Antibacterial Properties of *Clinacanthus nutans* Extracts Against *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans:* An *In-Vitro* Study

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Abstract

The aim of this study is to futher investigate and validate the antibacterial effect of *Clinacanthus nutans* plant extract against periodontal pathogens namely *Porphyromonas gingivalis* and *Aggregatibacter actinomycetamcomitans.*

Four samples of alcoholic extract of *C. nutans* leaves were used in different concentrations i.e. 100%, 50%, 10% ethanol and 100% chlorofom and Chlorhexidine 0.2% (CHX) was used as the positive control. The antibacterial activity of *C.nutans* extract were investigated using disc diffusion agar test for determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

In this study, 50% ethanol extract and 100% chlorofom extract were found to have antibacterial activity against *P.gingivalis*, while only 50% ethanol crude extract was found to have acceptable antibacterial activity against *A. actinomycetamcomitans* ($p<0.05$). The MIC and MBC tests showed that 50% ethanol extract had bacteriostatic activity against both *P.gingivalis* and *A. actinomycetamcomitans* while 100% chlorofom extract had bactericidal activity against *P. gingivalis*. These two findings were also found to be better than the activity of CHX.

C. nutans extract was found to have notable antibacterial activity against *P. gingivalis* and *A. actinomycetamcomitans* comparable to CHX 0.2%.

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Introduction

Clinacanthus nutans (C. nutans) is a small shrub that belongs to the family of *Acanthaceae.* It is well-known to the South East Asian countries particularly in Malaysia, Thailand and Indonesia[1]. The plant is found to be widely grown and distributed throughout the tropical regions as in South East Asia and China and has been used as an important medicinal herb[2]. In Malaysia, the plant is known as Sabah Snake Grass or Belalai Gajah[3], due to the slightly curved stem supporting the leaves which resembles the elephant's trunk[2]. The fresh leaves of *C. nutans* is consumed traditionally in Thailand as a cure for stings by snakes, mosquitos, millipedes, catfish, centipedes, hornets, jellyfish, ants, bees, scorpions and wasps[1]. In Indonesia, the plant is used to treat diabetes and dysentery[4]. Meanwhile in China, it is using to control menstrual function, relieve pain, anemia, jaundice and set fractured bones.

C. nutans plant has been found to have anti-venom, anti-inflammatory, analgesic, anti-diabetic, anti-rheumatism, anti-viral, and antioxidant properties [2]. The methanolic extract of *C. nutans* leaf was shown to possess antibacterial effect against *Staphylococcus aureus, Escherichia coli, Propionibacterium acnes, Staphylococcus epidermidis* and *Bacillus cereus* at a minimal concentration of 12.5 mg/mL[5]. The plant also provides various beneficial effect on oral pathological condition such as preventing and relieving radiation-induced oral mucositis in

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head and neck cancer patients, treatment of recurrent aphthous stomatitis by providing better healing compared to placebo and showed no toxicity effect on human gingival fibroblast cell line[6,7&8]. However, there was no study found regarding antibacterial effects of *C. nutans* against periodontal pathogens infection.

The prevalence of periodontal disease in Malaysia is high. Almost half of adult population i.e. about 11.5 million people suffer from either moderate or severe periodontitis [9]. It is known that CHX as commercial mouthwash used as chemical adjunctive therapy in periodontal treatment have various local side effects as including discoloration of teeth, altered taste sensation, and mucosal erosion. Due to this, researchers are now focusing to find natural products with antibacterial properties like *C. nutans* extracts, as an alternative to commercial drugs. Therefore, this study has been designed to determined antimicrobial activity of *C. nutans* extracts as compared to CHX against *P. gingivalis* and *A. actinomycetemcomitans.*

Materials and methods

Collection and preparation of plant extracts

The leaves of *C. nutans* extracts were taken from the Integrative Medicine Cluster, Advanced Medical and Dental Institute, USM Pulau Pinang.The leaves of *C. nutans* were dried at room temperature in a ventilated room, milled to a fine powder in a grinder (Model C14, Kesmac Sdn. Bhd., Malaysia) and stored in closed containers. The leaves (800 g) were extracted with 1 L ethanol (100%, 50% and 10%) and 100% chloroform. The mixture was vigorously shaken for 2 h on an orbital shaker (LM-530RD, Taiwan) and the extract was filtered using Whatman No. 1 filter paper. The collected solvent filtrate was evaporated using a rotary evaporator (Laborota 4000, Germany). The stock solution concentration of *C. nutans* extract was 100 mg/mL in 10% dimethyl sulfoxide (DMSO).

