Original Research Article

Antibacterial Activity and Minimum Inhibitory Concentration of Ethanolic Leaf Extract of *Clinacanthus nutans*

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ABSTRACT

Herbal medicine with antimicrobial properties has the potential to serve as an alternative therapeutic option to modern pharmaceuticals. *Clinacanthus nutans (C. nutans)*, a medicinal plant widely distributed across South East Asia, is known to possess a diverse range of bioactive compounds that give significant antioxidant, anti-inflammatory, anti-proliferative and antibacterial properties. Previous research has primarily investigated the antibacterial properties of *C. nutans* using water and methanol extractions. However, studies on ethanol extracts have been limited. Therefore, this study aimed to evaluate the antibacterial properties and minimum inhibitory concentration of ethanolic leaves extract of *C. nutans* against Gram-positive *(Staphylococcus aureus* and *Bacillus subtilis)* and Gram-negative *(Escherichia coli* and *Pseudomonas aeruginosa)* bacteria. The methodology involved ethanol extraction of *C. nutans* leaves, followed by antibacterial evaluations via disk diffusion test, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods. The yield obtained from the ethanol extraction was 7.1%. Results from the antibacterial evaluations showed that the extract was able to inhibit the growth of all tested bacteria, with the highest zone of inhibition against *S. aureus* (11.0 ± 0.6 mm) and the lowest against *E. coli* (6.5 ± 0.5 mm) at concentration of 500 mg/ml. The MIC and MBC values further confirmed this activity, with the extract showing a notable lower MIC/MBC for *S. aureus* (15.63/15.63 mg/ml) compared to *E. coli* (62.5/250 mg/ml). As a conclusion, the ethanolic extract of *C. nutans* has demonstrated antibacterial activity against all tested bacterial strains, including both Gram-positive and Gram-negative species. These findings support the potential of leaves of this plant to be used as a broad-spectrum natural antimicrobial agent for future therapeutic applications.

Keywords: *Clinacanthus nutans*, antibacterial activity, zone of inhibition, minimum inhibitory concentration

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1.0 Introduction

Clinacanthus nutans (*C. nutans*) is an herbaceous plant belonging to the Acanthaceae family that is highly regarded in traditional medicinal research due to its family's diversity, with 250 genera and 2,500 species known for their traditional medicinal uses (1). It is also locally known in Malaysia as "Belalai Gajah" due to its slightly curved stem and leaf arrangement resembling an elephant trunk. This plant thrives in tropical Asian countries such as China, Indonesia, Malaysia, Thailand and Vietnam (2). *C. nutans* is rich in secondary metabolites, which serve as defensive mechanisms and exhibit various pharmacological effects (3). It contains phenolic compounds, alkaloids, terpenoids, and saponins, with the yield of these metabolites can vary based on cultivation methods and extraction conditions. The presence of diverse phytochemicals makes *C. nutans* a promising candidate for therapeutic applications. It has been used to treat skin rashes, snake bites, herpes simplex lesions, diabetes mellitus, fever, and as a diuretic (4).

In recent years, the usage and research of plant-derived medications have increased as they have fewer adverse effects and are lower in cost compared with modern medicine (5). Additionally, the problem of antimicrobial resistance has caused the available antibiotics to rapidly lose their effectiveness against different bacterial strains (6). This issue is particularly concerning for *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*), which are major contributors to antibiotic-resistant infections and related deaths (7). Consequently, the ability of *C. nutans* to exhibit antibacterial properties, which was reported in many studies, has attracted many researchers to discover more about the potential of this plant to be an alternative antibacterial agent in clinical settings (8)

