

## RESEARCH ARTICLE

# Clinacanthus Nutans Hexane Extracts Induce Apoptosis Through a Caspase-Dependent Pathway in Human Cancer Cell Lines

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### Abstract

**Background:** *Clinacanthus nutans* (*C. nutans*) is a plant consumed as a cancer treatment in tropical Asia. Despite the availability of numerous anecdotal reports, evaluation of active anticancer effects has remained elusive. Therefore we here examined antiproliferative, reactive oxygen species (ROS)-inducing and apoptosis mechanisms of whole plant extracts in different cancer cell lines. **Methods:** Antiproliferative actions of five solvent extracts (hexane, chloroform, ethyl acetate, methanol and water) of *C. nutans* were tested on non-small cell lung cancer (A549), nasopharyngeal cancer (CNE1) and liver cancer (HepG2) cells using MTT assay. The most potent anticancer extract was then assessed by flow cytometry to study cell cycle changes. Intracellular levels of ROS were quantified by DCFH-DA assay. Involvement of the caspase pathway in induction of apoptosis was assessed using caspase assay kits. GC-MS analysis was performed to identify phytoconstituents in the extracts. **Results:** Hexane and chloroform extracts were antiproliferative against all three cell lines, while the ethyl acetate extract, at 300 µg/mL, was antiproliferative in the CNE1 but not A549 and HepG2 cases. Methanol and water extracts did not inhibit cancer cell proliferation. The most potent anticancer hexane extract was selected for further testing. It induced apoptosis in all three cell lines as shown by an increase in the percentage of cell in sub-G1 phase. Dose-dependent increase in ROS levels in all three cell lines indicated apoptosis to be possibly modulated by oxidative stress. At high concentrations (>100 µg/mL), hexane extracts upregulated caspases 8, 9 and 3/7 across all three cell lines. GC-MS analysis of the hexane extract revealed abundance of 31 compounds. **Conclusion :** Among the five extracts of *C. nutans*, that with hexane extract demonstrated the highest antiproliferative activity against all three cancer cell lines tested. Action appeared to be via ion of intracellular ROS, and induction of apoptosis via intrinsic and extrinsic caspase pathways.

**Keywords:** *Clinacanthus nutans*- cancer- apoptosis- caspase- reactive oxygen species

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### Introduction

Shifting from conventional therapy towards herbal cure would require empirical evidence with regards to efficacy and safety (Ernst, 2005). According to World Health Organization, cancer remains as the leading cause of death worldwide. Over the years, there is an increasing trend of using traditional Chinese medicine (TCM) to treat cancers, and since then TCM has been slowly gaining worldwide acceptance (Sagar et al., 2006; Sagar and Wong, 2008; Hsiao and Liu, 2010). Herbal extracts with anticancer property can act via diverse mechanisms, including inhibition of proliferation by apoptosis (Tsai et al., 2012; Moon et al., 2013), modulation of diverse cell signalling pathways (Nag et al., 2012), suppression of adhesion and migration of cancer cells (Ye et al., 2012),

inhibition of topoisomerase I (Lim et al., 2010), and cell cycle arrest (Huang et al., 2008; Yun et al., 2008).

*Clinacanthus nutans* (*C. nutans*) (Burm.f.) Lindau, also known as You Dun Cao in Chinese community (Siew et al., 2014), is a member of the Acanthaceae family that grows as tall, erect or sometimes rambling shrubs in Tropical Asia. A range of biological activities of this plant, including anti-inflammatory (Wanikiat et al., 2008), anti-virus (Charuwichitratana et al., 1996; Yoosook et al., 1999; Janwitayanuchit et al., 2003; Sakdarat et al., 2009), anti-venom (Uawonggul et al., 2006) and anti-cancer (Yong et al., 2013; Huang et al., 2015) effects have been reported. In particular, methanolic extracts of the whole plant are reported to have anti-inflammatory property (Wanikiat et al., 2008); whereas, β-galactosyl diglycerides (Janwitayanuchit et al., 2003) and 132-hydroxy-(132-

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R)-phaeophytin b, 132-hydroxy-(132-S)-phaeophytin a and 132-hydroxy-(132-R)-phaeophytin a (Sakdarat et al., 2009) from *C.nutans* leaves have inhibitory effect on herpes simplex virus types 1 and 2. The anti-venom screening test conducted by Uawonggul et al., (2006) has also suggested the anti-venom potency of *C.nutans*.

