

Short Communication

CYTOTOXICITY OF *CLINACANTHUS NUTANS* EXTRACTS ON HUMAN HEPATOMA (HepG2) CELL LINE

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ABSTRACT

Objective: *Clinacanthus nutans* is one of the herbs that has been used in Asia as a traditional medicine for the treatment of serious diseases. The aim of this study is to investigate the phytochemical constituents (flavonoids and phenolics) and cytotoxicity against human hepatoma (HepG2) cancer cell lines of *C. nutans* extracts.

Methods: The fractions from *C. nutans* were extracted from hexane, methanol, chloroform and ethyl acetate by the solvent-solvent extraction method. The crude extracts (10 mg/ml) were tested against HepG2 cell lines using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Phytochemical screening was done to determine the total phenolic content (TPC) and total flavonoid content (TFC).

Results: Methanol extract showed the strongest cytotoxic activity against HepG2 cell line with IC₅₀ of 43.9367 µg/ml after 24 h of treatment compared to chloroform extract and ethyl acetate, 55.6112 µg/ml and 62.0655 µg/ml, respectively. Hexane extract formed the lowest cytotoxicity activity with IC₅₀ of 68.3807 µg/ml. Total phenolic content (TPC) was found to be highest in chloroform, which was 119.29 mg of gallic acid equivalent (GAE) and total flavonoid content (TFC), methanol was performed the highest value, which is 937.67 mg of butylated hydroxytoluene (BHT).

Conclusion: Different active compounds present in the extracts may contribute different cytotoxicity effects of crude extracts. The relationship data of total phenolic, total flavonoid, and cytotoxic potential of *C. nutans*, indicates that these plants might contain valuable active compounds as a chemotherapeutic agent. Further investigations to elucidate the chemical structures of active compounds are necessary for potential compounds discovery in drugs.

Keywords: *Clinacanthus nutans*, Cytotoxicity, Human hepatoma (HepG2) cell line

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INTRODUCTION

Cancer is the most distressing and life-threatening disease that enforces severe death worldwide [1]. Moreover, Cancer remains as one of the major health threats to Malaysians with a yearly mortality rate of cancer patients that has consistently reached 10-11% [2]. Current treatments including radiotherapy and chemotherapy are mostly ineffective against advanced stages of cancer and associated with severe side effects. [1] In addition, these treatments are not selective of cancerous cells and their therapeutic efficiency is limited due to the damage they can cause to healthy cells and tissues. To avoid these side effects in cancer therapy, there is an urgent need to develop therapeutic modalities with no or minimal side effects to normal organs. Use of plants for therapy is not new; indeed plants have been considered a valuable source of bioactive compounds for the treatment of many conditions, including cancer, in almost all cultures and communities for thousands of years [3].

Nowadays, there is a lot of research being done on the importance of medicinal plants for healing process compared to the existence of medicinal synthesise from the chemical. Herbs are one of in the types of plants, and some herbs have potential to cure serious diseases. In Indonesia, where herbal medicine is popular, more than 1300 species are known as medicinal plants, called Jamu [4]. These traditional herbal medicines still play an important role in the treatment of illnesses. *C. nutans* is one of the herbs that has been used in Thailand as a traditional medicine for the treatment of serious diseases [5]. *C. nutans* belongs to the family of Acanthaceae, which are known to have medicinal properties. This plant can be found in Malaysia, Thailand and Vietnam [6].

Previous phytochemical studies from *C. nutans* led to the isolation of flavonoids, steroids, triterpenoids, cerebrosides, glycol glycerolipids, glycerides, sulfur-containing glyceride from this plant [5, 7]. *C. nutans* has a viral potential for treatment of skin rashes, insect, and snakebite, herpes simplex virus (HSV), and varicella-zoster virus

(VZ) [7, 8]. Moreover, previous studies reported that the chloroform extract from *C. nutans* are good in antioxidant against α -diphenyl- β -dipicryl-hydrazyl (DPPH) and galvinoxyl radicals and exert a high antiproliferative effect on human leukemia (K-562) cell line [2]. Although other researchers, but none of the reports has reported cytotoxicity of *C. nutans* in different doses, time and solvent extraction was tested on human hepatoma (HepG2) cell line. Apart from these reports, the correlation between cytotoxicity and phytochemicals contents may provide potential lead compounds in drug discovery. Thus, the aim of this work is to screen the total phenolic content (TPC), total flavonoid content (TFC) of the four different extracts and cytotoxicity toward human hepatoma (HepG2) cell line.

All solvents used were of analytical grade and included: methanol, chloroform, ethyl acetate, and hexane (Merck, Darmstadt, Germany). Folin-Ciocalteu phenol reagent, gallic acid, butylated hydroxytoluene, Roswell Park Memorial Institute medium (RPMI), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Ultraviolet (UV) spectra were recorded on a microplate reader (Tecan Infinite 200 Pro).