Bacteria culture

Both periodontal bacteria (*P. gingivalis and A. actinomycetemcomitans*) were taken from level 18, Laboratory of Microbiology Bank, Faculty of Dentistry, USIM. All the bacteria were cultured in blood agar at 37 °C under anaerobic condition**.** Glycerol stock of the bacteria was kept at −80 °C.

Disk diffusion assay

Bacteria were cultured on blood agar and incubated under 37°C at anaerobic environment for 24 h. Approximately 1 X 10^8 CFU/mL of bacteria densities corresponding to a 0.5 McFarland turbidity standard was used to inoculate bacteria into fresh Trypson soy broth and further incubated at 37°C overnight. A sterile cotton swab was used to spread the culture on blood agar before application of 5 x 6mm paper discs (Whatmann No. 1) impregnated with 20 µL *C. nutans* at concentration between 10-30 mg/mL. DMSO 10% was used as negative control and chlorhexidine 0.12% was used as positive control. Zones of inhibition was measured from the circumference of the disks to the circumference of the inhibition zone after incubating under 37°C for 24 h at anaerobic state.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was performed using the two-fold serial dilution method described by Alwash et al., (2013) [10]. MIC was done in a sterile 96-well plate. Test bacteria (100 µL) at 10^8 CFU/mL was added to various concentrations of *C. nutans* extract (1-100 mg/mL) diluted in fresh Trypson soy broth to a final volume of 200 µL/well. DMSO 10% (v/v) was used as negative control and CHX 0.12% was used as positive control. Following incubation at 37 °C under anaerobic condition for 24 h, MIC value was determined as the lowest concentration that inhibits the visible growth of bacteria. Minimum bactericidal concentration (MBC) was determined by culturing a 5 µL aliquot from wells that exhibited no bacterial growth in MIC wells onto sterile blood agar and incubated overnight at 37 °C and the MBC was defined as the lowest concentration preventing bacterial growth.

Statistical Analysis

The data was collected and analyzed using two-way ANOVA test where the p value is less than 0.05 ($p<0.05$).

Results

Table 1 shows results from the disc diffusion agar method. which the zone of inhibition observed when *C.nutans* extracts are treated against *P.gingivalis*.

C. nutans extract	Concentration (mg/mL)				DMSO 10%	CHX 0.2%
	12.5	25	50	100		
ETOH (100%)	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	9.80±1.57
ETOH (50%)	12.00±0.00*	13.33±1.15*	15.33±5.77*	17.33±2.32*	6.00±0.00	9.80±1.57
ETOH (10%)	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	9.80±1.57
$ChCl_3$	7.00±0.00*	7.00±0.00*	7.00±0.00*	8.00±0.00*	6.00±0.00	9.80±1.57
H_20	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	9.80±1.57

Table 1. The zone inhibition of *C. nutans* extracts against *P.gingivalis*. Values are represented as mean ±SD. Values on the same row followed by * asterisk, differ significantly (p<0.05,). Groups: positive control (CHX 0.2%), negative control (DMSO 10%), 12.5mg/mL, 25mg/mL, 50mg/mL, 100mg/mL of *C. nutans* extracts (100% EtOH, 50% EtOH, 10% EtOH, ChCl3 and H20).

The data are collected in millimeter measurement. Only 50% ethanol extract and 100% chloroform extract of C.nutans showed antibacterial activity against *P.gingivalis*. The 100% chloroform extract of *C.nutans* also has zone of inhibition slightly higher than other *C. nutans* extract but less than 50% Ethanol extract. Then, both of the *C. nutans* extracts with positive results against *P.gingivalis* which are 50% ethanol and 100% chloroform are used to find the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) values, where 50% ethanol of *C.nutans* extract is bacteriostatic against *P.gingivalis* while 100% chloroform of *C. nutans* extract is bactericidal against *P.gingivalis* (Table 2).

***C. nutans* extract**	**MIC (mg/mL)**	**MBC (mg/mL)**
Etoh (50%)	12.5±0.2	>100 (Bacteriostatic)
$ChCl_3$	12.4±0.1	12.5 (Bactericidal)

Table 2. The MIC and MBC values for 50% EtOH and ChCl3 extracts against *P.gingivalis*. Values are represented as mean ±SD.