Despite numerous anecdotal reports about the medicinal effects of this plant in South East Asian countries, studies on the anticancer property of this plant are scarce. Yong et al., (2013) reported the antioxidant properties and anticancer activity of *C.nutans* leave extract on a number of human cancer cell lines. However, the anticancer mechanism was not elucidated in that study. On the other hand, Huang et al., (2015) tested the ethanol extract of *C.nutans* on hepatoma grafted mice and postulated that the observed anticancer effect is mediated through upregulating immune response and apoptosis. In order to confirm whether the anticancer property of *C.nutans* is mediated through promoting oxidative stress and to determine the mechanism involved, we examined the anti-proliferative, reactive-oxygen species (ROS)-inducing and caspases pathway of *C.nutans* whole plant (stem and leaf) in non-small cell lung cancer (A549), nasopharyngeal cancer (CNE1), and liver cancer (HepG2) cell lines. Further, we also performed the preliminary phytochemical screening for the potential anti-cancer compounds with gas chromatography-mass spectra (GC-MS) analysis.

## Material and Methods

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, trypan blue, trichloroacetic acid, and penicillin-streptomycin were obtained from GIBCO Laboratories (GIBCO BRL, Grand Island, NY, USA). Phosphate buffer saline (PBS), hexane, chloroform, ethyl acetate, methanol (> 99.8%), dimethyl sulfoxide (DMSO), and sodium carbonate were purchased from Merck (Darmstadt, Germany). Propidium iodide, tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), RNase, and dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma Chemical (St. Louis, MO, USA). All chemicals were of the highest grade commercially available.

### Source and storage of plants

Dried plants (stem and leaf) of *C.nutans* were bought from Hoong Heng Acupuncture and Herbal Center (Kuala Lumpur, Malaysia). The botanical identity of *C.nutans* was determined and authenticated by a taxonomist from the Forest Research Institute Malaysia (Kuala Lumpur, Malaysia; sample number: PID 060114-04). Fresh plant materials (leaves and stems) were collected and dried in an oven at 40°C until a constant weight was obtained. The specimens were stored at room temperature in the dark prior to their extraction and subsequent testing.

### Preparation of *C. nutans* extracts for testing

Stock solutions of the five different extracts were prepared by dissolving them in different solvent/solvent

mixture: hexane extract in hexane-ethanol (5:1 v/v); chloroform extract in chloroform-ethanol (1:5 v/v); both ethyl acetate extract and methanol extract in DMSO; and water extract in water. For all experiments, stock solutions were filtered prior to use.

### Organic solvent extraction

Dried plant material (100 g) was grinded into powder. This powder was sequentially extracted with 500 ml of each of the solvents, viz. hexane, chloroform, ethyl acetate, and methanol. Extraction with hexane was considered as a typical procedure. All 100 g of powder was mixed with 500 mL of hexane and kept in dark for 72 h at room temperature. Undissolved solid was filtered off and subsequently extracted with the next solvent. Rotary evaporator was used to recover the dissolved material from each solvent extract. These yielded four samples of semi-solid which were collected in universal bottle and air dried in oven at 60 °C for 24 h.

### Water extraction

Fifty gram of dried plant material was grinded into powder and macerated in 500 mL of warm water (60 °C) and incubated in water bath at 60 °C for 4 h with continuous stirring. The plant extract was filtered by filter paper and pre-frozen at -80 °C for 24 h. The frozen extract was freeze dried using lyophilizer for 72 h to yield dry crystal. The crystals were grounded into powder and stored at 4 °C.

### Cell lines and culture

A549 (ATCC® CCL-185™) and HepG2 (ATCC® HB-8065™) cell lines were purchased from American Type Culture Collection. CNE1 is a kind gift from University of Malaya. All cancer cell lines (A549, CNE1 and HepG2) were cultured in DMEM with 10% FBS in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Cell lines used in the study are all within 40 passages at the time when the experiment was conducted.

### MTT assay

All cancer cell lines (4 x 10<sup>3</sup> cells/well) were incubated with *C. nutans* extracts (25, 50, 75, 100, 150, 200 µg/mL) in 96-well plates for 72 h at 37 °C with 5% CO<sub>2</sub>. MTT (5 mg/mL) was added to each well and incubated at 37 °C for a further 4 h. Supernatant was removed and DMSO was added. The amount of formazan formed is measured at wavelength of 570 nm with background subtraction at 630 nm using microplate reader (Dynex Opsys MR Microplate Reader, Dynex Technologies).

