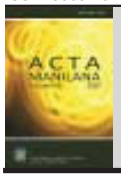
Acta Manilana 69 (2021), 45-52

ISSN: 0065-1370



Acta Manilana

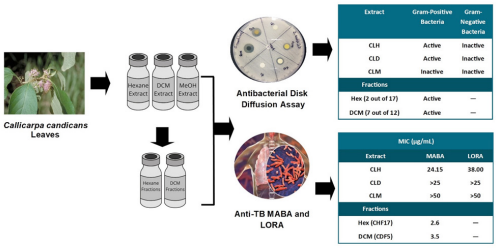
journal homepage: https://actamanilana.ust.edu.ph/

Evaluation of antibacterial and antitubercular activities of *Callicarpa candicans* (Burm f. Hochr.) leaf extracts

Gladys Ann B. Bautista[1], Mary Ann G. Santos[2,3], Baojie Wan[4], Scott G. Franzblau[4], & Alicia M. Aguinaldo[1,3*]

[1]The Graduate School, University of Santo Tomas, España, Manila, Philippines 1008
[2]Department of Biological Sciences, College of Science, University of Santo Tomas, España, Manila, Philippines 1008
[3]Research Center for the Natural and Applied Sciences, Thomas Aquinas Research Complex, University of Santo Tomas, España, Manila, Philippines 1008
[4]Institute for Tuberculosis Research, College of Pharmacy, University of Illinois at Chicago, Chicago, IL, USA

Graphical Abstract



Abstract

Plants from the genus *Callicarpa* have been reported for their ethnomedicinal uses such as treatment for skin diseases, anti-inflammatory, and antidiabetic. The ability of some *Callicarpa* species to heal skin diseases indicates the possibility of these species to have an antimicrobial property. The leaves of *Callicarpa candicans* were used in traditional medicine for wound healing and for treatment of tuberculosis. This study aims to evaluate the antibacterial activity and antitubercular activity of the leaf extracts (hexane, dichloromethane and methanol) of *C. candicans*. The antibacterial activity of *C. candicans* extracts was tested through disk diffusion assay using *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa* as the test organisms. The antitubercular activity was determined through Microplate Alamar Blue Assay (MABA) Minimum Inhibitory Concentration (MIC) on *Mycobacterium tuberculosis* H37Rv. The results of the disk diffusion assay showed that the hexane and DCM crude extracts and their fractions have antibacterial activity against gram positive *S. aureus* and *B. subtilis*, but not gram negative *E. coli* and *P. aeruginosa*. The same crude extracts and fractions from hexane and DCM showed high antituberculosis activity based on their MIC. The antibacterial and antitubercular constituents present in *C. candicans* have a semipolar nature since the active fractions were obtained from the semipolar DCM extract as well as the least nonpolar fractions of the hexane extract. Investigation on the active constituents from these fractions is in progress.

Keywords: *Callicarpa candicans*; antibacterial; antitubercular; *Mycobacterium tuberculosis* H37Rv

Corresponding author: amaguinaldo@ust.edu.ph
DOI: https://doi.org/10.53603/actamanil.69.2021.sgjl2556

Introduction

Callicarpa, commonly known as "beauty berry," are plants from the family of Lamiaceae, which are commonly planted in temperate and tropical regions. About 140 species of *Callicarpa* are distributed in the Caribbean Islands, Madagascar, China, Malesia, Australia, and the Pacific. In the Philippines, there are 27 species of *Callicarpa*, of which 19 are endemic [1]. *Callicarpa* is a rich source of bioactive natural products such as terpenoids [2], flavonoids [3], and phenolic acids [4]. These bioactive compounds are responsible for the multifunctional use of *Callicarpa* plants in traditional medicine [2].