Currently, studies on the antibacterial properties of *C. nutans* samples collected from Malaysia were based mainly on dichloromethane, diethyl ether, methanol, hexane and water extractions (9,10). In Thailand on the other hand, a recent study by Chiangchin *et al*. (2023) (11) reported findings on ethanol extract. However, thus far no similar studies were conducted on ethanolic extract from the same plant in Malaysia. Previous studies have shown that environmental conditions, such as soil type and altitude may significantly affect the secondary metabolites produced, hence resulting in different bioactivity. Therefore, this study aims to expand the investigation using ethanol extract of *C. nutans* from the local origin for its antibacterial properties. Ethanol has been widely preferred for plant extraction in antibacterial studies due to its capability to extract a wide range of bioactive compounds, combined with its low toxicity, affordability, and ease of handling (12). The data retrieved from this study will offer valuable information into the optimal solvent used for extraction and the potential of *C. nutans* to exert its antibacterial properties. These will assist other researchers in further discovering *C. nutans* as alternative antibacterial agents hence improving the management of bacterial infection.

We have performed the *C. nutans* leaves extraction using ethanol, followed by screening its antibacterial properties against selected pathogens. We finally determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract that can inhibit bacterial growth to be used as a guidance for the development of new antibacterial agent.

2.0 Materials and methods

2.1 Plant Material

The leaves of *C. nutans* were obtained from a local seller in Sepang, Malaysia. The plant was identified and authenticated at Forest

Research Institute Malaysia (FRIM), Kepong, Kuala Lumpur with voucher no. (SBID 007/21).

2.2 Plant Extraction

Preparation of *C. nutans* extract was conducted based on the previous method with slight modifications (13). Powdered leaves were soaked in 80% ethanol at a ratio of 1:10 (w/v) in a conical flask and then shaken constantly using orbital shaker for 48 hours at room temperature with rotation of 100 RPM. The mixture was filtered using Whatman No. 1 filter paper and then concentrated to dryness at 40°C for 30 minutes with a rotary evaporator. After complete drying, the weight of dry extract was recorded for the calculation of extraction yield. The crude extract was transferred into sterile containers and kept refrigerated at 4°C prior to experimentation. Finally, the extract was dissolved in 10% Dimethyl sulfoxide (DMSO) to obtain a stock solution of 1 g/ml.

2.3 Bacterial strain and growth condition

Four bacterial strains were used in this study: Gram-negative *Escherichia coli* (ATCC 10798) and *Pseudomonas aeruginosa* (ATCC 27853), Gram-positive *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (ATCC 27853). Prior to experimentation, an overnight subculture of the bacterium was prepared by incubating single colony of each bacterium into separate Mueller-Hinton broth (MHB) at 37ºC. Next, the subculture was standardized to 0.5 McFarland standard (1×106 CFU/mL) with sterile MHB in a new sterile bottle to be used for antibacterial evaluations (14).

2.4 Antibacterial activity

The disk diffusion method is a standard technique described in the Clinical and Laboratory Standards Institute (CLSI) guidelines for assessing antimicrobial susceptibility (15). Firstly, negative control (DMSO 10%) and test disks were prepared by pipetting 20 µL of the *C. nutans* extract at three different concentrations (75, 250, and 500 mg/mL) onto the sterile blank disks and left to dry. For the inoculation process, the standardized bacterial suspension that meets the 0.5 McFarland standard was gently swabbed over the agar plates using a sterile cotton swab and allowed to dry. Then, all prepared disks including positive control (gentamicin disc) were put on the surface of the inoculated plate using sterile forceps. All the agar plates were incubated at 37ºC for 24 hours, and the zone inhibition of the disks was measured. Measurements were performed in triplicate and the average reading was recorded.

2.5 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC of the *C. nutans* extract was determined via broth microdilution method (15). The starting extract concentration of 500 mg/mL was serially diluted using a two-fold method in a 96 well plates until the final concentration of 2.4 mg/ml. Positive and negative control used was gentamicin (30 mg/ml) and DMSO (1%). Next, the standardized bacterial suspension was further diluted to 1:100 and added to each well except for the sterility control wells in each respective plate. The plates were incubated at 37 °C for 24 hours and bacterial growth was assessed by observing turbidity. MIC value is determined as the lowest concentration of an antibacterial agent to inhibit the growth of the tested bacterial strains (18). MBC was determined by pipetting 10 µL of the suspension from the wells that showed no bacterial growth from the MIC plate onto the MHA and was incubated for 24 hr at 37ºC.

