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Original Research Article

In Vitro Screening of Cytotoxic, Antimicrobial and Antioxidant Activities of *Clinacanthus nutans* (Acanthaceae) leaf extracts

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Abstract

Purpose: *To evaluate the in vitro cytotoxic, antioxidant and antimicrobial activities of Clinacanthus nutans extracts and semi-fractions.*
Method: *The plant was subjected to cold solvent extraction to produce petroleum ether, ethyl acetate and methanol crude extracts, followed by isolation using bioassay-guided fractionation. The crude extracts (0.2 to 10.0 mg/ml) were tested against HeLa and K-562 cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and antioxidant activity using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay. Furthermore, fractions isolated from ethyl acetate leaf extract (0.02 to 10.0 mg/ml) were tested against Bacillus cereus (ATCC11778), Escherichia coli (ATCC25922), Salmonella enterica Typhimurium (ATCC14028) and Candida albicans (ATCC10231) using minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal (MBC/MFC) assays.*
Results: *Petroleum ether extracts demonstrated the strongest cytotoxic activity against HeLa and K-562 cells with IC50 of 18.0 and 20.0 µg/mL, respectively. Petroleum ether extracts also displayed the highest radical scavenging activity of 82.00 ± 0.02 %, compared with ascorbic acid and α-tocopherol corresponding values of 88.7 ± 0.0 and 86.6 ± 0.0 %, respectively. In MIC assay, all the crude extracts and fractions showed inhibition against all tested microorganisms. Fraction 7 displayed the lowest MIC and MBC/MFC values against B. cereus and C. albicans at 1.39 mg/ml, respectively. Ampicillin and amphotericin B displayed MIC value of 1.3 mg/ml.*
Conclusion: *The bioactive compounds from C. nutans are potential cytotoxic, antimicrobial and antioxidant agents.*

Keywords: *Clinacanthus nutans, Cytotoxic, Antioxidant, Antimicrobial*

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INTRODUCTION

Clinacanthus nutans, belonging to the family of Acanthaceae, is a well-known Thai medicinal plant [1]. This plant is a small shrub that can be found throughout South East Asia, primarily indigenous to Thailand, Indonesia and Malaysia [2]. It has been used traditionally as antivenom, anti-inflammatory, analgesic, antidiabetic, antirheumatism, antiviral and antioxidant [3-5]. The English name of this plant is Sabah snake grass because this plant is well-known and can be found easily in East Malaysia, Sabah [6]. However, in Thailand the plant is denoted as Saled pangpon tua mea (saliva of female

mongoose), Phaya-Yor, Phak Man Kai, Phak Lin Khiat [7].

In Thailand, scientists found that dysentery and fever can be treated by this plant. Due to its anti-cell lysis property, the plant has been used as anti-venom for snake and scorpion bites and also removes nettle rashes [3]. In China, this plant is used to treat inflammation such as hematoma, bruises on eye, anxieties, injuries and rheumatism [5]. This plant's natural minerals help in adapting normal menstrual function, relieving pain, anaemia, repairing of fractured bones and jaundice. In Indonesia, Sabah snake grass is used to treat diabetic patients, dysuria and dysentery by consuming the decoction from fresh leaves boiled with hot water [3].

Malaysia and other Asian countries broadly use this plant to treat uric acid, gout, urinates neuropathies, liver cancer, kidney syndrome, nasal cavity cancer and uterine fibroid. This plant has been endorsed for treatment of herpes simplex, herpes zoster and skin psoriasis in the Primary Health Care Programme [8]. Anti-inflammatory is another property of this plant which relieves major skin inflammation, skin rashes and insects bites [9]. Apart from these reports and preliminary studies, Malaysian species of *Clinacanthus nutans* may give potential lead compounds in drug discovery. Thus, this study is conducted to evaluate the biological properties of this plant.