The dried powder was percolated with hexane (Fraction I) followed by methanol for a day. Both extracted plant samples were sonicated in 30 min over three cycles. Hexane (Fraction I) and methanol extraction (Fraction II) were filtered. The filtrates were evaporated to dryness using a rotary evaporator at a temperature below than 60 °C and with low pressure. Next, methanol extract was mixed with water in a ratio 3:1 and activated charcoal is a function to remove excessive chlorophyll before fractionate by dissolving the mixture with chloroform producing chloroform phase (Fraction III) and an aqueous phase, thrice. Then, ethyl acetate was added into the aqueous phase, thrice to obtained ethyl acetate phase (Fraction IV) and an aqueous phase. The extracts were concentrated in vacuo and evaporated using a rotary evaporator to get crude extractions.

TPC of *C. nutans* extract was determined by a method developed by [9]. A stock solution of plant extracts was prepared in methanol and pipetted out into test tube and mixed with 0.5 ml of Folin-Ciocalteu phenol reagent was added (FC reagent was dissolved in distilled water with 1:1 ratio). Then 2.5 ml of 20% sodium carbonate was added in each tube and finally the mixture was mixed properly by using vortex and then the test tube was kept in the dark for 40 min. Absorbance spectra was recorded at 725 nm using glass cuvettes. To minimize standard error, the reaction was tested thrice and the results were expressed in milligrams of gallic acid equivalent (mg GAE).

Total flavonoid content of plant extracts was determined by [9]. Concisely, 100 μ l of each plant extract (1 mg/ml) was dissolved in corresponding solvents and then the extracts were made up to 1 ml by using distilled water followed by the addition of 75 μ l of 10% sodium nitrate solution. After a 6 min interval, 150 μ l of 5% aluminium chloride solution was added, and then 0.5 ml of 1 M NaOH was added in the test tubes. The mixture samples were added up to 2.5 ml by using distilled water and thoroughly mixed. The UV-V absorbance values were read immediately at 510 nm. The results were expressed in mg/g butylated hydroxytoluene (BHT) equivalents. Four different extracts, Fraction I, Fraction II, Fraction III and Fraction IV were tested on human liver hepatocellular carcinoma (HepG2) for their anticancer activity. The cell lines were stored in Roswell Park Memorial Institute medium (RPMI) medium containing 10% (v/v) fetal bovine serum (FBS) supplemented with penicillin (100 U/ml) and streptomycin (100 μ g/ml) under humidified atmosphere containing 5% CO₂ in the incubator.

1 mg of four extracts was dissolved in 1 ml of 0.1 % of DMSO (with distilled water) as a stock solution of 0.1 mg/ml respectively. The testing solutions were made by half series dilution from 0 μ g/ml to 100 μ g/ml (0, 6.25, 12.5, 25, 50, 100 μ g/ml). These crude extracts were used for MTT assay

For the MTT assays cells (10 μ l) were added into all the wells (104–105 cells per well) with various concentration of extracts and incubated in a 37 °C, CO₂ incubator for 24 h. A stock solution of 10 mg/ml MTT was prepared in PBS and MTT reagent (20 μ l) was added to the cell monolayer. During this period, the living cells produced insoluble blue formazan from yellow soluble MTT. The reaction was stopped by the addition of dimethyl sulfoxide (DMSO, 100 μ l) in each well. Control cells were incubated without the extract and with DMSO. The absorbance of samples was read at 540 nm using microplate reader.

Experiments were performed thrice. Results were expressed as percentage growth inhibition of control. The percentage of inhibition of cell lines was calculated as shown below:

$$\% \text{ Inhibition} = 100 - \left[\frac{Abs_{sample} - Abs_{blank}}{Abs_{control} - Abs_{blank}} \right] \times 100$$

The IC₅₀ values for each of the active extracts were determined by plotting the percentage inhibition values against the concentration of the extracts. A dose-response curve was used to enable the calculation of the concentrations that killed 50% of the HepG2 cells (IC₅₀). The results were expressed as mean \pm SD. One-way ANOVA was determined the concentration of extracts against the percentage of growth inhibition

Healthy HepG2 cells that achieved confluence were used in this assay. HepG2 cells exhibited epithelial-like structure to the bottom of the flask. The percentage inhibition of *C. nutans* extracts on HepG2 cell line after 24h of treatment as shown in table 1. The data shows that methanol extract performed the highest percentage of inhibition (74.17 %) against hepG2 cell line followed by ethyl acetate extract (69.67%), chloroform extract (64.80%) and hexane extract (61.67%) at a concentration of 100 μ g/ml.