The lowest well concentration with no bacterial growth was taken as MBC value.

2.6 Data Analysis

Data were presented as mean ± standard error of mean (SEM). The significant difference in the zone of inhibition data was analysed using Sigma Plot (version 15.0) for Windows. One-way Analysis of Variance (ANOVA) or Kruskal-Wallis with post-hoc Tukey test was conducted and performed at a significance of 95%, which is a $p < 0.05$, to show a significant difference.

3.0 Results

3.1 Extraction yield

Extraction of *C. nutans* using 80% ethanol with a ratio of 1:10 (w/v) have resulted in 7.1% yield as shown in Table 1. When compared with the previous paper by Alim et. al, it was reported that extraction of *C. nutans* with 70% ethanol have given the extraction yield of 11.22% (16). However, the weight of dry plant used in their study was 50 g which was higher compared to our study. Extraction yield can be affected by solvent to sample ratio, where using a higher solvent to solid ratio would increase extraction efficiency. This is because the extraction process will benefit from a high volume of solvent to maintain a steep concentration gradient between the solvent and the sample, allowing the solvent to hold more of the sample before reaching saturation. Additionally, using a higher weight of dry *C. nutans* can increase the surface area that are in contact with the solvent, which further enhances the extraction yield (17).

3.2 Antibacterial evaluation

Disk diffusion method was performed to evaluate the antibacterial properties of the *C. nutans* extract and compare its effectiveness against 4 bacterial types. Figure 1 showed the representative sample of MHA plates showing the zone of inhibition of the extract against tested bacteria in a dose dependent manner. Table 2 presents the mean diameter of zone of inhibition of the plant extracts, together with gentamicin and 10% DMSO. There is no inhibitory activity of the negative control against any of the tested bacteria, while the positive control showed the largest zone of inhibition for all bacterial types. The *C. nutans* extract was able to inhibit all tested bacteria at the highest concentration of 500 mg/mL, where inhibition towards *S. aureus* showed the largest zone of inhibition (11.0 ± 0.6 mm) and the smallest inhibition was observed against E. coli (6.5 ± 0.5 mm). Additionally, *S. aureus, B. subtilis and P. aeruginosa* was also susceptible to the extract at 200 mg/mL which was not observed on *E. coli* plate. Furthermore, the inhibition diameter of the 500mg/ml extract on *S. aureus* and *B. subtilis* is not statistically significant compared to positive control ($p>0.05$), suggesting that the extract is performing on a similar level as the potent antimicrobial agent, highlighting its potential as a natural antimicrobial source. Similar results were obtained from the previous study that showed a larger zone of inhibition diameter of *C. nutans* leaves methanol extract at 100 mg/mL against *S. aureus* (26.7 ± 3.51 mm) than *E. coli* (17.0 ± 2.00 mm). It was also observed that *C. nutans* extracts were more efficient to inhibit the growth of Gram-positive bacteria better than Gram-negative bacteria by both extraction solvents.

Table 1: Extraction yield of *C. nutans* leaves ethanol extract.