EXPERIMENTAL

Collection of plant material

The whole plant of *Clinacanthus nutans* were collected in January 2012 from Taiping, Malaysia. The plant was authenticated by Prof Ong, Institute of Biological Sciences, Faculty of Science, University of Malaya. A voucher specimen (12012) was kept at the Biomedical Science Laboratory, Universiti Tunku Abdul Rahman, Malaysia. The leaves and stems were separated, dried in the oven (40 ºC), powdered to a coarse consistency and stored at -20 ºC.

Plant extraction

The powdered leaves and stem barks were soaked separately in petroleum ether, ethyl acetate and methanol at room temperature for one week with occasional shaking. The extracts were filtered and evaporated using a rotary evaporator. The extraction process was repeated three times using the obtained recovery solvents. A gummy filtrate was obtained and dried in the oven at 40 °C.

Thin layer chromatography (TLC)

Pre-coated TLC plates were prepared by drawing the baseline and solvent front on the plate. A thin capillary tube was dipped into the sample solution and was spotted onto the baseline. The plate was then put into the developing chamber saturated with non-polar and polar solvents. The solvents used were n-hexane:ethyl acetate (70:30). The spots developed were visualised under ultra-violet lamp with both short and long wavelengths 254 and 365 nm, respectively.

Gravity column chromatography

The ethyl acetate extract of leaves were subjected to column chromatography using silica gel 60 which was packed in a glass column. The powdered ethyl acetate crude extracts were added and layered onto the top of the column. The column was eluted with n-hexane:ethyl acetate (70:30) and the polarity was increased by a stepwise gradient solvent system using ethyl acetate and then followed by methanol. Fractions were analysed using TLC. Fractions with similar profiles were pooled together and tested for antimicrobial assays. The dried fractions were kept at -20 °C.

Cell lines culture

HeLa and K-562 cell lines were cultured in RPMI1640 and DMEM complete medium, respectively, supplemented with 10 % foetal bovine serum (FBS) and 100 units/ml Penicillin-streptomycin. Both cells were maintained in a humidified atmosphere at 37 °C in a 5 % carbon dioxide (CO_2) incubator. The cell growth was observed daily to check for confluency. Subculture was performed once the cells reached 80 to 90 % confluency.

Microbial culture

The bacterial and fungal suspensions were streaked on MHA and SDA, respectively and the plates were incubated at 37 ˚C for 24 h for bacteria and 30˚C for 48 h for fungal. Few colonies of pure culture were suspended in 40 ml of fresh MHB or SDB. For bacterial culture, the inoculum was incubated in shaking incubator at 37 ˚C until it reached 0.5 McFarland standards which correspond to 1×10^8 CFU/ml, with absorbance of 0.08 to 0.10 at 625 nm [10]. Meanwhile, the fungal suspension was adjusted to achieve 0.5 McFarland standards with the

absorbance of 0.12 to 0.15 at 530 nm corresponding to 1×10^6 CFU/ml [11].

MTT assay

HeLa and K-562 cells were seeded into 96- well plates at 1×10^3 to 1×10^5 cells per well. Extracts at varying concentration, 1 % DMSO (negative control) and cisplatin (positive control) were added into respective wells. The treated plates were incubated for 24 and 72 h at 37 ºC in CO_2 incubator. After incubation, 50 µl of MTT reagent (5 mg/mL) was added to each treated well and incubated for 4 h at 37 °C in CO_2 incubator. MTT solution was then discarded and 100 µl of DMSO was added. The plates were gently rotated on orbital shaker to solubilise the formed purple crystal formazan [12]. The absorbance was measured using a microplate reader at a wavelength of 550 nm. The results were used to construct a graph of percentage cell viability against concentration of extracts. Cell viability (CV) was calculated using Eq 1 [13].

CV (%) = 100 - {(At-Ab)/(Ac-Ab)} x 100 .....(1)

where At is the absorbance of test sample, Ab is the absorbance of blank and Ac is the absorbance of negative control. The 50 % growth inhibition concentrations (IC_{50}) of the extracts were estimated from the graphical interpolation.