### Studies conducted on hexane extract

As the antiproliferative study showed that hexane extract has the highest activity among the five different extracts, we further determined the intracellular ROS level and study the apoptotic actions of hexane extract, followed by an attempt to identify the phytochemicals responsible for the anticancer activity.

### DCFH-DA assay

All cancer cell lines (8 x 10<sup>3</sup> cells/well) were incubated

with *C. nutans* extracts (25, 50, 75, 100, 150, 200  $\mu\text{g}/\text{mL}$ ) in 96-well plates for 1 and 3 h in a humidified atmosphere of 5%  $\text{CO}_2$ . Then, DCFH-DA (10  $\mu\text{M}$ ) was added and plates were incubated at 37  $^\circ\text{C}$  for another 30 min. The 96-well plates were measured at excitation wavelength of 485 nm and emission wavelength of 530 nm using microplate reader (Infinite<sup>®</sup>200 PRO NanoQuant, Tecan).

#### Cell cycle analysis using flow cytometry

Cells ( $8 \times 10^4$  cells/dish) of each type of cancer were incubated with *C. nutans* extract (25, 50, 75, 100, 150, 200  $\mu\text{g}/\text{mL}$ ) in petri dishes for 72 h in a humidified atmosphere of 5%  $\text{CO}_2$  at 37  $^\circ\text{C}$ . The cells were trypsinized and fixed with 70% ice cold ethanol overnight. The cells were washed twice with PBS. RNase and propidium iodide (20  $\mu\text{g}/\text{mL}$ ) were added and incubated in the dark for 15 min before the cells were analyzed by flow cytometer (BD FACSCalibur 4 Color Flow Cytometer, BD Bioscience).

#### Measurement of Caspase 8, 9, and 3/7 activities

The involvement of caspase pathway in the induction of apoptosis was examined using Caspase-Glo 8, 9 and 3/7 Assay kits (Promega, USA). Briefly,  $4 \times 10^3$  cells/well were seeded in white-walled 96-well plate and incubated at 37  $^\circ\text{C}$  incubator overnight. The cells were treated with plant extracts at six different concentrations (25, 50, 75, 100, 150, 200  $\mu\text{g}/\text{mL}$ ) at time points of 24 h. At the respective time points post-treatment, 100  $\mu\text{L}$  of Caspase-Glo<sup>®</sup> 3/7 Reagent, Caspase-Glo<sup>®</sup> 8 Reagent or Caspase-Glo<sup>®</sup> 9 Reagent were added to each well of a white-walled 96-well plate containing 100  $\mu\text{L}$  of blank, negative control cells or treated cells in culture medium. The contents of the wells were gently mixed and incubated at room temperature for 30 min. The luminescence of each sample was measured using a plate-reading luminometer (Panomics, USA).

#### Gas chromatography-mass spectrometry

GC-MS analysis of the plant extract was performed

using a Agilent technologies 7890A GC systems equipped with Agilent 5975c inert MSD with triple-axis as well as a capillary column (HP-5 ms) (length 30 m  $\times$  diameter 0.25 mm, film thickness 0.25  $\mu\text{m}$ ) packed with 5% phenyl methylpolysiloxane. The ion source temperature was maintained at 240  $^\circ\text{C}$ , and helium was utilized as a carrier gas. Samples were injected at a temperature of 250  $^\circ\text{C}$  with a splitless mode and a flow rate of 1 mL/min. Column oven temperature was programmed as 70-300  $^\circ\text{C}$  at a rate of 10 $^\circ\text{C}/\text{min}$  and maintained at 300  $^\circ\text{C}$  for 6 min. Mass spectra were taken at 70 eV with a scan fragments from 50 to 600 Da and the total MS running time was 29 min.

Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST). The mass spectrum of the unknown component was compared with the spectrum of the known compounds stored in the NIST library. Name and molecular weight of the compounds of the test materials were ascertained.

#### Statistical analysis

The experiments were performed in triplicate. The data are expressed as mean  $\pm$  standard deviation. Significant differences between groups were determined by using Student t-test. P value of less than 0.05 was considered significant.