Callicarpa candicans (Burm. f.) Hochr. locally known as "Tigau" in the Philippines [5], "Kembu-kembu" in Indonesia [6], and "Nang Nang" in Vietnam [7] is a plant with a high potential for antimicrobial constituents. In the Philippines, the Ati Negrito indigenous group of Guimaras Islands uses *C. candicans* to treat swollen muscles by applying ground leaves as a poultice [8]. The leaves of *C. candicans* are also utilized in Palau Island as fish poison [9]. In Sitio Marikudo, Isabela, Negros Occidental, the Ati community stated that Tigau is a shrub abundant in their sitio. It is found everywhere, from roadsides to grasslands, but not on the riverside. Livestock animals safely consume Tigau. For generations, the Ati Community used the Tigau leaves to stupefy fish in the river of their sitio and for traditional medicine. The sap is collected from the crushed leaves, and they let it flow along the river where fish are abundant and collect the fishes once they become immobile. They observed that fishes that consumed enough sap die, while some recover if they did not consume enough of it. Other animals such as shrimp and crabs are not affected by Tigau sap. As part of their traditional medicine practice, the sap collected from crushed Tigau leaves is applied to the wounds using cotton at least once a day. The wound is not covered to prevent the sap from sticking, which could hamper the healing process. The sap is said not to cause pain or irritation when applied to the wound. The healing process takes a few days, depending on the severity of the wound. Aside from wound healing, the leaves of Tigau were also used to treat tuberculosis (P. Cruz, personal communication, September 3, 2021). Another species of *Callicarpa* that has been reported for its anti-TB activity is *C. pilosissima*. The study on *C. pilosissima* demonstrated high anti-TB activity, wherein 15 out of 20 isolates exhibited inhibition of *M. tuberculosis* H37Rv [10]. *M. tuberculosis* is the etiologic agent in the disease, tuberculosis, which is one of the top public health threats and the leading cause of death from an infectious disease worldwide [11]. The emergence of drug-resistant TB forms suggests the need for potential new anti-TB drugs [12], such as those that can be developed from natural products [13].

According to Tu et al. [14], *C. candicans* is used to treat skin diseases such as scabies, eczema, and psoriasis. This skin disease healing property of *C. candicans* could indicate that it has an antimicrobial property. A study done by Nurtjahja et al. [6] showed that *C. candicans* extract has inhibitory activity against the growth of gram-positive and gram-negative bacteria. Because of the aforementioned reports, *C. candicans* was investigated by evaluating the antibacterial and antitubercular activities of its leaf extracts and the fractions from the active extracts.

Materials and Methods

Collection and Authentication of Plant Material. Fresh leaves of *C. candicans* were collected from the Quiet Place Farm Resort, Brgy. Tabunan, Bago City. The herbarium specimen was authenticated and assigned Certificate Acc. No. USTH 011767 at the UST Herbarium of the University of Santo Tomas Research Center for the Natural and Applied Sciences (UST-RCNAS), Manila.

Preparation of Extracts. The powdered air-dried leaves of *C. candicans* (1.37 kg) were extracted exhaustively in solvents of increasing polarity (hexane, dichloromethane, and methanol) at room temperature for 4-6 days. The extracts were separately concentrated in vacuo at <40°C. The hexane and dichloromethane crude extracts were separated using vacuum liquid chromatography by adsorbing separately in Celite 545 and packed on top of the silica bed (silica gel 60H, particle size <55µm, CAS No. 1.07736, Merck) packed in sintered glass column that is connected to a vacuum pump. While the set up was still under vacuum, gradient elution was carried out using solvent systems of increasing polarity. The hexane crude extract was fractionated using the following solvent systems: petroleum ether, petroleum ether-dichloromethane (3:1), petroleum ether-dichloromethane (1:1), petroleum ether-dichloromethane (1:3), dichloromethane. Whereas the dichloromethane crude extract was fractionated using the following solvent systems: hexane-dichloromethane (3:1), hexane-dichloromethane (1:1), hexane-dichloromethane (1:3), dichloromethane, dichloromethane-methanol (4:1). Fraction collection was done for every 200 mL per flask. The fractions were subjected to thin layer chromatography using hexane-dichloromethane and dichloromethane-ethyl acetate solvent systems, and their profiles were compared. The fractions that showed similar TLC profiles were pooled together and labeled accordingly.