Weight of dry plant (g)	Weight of dry extract (g)	Extraction yield (%) *
20.0	1.42 ± 0.08	7.1

*Extraction yield = Weight of dry extract (g) / Weight of dry plant (g) x 100

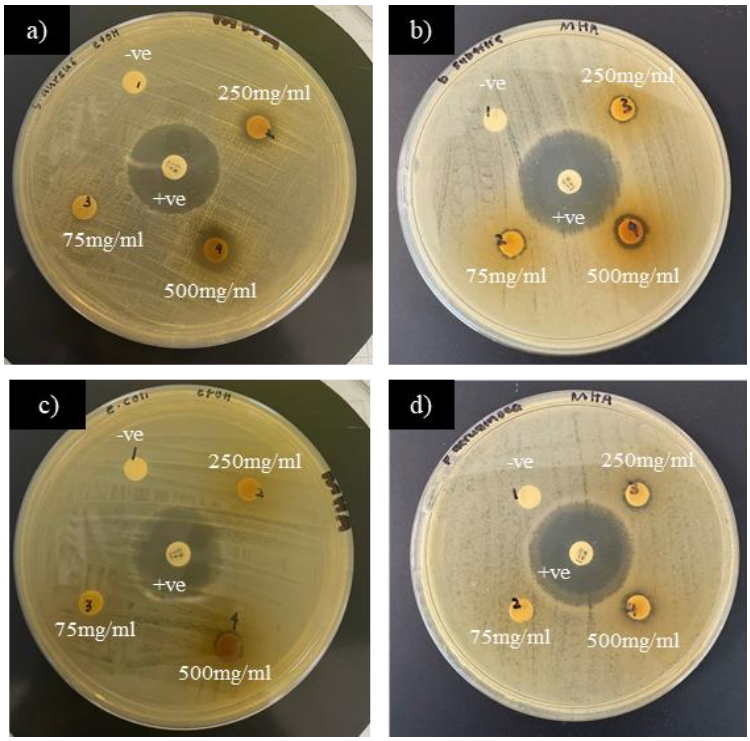

Figure 1: Representative sample agar plates showing the zone of inhibition for *C. nutans* ethanol extract against *S. aureus* (a), *B. subtilis* (b), *E. coli* (c) and *P. aeruginosa* (d).

Table 2: Diameter of zone of inhibition of *C. nutans* leaves ethanol extract against *S. aureus* and *E. coli.*

Bacteria[a]	Diameter of Zone of Inhibition (mm)					
	10% DMSO	Gentamicin (10 µg)	500 mg/mL	250 mg/mL	75 mg/mL	P-value*
S. aureus	N/A	22.3 ± 0.33	11.0 ± 0.58[b]	8.3 ± 0.33	N/A	0.004
B. subtilis	N/A	23.0 ± 0.24	9.1 ± 0.01[b]	7.4 ± 0.03	7.1 ± 0.06[c]	0.015
P. aeruginosa	N/A	24 ± 0.30	7.6 ± 0.41[c]	6.9 ± 0.22[c]	N/A	<0.001
E. coli	N/A	20.0 ± 0.00	6.5 ± 0.50[c]	N/A	N/A	<0.001

NA: no activity, Results were expressed as mean ± standard error of mean (SEM) (n = 3), *Results within groups, [a] Significantly different between all groups ($p < 0.001$), [b] Not significantly different compared to positive control ($p > 0.05$), [c] Significantly different compared to positive control ($p < 0.05$).

3.3 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The disk diffusion results alone cannot be used to estimate the MIC of a sample since it is impossible to quantify the amount of the antibacterial agent diffused into the agar medium. Further testing was performed to determine the bacteriostatic (MIC) and bactericidal (MBC) value of an extract. Bacteriostatic can be defined as the agent that prevents the growth of the tested bacteria strains while bactericidal means it kills the bacteria completely (18).

Results in Table 3 shows that the extract demonstrated bacteriostatic and bactericidal effects against all four tested bacteria, though at much higher concentrations than the positive control, gentamicin. The MIC and MBC values of the extract are the same for *S. aureus* and *B. subtilis*, with 15.63 mg/ml and 31.26 mg/ml respectively, indicating that the concentration required to inhibit growth is the same as the concentration required to kill the bacteria. This is in line with the disk diffusion results, where the antibacterial activity against *S. aureus* required much lower concentration to exert its bacteriostatic effect than the other tested bacteria. Meanwhile, the MIC (62.50 mg/ml) and MBC (250 mg/ml) values of the extract against *P. aeruginosa* and *E. coli* are the same, indicating that a much higher concentration is needed to kill both these bacteria than to inhibit its growth. Previous study using *C. nutans* leaves methanol extract showed MIC value of 12.50 mg/mL against *S. aureus*. (9). Yet in another study, nanoparticles of *C. nutans* extract were able to inhibit the growth of *E. coli* with a MIC value of 125 mg/mL (19).