## Results

#### Antiproliferative effects of *C. nutans* extracts

A549, CNE1, and HepG2 cancer cells were treated with increasing concentrations of each of the five extracts. The half maximal inhibitory concentration ( $\text{IC}_{50}$ ) values are tabulated in Table 1 and antiproliferative effects are illustrated in Figure 1. Among the four organic solvent extracts, only those of hexane (Figure 1A), and chloroform (Figure 1B) had significant antiproliferative effect ( $25 < \text{IC}_{50} < 200$   $\mu\text{g}/\text{mL}$ ) in A549, CNE1 and HepG2 cells. With an  $\text{IC}_{50}$  value greater than 250  $\mu\text{g}/\text{mL}$ , other

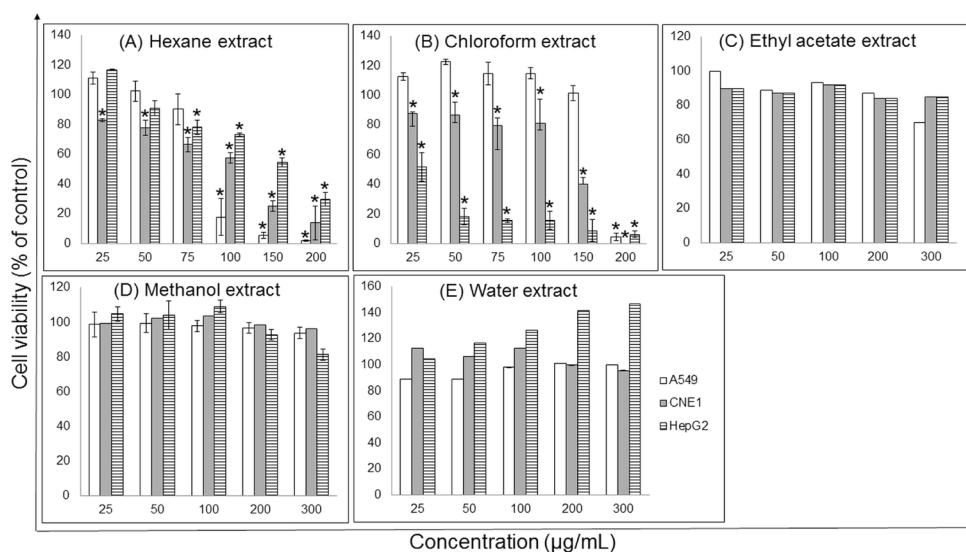


Figure 1. Antiproliferative Effects of *Clinacanthus Nutans* Extracts on A549, CNE1 and HepG2 Cancer Cell Lines. The cancer cells were incubated with increasing concentration of different solvent extracts (hexane, chloroform, ethyl acetate, methanol and water) for 72 h and cell viability was determined by MTT assay. All values given were the means  $\pm$  SDs of 3 independent tests. \* denotes statistical significance at  $P < 0.05$  when compared to the untreated control (100% viability).

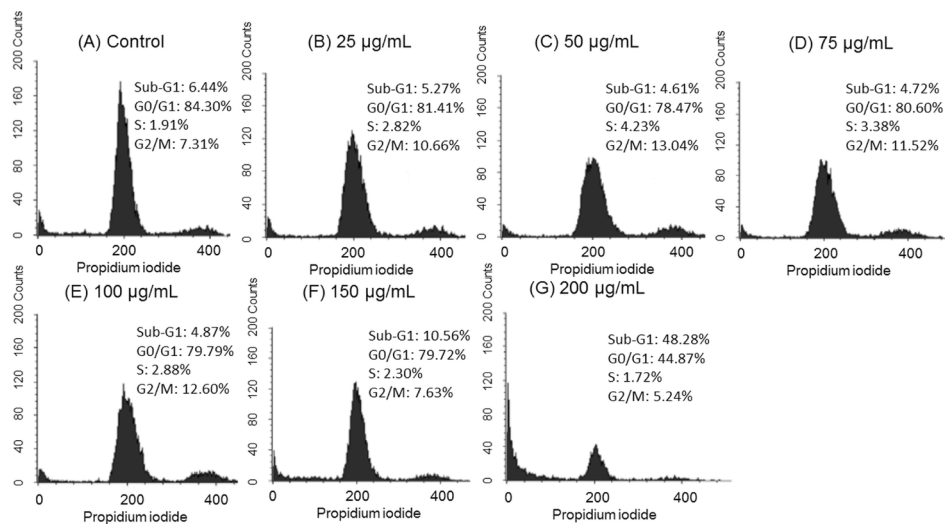


Figure 2. Flow Cytometric Cell Cycle Analysis of A549 Cells with Staining by Propidium Iodide. A549 cells were treated with different concentrations of *Clinacanthus nutans* hexane extract for 72 h.