Disk Diffusion Assay. The following bacterial samples were used for the disk diffusion assay - *S. aureus* (ATCC 25923), *B. subtilis* (UST-CMS 1011), *E. coli* (ATCC 25922), and *P. aeruginosa* (ATCC 27853). The bacterial samples were cultured in Mueller-Hinton Agar slants and incubated at 37ºC for 24 hours. After incubation, the bacteria were transferred to sterile Mueller-Hinton Broth and its turbidity were adjusted using 0.5 McFarland standard solution. Blank antibiotic disks were loaded with 20 µL of each of the three concentrations (1 mg/mL, 10 mg/mL and 100 mg/mL) of the *C. candicans* crude extracts, the positive control and the negative control and dried in vacuum oven. The dried discs were placed on plates inoculated with the bacteria and incubated at 37ºC for 24 hours. The plates were checked for zone of inhibition, the diameter of the zone was measured and recorded. The assay was done in triplicate. The same procedure was done on the *C. candicans* hexane and DCM fractions using 10 mg/mL, 50 mg/mL and 100 mg/mL as the concentrations and *S. aureus* and *B. subtilis* as bacterial strains [15].

Anti-tuberculosis assays (MABA and LORA). The anti-TB assay used the virulent strain *Mycobacterium tuberculosis* H37Rv (ATCC 27294, American Type Culture Collection, Rockville, MD). The activity against replicating *M. tb* H37Rv was determined using a fluorescence readout in the Microplate Alamar Blue Assay (MABA) [16]. following incubation for one week with the test compounds in glycerol-alanine-salts (GAS) medium and in a medium without added iron but with Tween 80 (GAST).

The MIC is defined as the minimum concentration inhibiting fluorescence by 90% relative to bacteria-only controls. The Low Oxygen Recovery Assay consisted of having a low-oxygen adapted culture of recombinant H37Rv (pFCA-luxAB), expressing a *Vibrio harveyii* luciferase gene with an acetamidase promoter, that was grown in a BiostatQ fermentor. Cells were collected on ice, washed in PBS, and stored at -80 °C. Circa 10^5 cfu/mL of thawed NRP cells were exposed to 2-fold serial dilutions of test compound in 7H9 broth in black 96-well plates, which were incubated for 10 days anaerobically at 37°C. Luminescence readings were obtained following a 28 h recovery in an aerobic environment (5% CO_2). The data were analyzed graphically, and the lowest concentration of test compound preventing metabolic recovery (90% reduction relative to untreated cultures) were determined [17].

Results and Discussion

The results for the antibacterial activity of the *C. candicans* crude extracts determined by disk diffusion assay are shown in Table 1. The bacterial growth inhibition results showed that only the gram-positive bacteria – *S. aureus* and *B. subtilis* – are susceptible to hexane and DCM crude extracts. The methanol crude extract was not able to inhibit the bacteria at all concentrations used. The resistance of gram-negative bacteria could be attributed to its distinctive cell wall structure. The gram-negative bacterial cell wall consists of three layers. The outer membrane, which is the first layer, serves as a protective barrier. This layer is unique to gram-negative bacteria and distinguishes it from gram-positive bacteria. The outer membrane is composed of lipopolysaccharides which can restrict the plant extracts' penetration [18-19]. The lack of an outer membrane layer in gram-positive bacterial cell wall makes it more susceptible to permeation of the plant extracts. Routine phytochemical investigations require a separation of the plant constituents by polarity.

