



Phytochemical Analysis and Antibacterial activity of Methanolic extract of *Clinacanthus nutans* Leaf

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Abstract: *Clinacanthus nutans* is a medicinal herb found in South East Asia. It has been traditionally used for skin infections. Hence, this study aimed to investigate the antibacterial effect of *C. nutans* against selected bacteria. *C. nutans* leaf was extracted with methanol. The dried methanolic extract was qualitatively screened for the presence of phytochemicals and quantitatively analysed for total phenols and total flavonoids. The strains of *Propionibacterium acnes* (ATCC 11827), *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Bacillus cereus* (ATCC 10876) and *Escherichia coli* (ATCC 25922) were selected for the study. The antibacterial effect was tested by microbroth dilution method in a 96-well microtiter plate and minimum inhibitory concentrations (MIC) were determined. The yield of methanolic extract of *C. nutans* leaves was 15.29 %w/w. Total phenolic content was 1.77±0.008 mg gallic acid equivalent/g. Total flavonoid content was 0.04±0.001 mg quercetin equivalent/g. Phytochemical screening revealed the presence of saponins, phenolics, flavonoids, diterpenes and phytosterols. The *C. nutans* extract exhibited antibacterial effect against *Propionibacterium acnes* (MIC > 12.5 mg/ml), *Staphylococcus aureus* (MIC 12.5 mg/ml), *Staphylococcus epidermidis* (MIC > 12.5 mg/ml), *Bacillus cereus* (MIC > 12.5 mg/ml) and *Escherichia coli* (MIC 12.5 mg/ml). Our study indicated that *C. nutans* possess significant antibacterial effect against *S. aureus* and *E. coli*. Further studies are required to identify the antibacterial compounds.

Keywords: *Clinacanthus nutans*, Phytochemical Screening, Total Phenolic Content, Total Flavonoid Content, Antibacterial Activity, Microbroth Dilution

Introduction

Clinacanthus nutans (Burm.) Lindau (Family: Acanthaceae) is a shrub and found in deciduous forests in Malaysia, Indonesia, Thailand, Vietnam and parts of China. It has been used traditionally to treat envenomation, dysentery, burns, scalds, fever, herpes skin infections and diabetes^[1, 2]. Recent studies have shown that *C. nutans* has significant antiviral activities against herpes simplex virus type-2, varicella-zoster virus, antioxidant activity and anti-inflammatory

activity^[3-6]. The chemical constituents of *C. nutans* are flavonoids, stigmasterol, β -sitosterol, lupeol, betulin, C-glycosyl flavones, vitexin, isovitexin, shaftoside, isomollupentin, 7-O- β -glucopyranoside, orientin, isorientin, cerebrosides, monoacylmonogalactosylglycerol and sulfur-containing glucosides^[7-9].

There are various skin diseases which are caused by bacterial colonisation particularly by the normal microflora of the skin. Among these, acne is one of the most common disorders of the skin^[10]. Antibiotics such as erythromycin and clindamycin

are topically used against acne. Tetracyclines are used as oral antibiotics against acne. *Propionibacterium acnes* is the bacterium which causes inflammation of sebaceous glands and cause acne^[11-13]. The emergence of antibiotic-resistant *P. acnes* bacteria represents a growing problem worldwide^[14]. Also, *Staphylococcus epidermidis* causes biofilms to grow on plastic devices placed within the body^[15, 16]. *S. epidermidis* shows resistance to various antibiotics^[17]. Similarly, infections caused by bacteria such as *S. aureus*, *B. cereus* and *E. coli* are also difficult to treat with current antibiotics^[18]. Hence, development of newer antibacterial agents is required and it is the need of the time. Since, *C. nutans* has been used in Thai traditional medicine against various skin infections^[1], we hypothesized that *C. nutans* may possess antibacterial activity against skin pathogens such as *Propionibacterium acnes* and *Staphylococcus epidermidis*. Therefore, our study evaluated the effectiveness of *C. nutans* against these skin pathogens and other selected clinically important pathogens.

Materials and Methods

Preparation of Plant Extract

Clinacanthus nutans was obtained from Yik Poh Ling Herbal Farm, Seremban, Malaysia. Authentication of the plant was done by Negeri Sembilan Forestry Department, Seremban, Negeri Sembilan, Malaysia. The leaves were separated, dried and powdered. The powder (255 g) was macerated with 1 litre methanol in a conical flask under room temperature for three days with frequent stirring using a magnetic stirrer. After three days, the mixture was filtered and fresh methanol was added to the residue. This process

was repeated several times until a clear colourless solution was obtained. The filtrates were combined and evaporated under reduced pressure at 40 °C using a rotary evaporator (BUCHI Rotavapor R-200, Switzerland). After evaporating the methanol, the dried extract was lyophilized with a freeze dryer (CHRIST Alpha 1-4 LD plus Freeze Dryer, Germany) at -50 °C to remove all traces of moisture.

Preliminary Phytochemical Screening

The crude methanolic extract of *C. nutans* was tested for the presence of phytochemicals by using specific reagents as described previously^[19].