MIC, MBC and MFC assays

Microdilution method was used in MIC assay with some modification using sterile round bottom 96-well plates. The fresh suspension (bacteria or fungus) were cultured to match the turbidity of 0.5 McFarland standards (1×10^8 CFU/ml for bacteria and 1×10^6 CFU/ml for fungus). Initially, the wells were filled with 50 µl stock fractions of 10 mg/ml. These fractions were serially diluted and then 50 µl of inoculums were added into the fractions well. The final concentrations of fractions were ranging from 0.08 mg/ml to 5 mg/ml. Positive controls used were ampicillin for antibacterial assay and amphotericin B for antifungal assay. Meanwhile, negative control was 1 % DMSO, growth control was 100 µl of inoculums only and sterility control was 100 µl of fresh broth without inoculum.

The plates were incubated for 24 h at 37 ˚C for bacteria and 48 h at 30 ˚C for fungus. After incubation, the absorbance was measured using microplate reader at wavelength of 625 nm (bacteria) and 530 nm (fungus), respectively. Then, 50 µl of 0.4 mg/ml INT dye was added into each well aseptically in a dark laminar flow. The plates were gently agitated and incubated for another 30 min. The colour changes was observed and recorded. The MIC value was determined by observing the absence of colour change (remains yellow) with the lowest concentration of extract that showed inhibition. The colour changes from yellow to pink indicate the presence of bacterial or fungal growth. The lowest concentrations of the inoculated MIC wells which showed inhibition was recorded. MBC or MFC values are determined by observing the lowest concentration of the extracts that showed no or less cell growth on the agar surface.

DPPH assay

About 4 ml of different extracts concentration, methanol, ascorbic acid and α- tocopherol were added to 1 ml of DPPH in test tubes. These test tubes were incubated at room temperature in the dark for 30 min at room temperature. After incubation, the absorbance was measured at 517 nm and the results were recorded. A graph of percentage of radical scavenging activity versus concentration was plotted and IC50 values were determined for each extract. The DPPH radical scavenging activity (D) was calculated using Eq 2 [14].

D (%) = (A0 – A1 / A0) × 100..... (2)

where A0 is the absorbance of the control and A1 is the absorbance of extracts or standard sample.

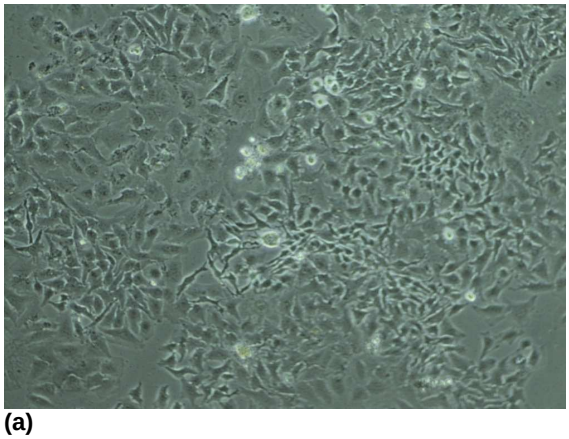
Data analysis

Cytotoxic, DPPH and MIC data were expressed as mean ± standard deviation (SD, n = 3) using SPSS (version 11).