Using appropriate solvents, the nonpolar constituents can be separated from the semipolar constituents and the polar constituents. Since the hexane (nonpolar) and DCM (semipolar) crude extracts exhibited antibacterial activity, they were further separated, to obtain fractions that were pooled based on chromatographic homogeneity. The 17 pooled hexane fractions and the 12 pooled DCM fractions were then tested only against the gram-positive bacteria, since earlier results with the extracts were already negative for the gram-negative bacteria. The zones of inhibition for the hexane and DCM fractions are shown in Table 2 and Table 3, respectively. Out of 17 hexane fractions, only fractions 16 and 17 effectively inhibited the growth of *S. aureus* and *B. subtilis* at all concentrations used. On the other hand, 6 out of 12 DCM fractions are active against the gram-positive bacterial strains. It is observed that the zone of inhibition is not directly proportional to the increase of the fraction concentration.

The results obtained in this study showed that the extracts were active against gram-positive bacteria and not gram-negative bacteria. It disproves the report by Nurtjahja et al. [6] that the leaf methanol extract was antimicrobial against both gram-positive and gram-negative bacteria.

Table 1. Zone of inhibition *C. candicans* crude extracts against four bacterial strains

Bacteria	Zone of Inhibition (mm)											
	CLH			CLD			CLM			Benzyl penicillin	Streptomycin	DCM
	1 mg/mL	10 mg//mL	100 mg/mL	1 mg/mL	10 mg/mL	100 mg/mL	1 mg/mL	10 mg/mL	100 mg/mL	10 units/uL	10 units/uL	-
B. subtilis	6.00	9.66	9.33	6.00	12.66	13.66	6.00	6.00	6.00	18.66	-	6.00
S. aureus	8.00	8.66	10.00	8.00	9.66	12.66	6.00	6.00	6.00	31.66	-	6.00
E. coli	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	-	17.33	6.00
P. aeruginosa	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	-	11.33	6.00

*Diameter of disk is 6mm; Values presented are average of three replicates

Table 2. Zone of Inhibition of *C. candicans* hexane fractions against gram-positive bacterial strains

Gram-positive bacteria	Zone of Inhibition (mm)							
	CHF16 (mg/mL)			CHF17 (mg/mL)			Benzyl penicillin	DCM
	10	50	100	10	50	100	10 units/µL	-
B. subtilis	15.16	16.00	16.50	16.33	17.16	18.00	20.00	6.00
S. aureus	13.00	13.66	13.83	13.83	14.33	15.00	28.66	6.00

*Diameter of disk is 6mm; Values presented are average of three replicates

Table 3. Zone of Inhibition of *C. candicans* DCM fractions against gram-positive bacterial strains

Bacteria	Zone of Inhibition (mm)											
	CDF3			CDF4			CDF5			CDF6		
	10 mg/mL	50 mg/mL	100 mg/mL	10 mg/mL	50 mg/mL	100 mg/mL	10 mg/mL	50 mg/mL	100 mg/mL	10 mg/mL	50 mg/mL	100 mg/mL
B. subtilis	7.66	9.83	10.66	13.66	14.33	15.00	16.16	17.00	17.66	14.50	15.16	15.50
S. aureus	7.50	8.16	8.66	11.66	12.33	12.50	14.33	15.50	16.00	12.66	13.83	13.83
	CDF7			CDF8			CDF9			Benzyl penicillin	DCM	
	10 mg/mL	50 mg/mL	100 mg/mL	10 mg/mL	50 mg/mL	100 mg/mL	10 mg/mL	50 mg/mL	100 mg/mL	10 units/µL	-	
B. subtilis	12.16	12.66	12.66	8.83	11.66	12.66	7.33	12.16	13.00	20.00	6.00	
S. aureus	10.00	10.83	11.33	7.33	10.00	10.33	6.00	10.00	11.00	28.66	6.00	

