhlet extraction was carried out at the boiling point of methanol which was 64.7 °C. The PLE extraction was carried out at temperatures above the boiling point as well as at an elevated pressure. In this instance, extraction yields would be increased for several reasons. (1) The dielectric constant of methanol is reduced at elevated temperature and pressure resulting in lowering of the solvent polarity. Hence, the spectrum of compounds extracted by methanol in PLE would be markedly enhanced compared with Soxhlet extraction. (2) The high pressures employed in PLE allowed the solvent to be driven into the matrix of the fruit peel. The -OH group of methanol would bind to polar matrix sites causing swelling of the plant matrix. This improves accessibility to the solvent and improved dissolution of compounds in the extract. (3) The high temperatures employed improve the mass transfer rates as well as the desorption of compounds from the matrix [3].

The thin-layer chromatography results presented in Table 3 indicate the presence of moderately to highly polar phenolic compounds in the methanolic extract whereas less polar compounds were found in *n*-hexane extract.

The crude extracts obtained by PLE demonstrated promising antimicrobial activities against *Staphylococcus aureus,* and *Mycobacterium smegmatis.* The experiments were performed with the corresponding positive controls, methicillin 5 µg and streptomycin 10 µg, respectively, and the ratio of the mean inhibition zones of the specimen discs over the control discs was calculated to compensate for the error due to the micro-organism diffusion rate. The methanolic crude extract gave higher zones of inhibition in comparison with the *n*-hexane extracts indicating that the antimicrobial agents of *Calophyllum inophyllum* consisted of moderately to highly polar compounds. Earlier studies confirmed that several phenolic constituents from the root bark and the nut of *Calophyllum inophyllum* exhibited antimicrobial activities against *Staphylococcus aureus* [7]. Both polar and non-polar extracts demonstrated good activity against *M. smegmatis* and this result is promising as *M. smegmatis* is closely related to *Mycobacterium tuberculosis*, which is the pathogen involved in tuberculosis. It is likely that the *n*-hexane fraction also possessed activity as lipophilic compounds are more likely to permeate through the mycobacterium membrane. None of the extracts inhibited *Ps. aeruginosa*, as this Gram-negative bacteria possess

an additional outer lipid membrane. It is likely that the antimicrobial compounds from the extracts were unable to penetrate this membrane and no appreciable antimicrobial activity was obtained.

Conclusion

PLE methanolic and *n*-hexane extracts of discarded *Calophyllum inophyllum* fruit peel demonstrated promising activity against *Staphylococcus aureus* and *Mycobacterium smegmatis*. This indicates that this material is a valuable source of antimicrobial compounds. Modern extraction methodologies such as PLE, having shorter extraction times, will preserve these antimicrobial compounds better than conventional solvent extraction techniques. Further investigations are underway to compare the performance of PLE with conventional extraction methods and to identify the phenolic compounds which are responsible for the observed antimicrobial activity.

References

[1] Medicinal plants in the south pacific, WHO regional publications, Western Pacific Series No.19, 1998, 29.
[2] Drug Strategies in Antibacterials, Marcel Dekker, Inc., 2000, 115.
[3] Waldeback M. Pressurized Fluid Extraction, PhD thesis-Uppsala Sweden, 2005, 25.
[4] Buelga CS, Williamson G, Methods in Polyphenol Analysis, UK, TheRoyal Society of Chemistry, 2003, 2, 12.
[5] Dweck AC, Meadows T. Tamaru (Calophyllum inophyllum) - The African, Asian, Polynesian and Pacific Panacea, Blackwell Science Ltd., 2002, 1.
[6] US Pharmacopoeia 30, 2007, 318-20.
[7] Yimdjo MC, Azebaze AG, Nkengfack AE, Meyer AM, Bodo B, Fomum ZT, Antimicrobial and cytotoxic agents from Calophyllum inophyllum, Phytochemistry 2004, 65, 2789-95.