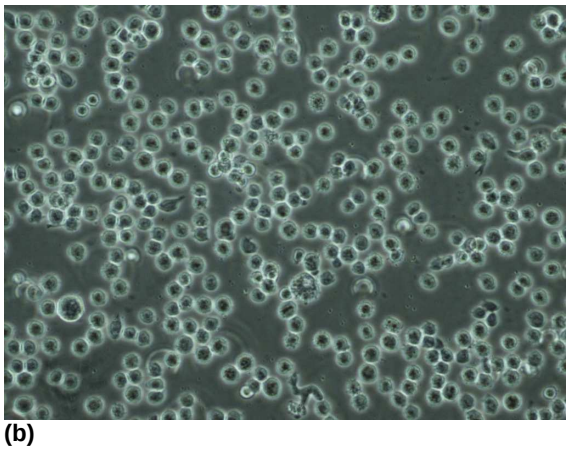
RESULTS

Cytotoxic assay

Healthy HeLa and K-562 cells that achieved confluency were used in this assay. HeLa cells exhibited epithelial-like structure and adherent to the bottom of the flask as shown in Fig 1 (a), meanwhile K-562 are non-adherent and rounded cells as Fig 1 (b). The IC_{50} values of leaves and stem extracts were summarised in Table 1. The percentage cell viability of HeLa cells were fluctuating as the extracts concentration increases. Petroleum ether leaves extract exhibited higher cytotoxicity on HeLa cells after 72 h incubation. The proliferation of K-562 cells was reduced by petroleum ether leaves extract at concentration of 20.0 µg/ml with an IC_{50} of 20.0 µg/ml after 72 h incubation.



(a)



(b)

Fig 1: (a) Morphology of HeLa cells cultured in RPMI 1640 (100x); **(b)** Morphology of K-562 cells cultured in DMEM (200x)

Antimicrobial assay

In MIC and MBC or MFC assays, F7 displayed the strongest antimicrobial activity against *B. cereus*, followed by *C. albicans* at 1.39 mg/ml. Meanwhile, the crude extracts showed the lowest MIC and MBC/MFC values of 6.31 mg/ml against *B. cereus* and *C. albicans*. Ampicillin and amphotericin B showed MIC and MBC or MFC values at 0.06 mg/ml (Table 2). The INT dye used changes from yellow to pink in the presence of microbial growth and remains yellow if there is inhibition. As the concentration of fraction increases, the growth of cells decreases and the INT remains yellow (Fig 2).

Antioxidant assay

Petroleum ether leaves extract showed the highest radical scavenging activity of 82.0 % at concentration of 4.0 mg/ml, while methanol stem extracts displayed percentage of radical scavenging activity of 70.0 % at 10.0 mg/ml (Table 3). Meanwhile, the percentage of radical scavenging activity of ascorbic acid and α-tocopherol was 88.7 % and 86.6 % respectively at 0.05 mg/ml (Table 4).

Table 4: Percentage radical scavenging activity of α-tocopherol and ascorbic acid

Concentration (mg/ml)	Radical scavenging activity (%)	
	Ascorbic acid	α-tocopherol
0.02	57.6±0.010	25.3±0.013
0.03	87.5±0.016	38.3±0.004
0.06	88.0±0.004	78.3±0.011
0.13	88.8±0.005	80.8±0.016
0.25	88.5±0.007	85.5±0.103
0.50	88.7±0.004	86.6±0.005

**Results are expressed as mean ± standard deviation (SD) (n=3)*

Table 1: IC_{50} values of *Clinacanthus nutans* crude extracts against HeLa and K-562 cell lines at 24 and 72 h incubation

Cell line	Sample	Extracts	IC_{50} values (µg/ml)	
			24 h	72 h
Hela	Leaves	Petroleum ether	20.0	**18.0**
		Ethyl acetate	50.0	92.0
		Methanol	74.0	66.0
	Stem	Petroleum ether	28.0	>100
		Ethyl acetate	18.0	20.0
		Methanol	96.0	56.0
		Cisplatin	43.5	>100
K-562	Leaves	Petroleum ether	80.0	**20.0**
		Ethyl acetate	>100	>100
		Methanol	>100	>100
	Stem	Petroleum ether	>100	>100
		Ethyl acetate	>100	>100
		Methanol	>100	>100
		Cisplatin	>100	>100