*Diameter of disk is 6mm; Values presented are average of three replicates

Table 4. Antituberculosis Assay Results of the *C. candicans* crude extracts

C. candicans	**MIC90 (µg/mL)**	
Crude Extract	**MABA**	**LORA**
CLH	24.15^{M}	38.00^{M}
CLD	$>25^{M}$	$>25^{M}$
CLM	$>50^{W}$	$>50^{W}$
Anti-TB Drugs		
RMP	0.01	0.13
INH	0.94	>128
LIZ	1.80	1.91
MOX	0.23	-
MET	-	>512
PA824	0.22	5.70
TMC207	0.06	0.10

RMP – rifampin; INH – isoniazid; LIZ – linezolid; MOX – moxifloxacin; MET - metronidazole; Drugs under clinical trials: PA824, TMC 207
Extracts: Highly active < 20 ug/mL. Moderately active 21-50 ug/mL. Weakly active 51-100 ug/mL. Inactive > 100 ug/mL

In this study, MABA and LORA assays were used to screen the *C. candicans* crude extracts and fractions for their anti-TB activity. MABA quantitatively determines the drug susceptibility of replicating *M. tuberculosis*, whereas LORA determines non-replicating *M. tuberculosis* [16]. The anti-TB activity of the crude extracts and fractions expressed as minimum inhibitory concentration (MIC) is shown in Table 4 and Table 5, respectively.

Table 5. Antituberculosis Assay of the *C. candicans* hexane and DCM fractions

C. candicans Fractions	MIC90 (µg/mL) MABA	Remarks
HEXANE FRACTIONS		
CHF16	4.5	Highly active
CH17	2.6	Highly active
DCM FRACTIONS		
CDF3	35.5	Weakly active
CDF4	18.3	Moderately active
CDF5	3.5	Highly active
CDF6	5.8	Highly active
CDF7	10.1	Highly active
CDF8	21.2	Weakly active
CDF9	24.6	Weakly active
Anti-TB Drugs		
RMP	0.01	
INH	0.36	
LIZ	0.95	
MOX	0.17	

Fractions: Highly active <10 ug/mL. Moderately active 11-20 ug/mL. Weakly active 21-50 ug/mL Inactive > 50 ug/mL

The MABA MIC90 of the hexane crude extract (CLH) is less than 25 µg/mL, whereas the CLD is >25 µg/mL and CLM is >50 µg/mL. It can be inferred that CLM has weak anti-TB activity compared to the moderately active CLH and CLD. The MABA MIC90 of the fractions showed that 2 out of 17 hexane fractions and 7 out of 12 DCM fractions tested have potential anti-TB activity. These fractions were also the same fractions that can inhibit the growth of *S. aureus* and *B. subtilis*, except for CDF3. Out of the nine active fractions, CHF16, CHF17, CDF5, and CDF6 have the highest anti-TB activity.

Conclusions

Based on the results of the study, it can be concluded that *C. candicans* has antibacterial activity against gram-positive bacteria *S. aureus* and *B. subtilis*, no activity against gram-negative *E. coli* and *P. aeruginosa*, and has antimycobacterial activity against *M. tuberculosis* H37Rv. It can be a potential source of new antibacterial and antitubercular compounds. The active compounds in *C. candicans* are semipolar in nature since these were obtained from the semipolar DCM extract as well as the least nonpolar fractions of the hexane extract. Investigation on the active constituents from these fractions is in progress. The compounds will undergo purification and structure elucidation.

Acknowledgement

This work was funded through the thesis grant of GAB Bautista by the Department of Science and Technology – Science Education Institute Accelerated Science and Technology Human Resource Development Program - National Science Consortium. G.A.B. Bautista gratefully acknowledges the DOST-SEI for the scholarship. The authors thank Mr. Philip Cruz for the plant samples and ethnomedicinal information, and Ms. Luisa Choa for the preliminary work on the plant.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, AMA; methodology, GAB, MAS, SGF; data collection, GAB, MAS, BW, SGF; analysis and interpretation of data, GAB, MAS, BW, SGF; original draft preparation, GAB, AMA; review and editing of the draft, GAB, MAS, AMA. All authors have read and agreed to the final version of the manuscript.

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