Estimation of Total Phenolic Content

The dried methanolic extract of *C. nutans* was dissolved in methanol and made to the concentration of 10 mg/ml. Six concentrations of gallic acid standard solution ranging from 0.1 to 0.6 mg/ml were prepared in methanol. The total phenolic content in extracts was determined by using Folin-Ciocalteu reagent (Merck, Germany) and external calibration with gallic acid (GA) as per the previously reported method^[20]. Briefly, 0.2 ml of extract solution or 0.2 ml of gallic acid solution in a test tube and 0.2 ml of Folin-Ciocalteu reagent were added and the contents mixed thoroughly. After 4 min, 1 ml of 15% Na₂CO₃ was added, and then the mixture was allowed to stand for 2 h at room temperature. The absorbance was measured at 760 nm by using LAMBDA 25 UV-vis spectrophotometer (Perkin-Elmer, USA). The content of total phenolics was calculated by using gallic acid (GA) calibration curve. The results were expressed as mg gallic acid equivalents / g extract.

Estimation of Total Flavonoid Content

The dried methanolic extract of *C. nutans* was dissolved in methanol and made to the concentration of 10 mg/ml. Six concentrations of quercetin (Sigma-Aldrich, USA) standard solution ranging from 0.2 to 0.8 mg/ml were prepared in methanol. Determination of total flavonoid content was done by following previously reported method^[21] by mixing 0.1 ml of 10 mg/ml extract or 0.1 ml of quercetin solution with 0.3 ml of deionised water followed by 0.3 ml of 5% sodium nitrite, NaNO₂, (Acros Organics, USA). After 5 min, 0.3 ml of 10% aluminium chloride was added. At sixth min, 2 ml of 1M NaOH (Merck, Germany) was added and the final volume was made up with deionised water. Absorbance of each sample was measured at 510 nm against the prepared reagent blank using LAMBDA 25 UV-vis spectrophotometer (Perkin Elmer, USA). All tests were performed three times and averaged. Total flavonoid content of *C. nutans* methanolic extract was expressed as milligrams of quercetin equivalents per 1 g of plant extract (mg quercetin equivalents / g extract).

Microbroth Dilution Test

The antibacterial effect was tested by microdilution assay based on the method published by Zgoda and Porter with modifications^[22]. The strains of *Propionibacterium acnes* (ATCC 11827), *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Bacillus cereus* (ATCC 10876) and *Escherichia coli* (ATCC 25922) were obtained from All eights (M) Sdn Bhd. Based on CLSI guideline, microorganisms were suspended in saline and the turbidity is adjusted to 0.5 McFarland standard which corresponds to 1×10^8 CFU/ml^[23, 24]. The dried crude extract of *C. nutans* was reconstituted

with 10 %v/v dimethyl sulfoxide (DMSO) in sterile water and tween 80 up to 2% to give a stock solution of 100 mg/ml. From this stock solution, dilutions of 10 and 1 mg/ml were prepared. From these solutions, concentrations ranging from 0.1 to 12.5 mg/ml were prepared in the wells of microplate by diluting them with Mueller-Hinton broth based on CLSI guideline^[25]. The antibiotic positive controls (erythromycin and chloramphenicol) were prepared by dissolving the antibiotic powder in solvents recommended by CLSI^[24]. Erythromycin and chloramphenicol powder were separately dissolved in 95% ethanol and subsequently diluted with Mueller-Hinton broth to make the dilutions ranging from 0.125 to 256 µg/ml and added to the wells of the microplate. The negative control wells are filled with only 100 µl of broth. Growth control wells are filled with 100 µl of broth and 100 µl of inoculum. Then, 100 µl of inoculum was added to the wells which contained positive controls and extract. The microplates were covered and incubated at 37 °C for 24 hr^[26]. The microplates were then read with an ELISA plate reader (BioTek ELx800 absorbance microplate reader, United States) at 620 nm. The concentration at which sharp decrease of absorbance or zero absorbance occurs was considered as minimum inhibitory concentration (MIC)^[27, 28].

Results

The yield of methanolic extract of *C. nutans* leaves was found to be 15.29 %w/w with respect to the dry weight of leaf powder. The results of phytochemical screening are summarized in Table 1. Total phenolic content was found to be 1.77 ± 0.008 mg gallic acid equivalent/g. Total

flavonoid content was found to be 0.04 ± 0.001 mg quercetin equivalent/g.

Table 1: Phytochemical screening data of crude methanolic extract of *Clinacanthus nutans* leaf

	Crude methanolic extract of <i>C. nutans</i> leaf
Alkaloids - Mayer's reagent - Modified Dragendorff's reagent	- -
Saponins - Foam test	+
Phenolic compounds - FeCl_3 test	+
Tannins - Gelatin test	-
Flavonoids - Alkaline reagent test - Lead acetate test	+ +
Diterpenes - Copper acetate test	+
Phytosterols - Salkowski's test - Liberman Burchard's test	+ +

- indicates absence; + indicates presence

The MIC's of erythromycin, chloramphenicol and methanolic extract of *C. nutans* were recorded and presented in Table 2.