Table 1: Percentage inhibition of *Clinacanthus nutans* extracts on HepG2 cell line after 24h of treatment

Extracts	% inhibition at different concentration (μ g/ml)				
	6.25	12.5	25	50	100
Hexane	11.67 \pm 0.24	20.00 \pm 0.32	42.50 \pm 0.83	43.75 \pm 0.06	61.67 \pm 0.75
Methanol	10.58 \pm 0.27	24.17 \pm 0.39	47.63 \pm 0.68	60.00 \pm 0.81	74.17 \pm 0.50
Chloroform	10.23 \pm 0.75	28.10 \pm 0.45	34.92 \pm 0.60	54.16 \pm 0.75	64.80 \pm 0.05
Ethyl Acetate	10.10 \pm 0.57	22.86 \pm 0.38	39.67 \pm 0.73	48.46 \pm 0.91	69.67 \pm 0.44

n=3; Values have been expressed as mean \pm standard deviation

The evaluation of cytotoxicity was done using the dose-response curve obtained by non-linear regression analysis. The IC₅₀ values of all extracts are summarized in table 2. The percentage of inhibition of HepG2 cells is directly proportional as the extracts concentration increases. Methanol extract exhibited higher cytotoxicity on HepG2 cells after 24 h. The results obtained

shows that methanol extract exhibited the strongest cytotoxicity activity against HepG2 cells line after a 24 hr treatment with IC₅₀ of 43.9367 μ g/ml followed by chloroform extract and ethyl acetate, 55.6112 μ g/ml and 62.0655 μ g/ml, respectively. Hexane extract performed the lowest cytotoxicity activity with IC₅₀ of 68.3807 μ g/ml.

Table 2: IC₅₀ values of *Clinacanthus nutans* crude extracts against HepG2 lines at 24 h incubation

Cell line	Extracts	IC ₅₀ values (μ g/ml)
HepG2	Hexane	68.38 \pm 0.07
	Methanol	43.93 \pm 0.05
	Chloroform	55.61 \pm 0.03
	Ethyl Acetate	62.06 \pm 0.06

n=3; Values have been expressed as mean \pm standard deviation

Table 3: Total phenolic content (TPC) and total flavonoid content (TFC) of *C. nutans* from four different type of solvent

Solvent extracts	Total phenolic content (mg/GAE)	TotalFlavonoid content (mg/g)
Standard	250.57 \pm 0.07*	533.22 \pm 0.04#
Hexane	28.25 \pm 0.02	586.67 \pm 0.01
Ethyl Acetate	22.17 \pm 0.02	532.33 \pm 0.02
Chloroform	119.29 \pm 0.07	428.67 \pm 0.03
Methanol	53.91 \pm 0.03	937.67 \pm 0.02

n=3; Values have been expressed as mean \pm standard deviation, *Gallic acid# α -BHT

Total phenolic content (TPC) and total flavonoid content (TFC)

The total of phenolic content (TPC) was found to be highest in the chloroform extract, which is 119.29 mg of GAE/g compared to other extracts. Total of flavonoid content (TFC), methanol crude performed the highest value, which is 937.67 mg of BHT/g compared with hexane and Ethyl Acetate, which are 586.67±0.001 mg/g and 532.33±0.0002 mg/g respectively. This chloroform extract had the lowest value (428.67±0.003 mg/g) (refer table 3)

The methanol extract from *C. nutans* showed the highest cytotoxicity effect compared to hexane, chloroform, and ethyl acetate extracts. Possibly, the activity might be due to the total flavonoid content which are highest in methanol extract. Methanol extracts treated with HepG2 cells, the IC₅₀ values was 43.9367 µg/ml, which was above the recommended IC₅₀ value by National Cancer Institute of America, for crude extract, which is <30 µg/ml [10]. Although this data suggests that methanol extract from *C. nutans* may not be a strong anticancer regimen, cancer inhibitory properties shown in this experiment may still support the use of *C. nutans* as an alternative adjunctive therapy for cancer prevention or treatment.

A similar study was reported by [6] that methanol extract from *C. nutans* yield sub-fraction F-III. Sub-fraction F-III inhibited the growth of all 4 cell line HepG2, NCI H460, MCF-7 and Hela. Subfraction F-III got the highest cytotoxicity with IC₅₀ of 36.80µg/ml against HepG2 cell line compared to other cell lines.

In previous studies, Muhammad (2014) [5] claimed that six known C-glycosyl flavones were found in methanol extract that has been isolated earlier in the study. Flavonoid is a sub-class of phenolic and known with its polyphenolic structures. Flavonoids play an important role in protecting biological systems against harmful effects of oxidative processes on macromolecules [11]. Apart from the ability of a substance to modulate several biological activities, its direct interaction with cells *in vivo* can result in toxic effects [12].

Different flavonoids and phenolic compounds react with free radicals to reduce the degradation of membranes by preventing the reaction between free radicals and phospholipids [13]. Flavonoids are reported to possess antiulcer activity, hepatoprotective activity, anti-inflammatory activity, antidiabetic effects, vasorelaxant process, anti-atherosclerotic effects, anti-thrombogenic effects, cardio-protective effects and anti-neoplastic activity. Flavonoids are also known to possess antibacterial properties.

In conclusion, the methanol extract exhibited the strongest cytotoxic activity against HepG2 amongst all the *C. nutans* extracts with a high total flavonoid content. Based on these results, study are ongoing to determine the chemical profiling of flavonoids in methanol extract by using UPLC/QToF-MS. A major content of flavonoids may be utilized as an anticancer pharmaceutical application.

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CONFLICTS OF INTERESTS

Declared none

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