**IC_{50} >100 µg/ml indicating possible cytotoxic effect using higher concentration of crude extracts*

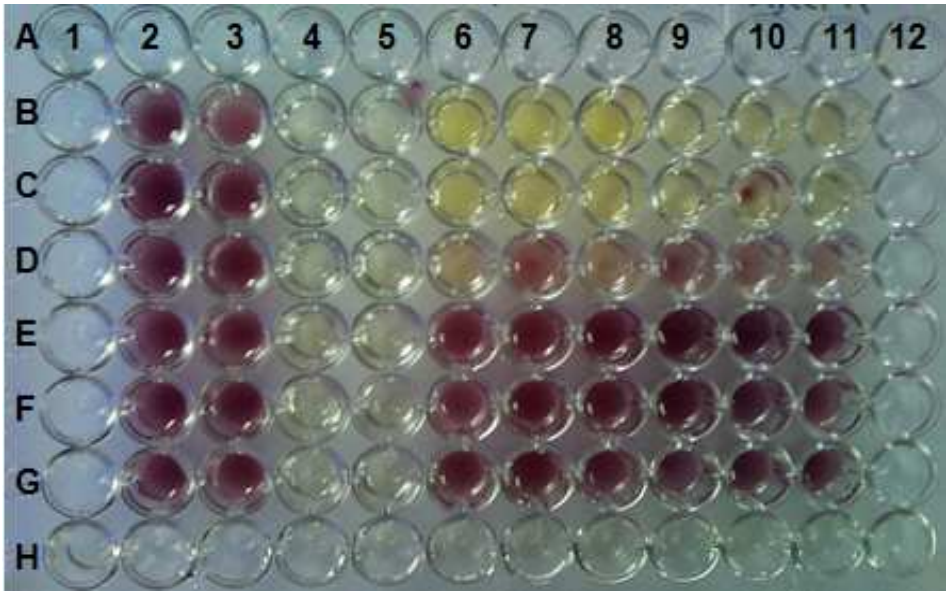


Fig 2: The MIC determination against *Bacillus cereus* using fraction 7(column 6 to 11), negative control (column 2 to 3) and positive control (column 4 to 5) in which yellow colour showed inhibition

Table 2: MIC and MBC/MFC values of *Clinacanthus nutans* crude extracts, fractions and positive controls

Samples	**Minimum inhibitory concentration (MIC) (mg/ml)**			
	Bacillus cereus	***Escherichia coli***	***Salmonella enterica* Typhimurium**	***Candida albicans***
Crude extracts	6.31	>100	>100	6.31
F1	1.44	1.44	1.44	1.44
F2	1.73	1.73	1.73	1.73
F3	1.91	1.91	1.91	1.91
F4	1.93	1.93	1.93	1.93
F5	1.72	1.72	1.72	1.72
F6	1.51	1.51	1.51	1.51
F7	**1.39**	**1.39**	**1.39**	**1.39**
Ampicillin	0.06	0.06	0.06	-
Amphotericin B	-	-	-	0.06

**MIC value of >100 mg/ml indicating possible antibacterial activity using higher concentration of crude extracts.*
** - Not applicable*

Table 3: Radical scavenging activity (%) of leaves and stem extracts of *Clinacanthus nutans* at various concentrations

Part	**Extracts**	**Radical scavenging activity of *Clinacanthus nutans* extracts (%)**				
		2 mg/ml	**4 mg/ml**	**6 mg/ml**	**8 mg/ml**	**10 mg/ml**
Leaves	Petroleum ether	69±0.004	**82±0.016**	75±0.006	70±0.014	66±0.024
	Ethyl acetate	50±0.088	65±0.061	71±0.020	71±0.006	71 ±0.001
	Methanol	37±0.023	65±0.049	68±0.013	70±0.013	76±0.0 23
Stem	Petroleum ether	9±0.001	13±0.037	23±0.054	33±0.139	52±0.047
	Ethyl acetate	27±0.021	44±0.102	51±0.041	57±0.015	60 ±0.001
	Methanol	32±0.028	48±0.126	64±0.030	67±0.018	70±0.0 19

**Results are expressed as mean ± standard deviation (SD) (n=3)*

DISCUSSION

Based on the previous investigations, the essential metabolites found in *Clinacanthus nutans* were flavonoids, stigmasterol, B-sitosterol, lupeol, botulin, C-glycosyl flavones, vitexin, isovitexin, shaftoside, isomollupentin, 7-gluco pyranoside, orientin, isoorientin and sulfur-containing glucosides [15]. These chemical constituents have effective medicinal values with

limited side effects, less toxic and enhance health for a longer period.