Similar method was used in determining the antibacterial activity of different sample of *C.nutans* extracts against *A. actinomycetemcomitans* using disc diffusion agar, minimum inhibitory concentrations (MIC), and minimum bactericidal concentrations (MBC) antibacterial susceptibility tests. Table 3 shows results of disc diffusion agar tested against *A. actinomycetemcomitans* where only 50% ethanol of *C. nutans* extract shows antibacterial activity by presence of zone of inhibition. The other sample of *C. nutans* extracts have no antibacterial activity against *A. actinomycetemcomitans*. 50% ethanol of *C. nutans* extracts was also found to be a bacteriostatic agent against *A. actinomycetemcomitans* based on its MIC and MBC result in Table 4.

C. nutans extract	Concentration (mg/mL)				DMSO 10%	CHX 0.2%
	12.5	25	50	100		
ETOH (100%)	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	8.06±0.80
ETOH (50%)	17.00±1.00*	15.33±1.53*	14.00±2.00*	12.00±0.00*	6.00±0.00	8.06±0.80
ETOH (10%)	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	8.06±0.80
$ChCl_3$	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	8.06±0.80
H_20	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	8.06±0.80

Table 3. The zone of inhibition of *C. nutans* extracts against *A. actinomycetemcomitans* (mm). Values are represented as mean ±SD. Values on the same row followed by * asterisk, differ significantly (p<0.05,). Groups: positive control (CHX 0.2%), negative control (DMSO 10%), 12.5mg/mL, 25mg/mL, 50mg/mL, 100mg/mL of *C. nutans* extracts (100% EtOH, 50% EtOH, 10% EtOH, $ChCl_3$ and H_20).

***C. nutans* extract**	**MIC (mg/mL)**	**MBC (mg/mL)**
Etoh (50%)	6.26±0.05	>100 (Bacteriostatic)

Table 4. The MIC and MBC values for 50% EtOH extract against *A. actinomycetemcomitans.* Value are represented as mean ±SD.

Discussion

In this study, the stem leaf of *C. nutans* were extracted using solvents of differing polarities and we found that the extract produced using 50% ethanol and chloroform as solvent exhibited good antibacterial activity compared to

other extracts using different solvents. Most of the extracts use ethanol solvent which exhibit antibacterial activity of the plant. This corresponds with our method of study that uses ethanolic solvent to study about antibacterial activity of *C. nutans* leaves extract. MIC determination is important in diagnostic laboratories in order to determine the activity of new antimicrobial agents and also can be used to confirm resistance of microorganisms to an antimicrobial agent. Palombo (2011) stated that a garlic extract was active toward gram-negative pathogens including *P. gingivalis* with MIC = 1.1–17.4 mg/mL[11]. *C. nutans* extract in present study has MIC result within the range showing similar antimicrobial activity. Another medicinal plant, *Sagittaria sagittifolia (Alismaceae)* exhibited antibacterial activity against *S. mutans* and *Actinomyces naeslundii* with MIC values of between 62.5 and 125 µg/mL, but no activity against *A. actinomycetemcomitans* [11].

This study is the first to report on *C. nutans* activity against periodontal pathogens specifically *P. gingivalis* and *A. actinomycetemcomitans*. The antibacterial agents were usually regarded as having bactericidal activity if the value of MBC was no more than four times the value of MIC[12]. The MBC results showed that the chloroform type of *C. nutans* extract (12.5 mg/mL) had bactericidal activity against *P. gingivalis* while 50% ethanol of *C. nutans* extract (more than 1000 mg/mL) had bacteriostatic activity against both *P. gingivalis* and *A. actinomycetemcomitans*. MBC value can be subject to technical variation and several theoretical limitations. In the study, Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentration (MBC) test results both proposed that 50% ethanol extracts of *C. nutans* plant is a bacteriostatic agent against both *P. gingivalis* and *A. actinomycetemcomitans*, while chloroform extracts of *C. nutans* plant is a bactericidal agent against *P. gingivalis*. As conclusion, *C. nutans* extracts was found to have potential as antibacterial agent against *P. gingivalis* and *A. actinomycetemcomitans.*

Conclusions

C. nutans extract 50% Etoh was found to have notable antibacterial activity against *P. gingivalis* and *A. actinomycetamcomitans* comparable to CHX 0.2% . Meanwhile, *C. nutans* extract $ChCl_3$ was found to have notable antibacterial activity against *P. gingivalis* only.

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Declaration of Interest

The authors report no conflict of interest

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