The MIC results are much lower compared to the findings of Chiangchin *et al*. (2023) (11), who found that the extract from a specific location in Thailand had MIC values of 250 mg/mL for S. aureus and 500 mg/mL for E. coli (11). This suggests that the extract used in this experiment is more potent against these bacterial strains. The variation in the results could be due to several factors, including the geographical location of the plant, different extraction methods, and variations in the plant's phytochemical composition (20). These factors can influence the concentration and types of active compounds present in the final extract.

Overall, the difference results observed between the two bacterial types is probably due to easier penetration of the *C. nutans* active compound into Gram-positive bacteria than Gram-negative bacteria that has an additional outer membrane (6). It only needs a neutral and nonpolar compounds to pass the cytoplasmic membrane of Gram-positive bacteria (19) while to be able to penetrate Gram-negative bacteria, the compound needs to be positively charged as the outer membrane is full of negative charge (20).

According to the previous study, the proposed mechanism of action of antibacterial activity of *C. nutans* leaves includes the presence of flavonoid which is derived from phenolic compounds (21,22). It has the ability to interact with lipid bilayers and disrupt the normal functions of the bacteria's plasma membrane, leading to cell death. In addition, *C. nutans* is renowned for its pharmacological activity as antioxidants, which can induce oxidative stress in bacteria (23). Oxidative stress is defined as the overproduction of reactive oxygen or nitro-

Table 3: MIC and MBC of *C. nutans* leaves ethanol extract.

Bacteria	MIC (mg/mL)		MBC (mg/mL)	
	Gentamicin	***C. nutans***	**Gentamicin**	***C. nutans***
S. aureus	0.00025	15.63	0.00025	15.63
B. subtilis	0.00025	31.26	0.00025	31.26
P. aeruginosa	0.002	62.50	0.002	250
E. coli	0.002	62.50	0.002	250

gen species (ROS/RNS), which can lead to cellular death. Increased levels of reactive oxygen species/reactive nitrogen species in cells will cause disruption of lipid membranes, resulting in increased fluidity and permeability. This additionally leads to malfunctioning of proteins and damage to DNA, finally resulting in cellular damage and apoptosis (11,24). The antioxidant properties can be due to the presence of cinnamic acid, protocatechuic acid, caffeic acid, ferulic acid and chlorogenic acid which were identified from phenolic composition analysis of *C. nutans* extract (24).

4.0 Conclusion

From this study, we found that the ethanol extract from *C. nutans* leaves has showed an effective antibacterial activity, where the inhibitory effect is more potent towards Gram-positive compared to Gram-negative bacteria. This is the first study in Malaysia that reported on the ethanol extract testing on both bacteria simultaneously, and the results have supported the previous findings on antibacterial studies that used other solvents. To further elucidate the specific compounds responsible for this activity, bioassay-guided fractionation is recommended for future studies to isolate and characterize the bioactive constituents. This study can be the starting point for further research on *C. nutans* leaves ethanol extract to be developed as an alternative antibacterial agent within healthcare settings and hence improve the clinical management in bacterial infection.

Authorship contribution statement

RS & QK: Methodology, Experimentation, Data analysis, Writing - original draft. **NSH:** Writing - editing. **NAL:** Resources, Methodology. **MM:** Supervision, Funding acquisition, Writing - review & editing.

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Conflict of Interest

The authors declared that they have no conflicts of interest to disclose.

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