Table 2: MIC's of erythromycin, chloramphenicol and methanolic extract of *C. nutans* leaf against selected bacteria

Bacteria	Erythromycin ($\mu\text{g/ml}$)	Chloramphenicol ($\mu\text{g/ml}$)	Methanolic extract of <i>C. nutans</i> leaf (mg/ml)
<i>Propionibacterium acnes</i>	2.000	n.t.	> 12.50
<i>Staphylococcus aureus</i>	0.250	n.t.	12.50
<i>Staphylococcus epidermidis</i>	0.250	n.t.	> 12.50
<i>Bacillus cereus</i>	0.125	n.t.	> 12.50
<i>Escherichia coli</i>	n.t.	2.000	12.50

n.t. – not tested

Discussion

MIC of erythromycin against *P. acnes* was found to be $2 \mu\text{g/ml}$ which is higher than the breakpoints

($0.5 \mu\text{g/ml}$) set by EUCAST^[29]. Hence, *P. acnes* used in our study can be considered as nonsusceptible to erythromycin. In our study, MIC of erythromycin against *S. aureus* was $0.25 \mu\text{g/ml}$ and MIC of erythromycin against *S. epidermidis* was $0.25 \mu\text{g/ml}$. These results are in agreement with the clinical breakpoints ($1 \mu\text{g/ml}$) set by EUCAST and CLSI^[25, 29]. MIC of chloramphenicol against *E. coli* was found to be $2 \mu\text{g/ml}$ which is consistent with the clinical breakpoints set by CLSI^[25]. MIC of erythromycin against *B. cereus* was found to be $0.125 \mu\text{g/ml}$, which is within the previously reported range of 0.032 - $3 \mu\text{g/ml}$, as reported by Turnbull *et al*^[30]. DMSO was used to reconstitute the *C. nutans* extract. In our experiment (data not shown), DMSO showed inhibitory action at concentrations of $\geq 5\%$ v/v. However, the final concentration of DMSO in all the microdilution wells was less than 0.125% v/v. Hence, the antibacterial effect observed in this study can be attributed to the plant extract.

The *C. nutans* extract did not exhibit any significant inhibitory activity against *P. acnes* at the concentrations employed (MIC > 12.5 mg/ml). In a previous study by Chomnawang *et al*, *C. nutans* exhibited MIC and MBC of $>5 \text{ mg/ml}$ against the strain of *P. acnes* (ATCC 6919) used in their study^[31]. However, this study did not include information on the part of the plant used and solvent used for extraction. Hence, a direct comparison cannot be made. In this study *C. nutans* leaf extract exhibited MIC on *S. aureus* at 12.5 mg/ml . The results appear similar to a study conducted by Hussain *et al* on antibacterial activity of methanol extracts of various plants with agar dilution and agar well diffusion methods. In their study, the antibacterial activity of various plant extracts on *S. aureus* was observed at concentrations of 6.25 mg/ml and above^[32].

Chomnawang *et al* has also studied the antibacterial effects of ethanolic extracts of various Thai medicinal plants, one of them being *C. nutans*, against *S. aureus* and methicillin resistant *S. aureus* (MRSA)^[33]. Their study reported the MIC of 5 mg/ml and above for *S. aureus* and MRSA respectively. Although the ATCC strain of *S. aureus* used in their study differed from our study, the MIC of 12.5 mg/ml observed in our study is similar to their findings.

In our study, the crude extract of *C. nutans* exhibited MIC's of 12.5 mg/ml and above for all the organisms studied. Amongst the five organisms tested in our study, *S. aureus* and *E. coli* showed higher susceptibility to *C. nutans* extract. The higher MIC value may be attributed to the reason that the extract is a crude extract. Further fractionation of extract and isolation of compounds may give better MIC values.

In our study, preliminary phytochemical screening indicated the presence of saponins, phenolic compounds, flavonoids, diterpenes and phytosterols. Total amount of phenolics and flavonoids were also quantitated. These results are in agreement with the compounds isolated from this plant^[7-9]. Lupeol and β -sitosterol were reported to exhibit inhibitory action against *S. aureus*^[34]. Also, few studies showed that lupeol possessed inhibitory activity against *E. coli*^[34-36]. Similarly, flavonoids and terpenoids were reported to possess antibacterial property^[34, 37, 38]. All these compounds were reported to be present in *C. nutans*^[7-9]. Hence, the observed antibacterial activity may be attributed to the presence of these compounds. However, this cannot be concluded from this study. Further studies are required to identify the compounds responsible for antibacterial activity.

Conclusion

The study explored the potential antibacterial effect of *C. nutans* against selected skin pathogens and other organisms. Our study found out that methanolic extract of *C. nutans* leaf possessed antibacterial effect against *S. aureus*, *E. coli*, *P. acnes*, *S. epidermidis* and *B. cereus* with MIC's \geq 12.5 mg/ml.

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