According to a study, the antioxidant and antiproliferative effects were exhibited by chloroform, methanol and water extracts of *Clinacanthus nutans* using DPPH and galvinoxyl radical scavenging activity assays [16]. In this study, petroleum ether extracts, that is a non-polar solvent, actively exhibited stronger biological activities against test samples. Non-polar solvents have both sides charged, lack of an O-H end and are able extract out compounds such as alkaloids, terpenoids, coumarins [17,18].

Petroleum ether extract from leaves showed the greatest cytotoxic effect compared to ethyl acetate and methanol extracts. Possibly, the activity might be due to its various phytochemical constituents and other types of antitumour compounds which are present in the petroleum ether extracts. Interaction that occurs among different classes of chemical compounds contributes to changes in protein-protein interaction, for example, in phytosterols [19]. Phytosterols are soluble in polar and dipolar solvents while phytostanols, a derivative of phytosterols that are present in non-polar solvent extraction like petroleum ether. In this plant, some of the phytosterols present are stigmasterol, lupeol and β-sitosterol. Phytosterols in combination with other bioactive compounds are able to exert cytotoxic effect against cancer cell lines [20], particularly HeLa and K-562 cells in this research. Another mechanism that may responsible for the cytotoxic effect could be the synergistic effect. Due to the immense amount of bioactive compounds present in the extracts, the synergistic effect could have taken place leading to the cytotoxic activity and therefore reduced cell viability of HeLa and K-562 cells.

In DPPH assay, the results obtained were higher compared to the previous study by Pannangpetch *et al* [21]. The possible reason may due to different geographical area that influences the amount of phyto-constituents in the plant [22]. The presence of alkaloids, flavonoids and flavones [23] may also a possible reason for higher radical scavenging activity. This action generally proceeds via hydrogen atom transfer or electron donation. These chemical constituents also may eliminate toxic metals produced by reactive species through metal chelation and interaction with other antioxidants (cooperative actions), localisation and mobility of the antioxidant at the microenvironment [24].

Fraction 7 demonstrated antimicrobial activity against the test microorganisms with the lowest inhibition values. This could be due to the presence of flavonoids that may have a bacteriostatic (inhibitory) or bactericidal (killing) effect on microorganisms [25]. Both extracts and fractions were more potent to Gram-positive bacteria (*Bacillus cereus*) than the Gram-negative strains (*Escherichia coli* and *Salmonella enterica* Typhimurium). *B. cereus* has a peptidoglycan layer which enables the entry of foreign substances and hence there is no barrier to stop the penetration of foreign materials. Meanwhile, Gram-negative bacteria have lipopolysaccharide (endotoxin) which serves as a protective barrier for the cells from complement-mediated lysis or the action of lysozyme from wide range of antibiotics. These components make the bacteria less susceptible to the plant extracts [26] and therefore, Gram-negative bacteria are less susceptible towards inhibition.

According to Rathee *et al*, flavonoids are phenolic compounds that are synthesised universally in medicinal plants that can induce antibacterial response due to the presence of carbonyl group [27]. These carbonyl groups act by blocking important enzymes such as aldose reductase, xanthine oxidase, phosphodiesterase and ATPase that inhibit the pathogen surveillance. So, maybe the presence of flavonoids in this plant could inhibit the microorganisms. Further studies are on-going to elucidate the structure of the bioactive constituents and to confirm these compounds for biological activities.

CONCLUSION

The bioactive compounds of Clinacanthus nutans contain potential cytotoxic, antioxidant and antimicrobial agents. Further studies are currently in progress to elucidate the chemical structures of the purified bioactive compounds.

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