Table 1. IC<sub>50</sub> (Mg/MI) of Various *Clinacanthus Nutans* Extracts for A549, CNE1 and Hepg2 Cancer Cell Lines

Extraction solvent	IC <sub>50</sub> * (µg/mL)		
	A549	CNE1	HepG2
Hexane	74	116.7	150
Chloroform	164.1	202.1	25
Ethyl acetate	>300	>300	>300
Methanol	>300	>300	>300
Water	>300	>300	>300

\*IC<sub>50</sub>, The half maximal inhibitory concentration

extracts (ethyl acetate, methanol, water) did not possess much inhibitory effects on the growth of any cancer cells. At 300 µg/mL, ethyl acetate extract reduced the cell viability of A549 cells to 70% but had very minimal antiproliferative effects on the CNE1 and HepG2 cells (Figure 1C). Similarly, methanol extract was able to reduce cell viability of HepG2 cells to 80% but posed minimal effects on A549 and CNE1 cells (Figure 1D). On the other hand, water extract had minimal effects on

A549 and CNE1 cells but surprisingly increased the cell viability of HepG2 cells to more than 140% (Figure 1E).

#### Cell cycle analysis by flow cytometry

As the hexane extract of *C.nutans* shows the most promising anticancer activity towards all cancer cell lines tested, it was chosen for further mechanistic study. Cells were incubated with increasing concentration of this extract (25, 50, 75, 100, 150, 200 µg/mL) for 72 h and the results of their effect on cell cycle are shown in Figures 2, 3, and 4. The results showed that hexane extract induced apoptosis as the sub-G1 population of all cell types increased in a dose dependent manner while the G0/G1 and G2/M population showed a corresponding decrease. At 200 µg/mL of extract, the percentage of cell at sub-G1 phase for CNE1, HepG2, and A549 were 90%, 81% and 48%, respectively.

#### DCFH-DA assay

ROS levels, as an indicator of intracellular oxidative stress build-up, in A549, CNE1 and HepG2 cancer cells

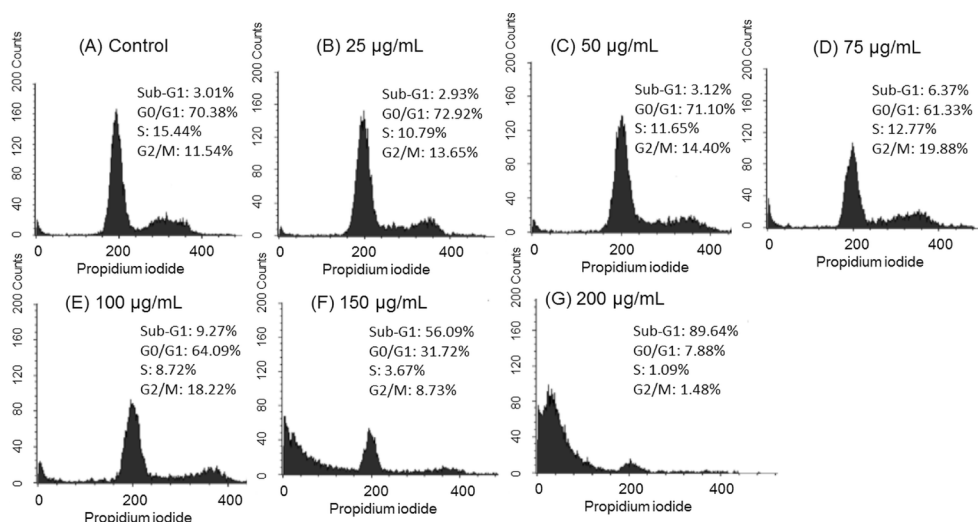


Figure 3. Flow Cytometric Cell Cycle Analysis of CNE1 Cells with Staining by Propidium Iodide. CNE1 cells were treated with different concentrations of *Clinacanthus nutans* hexane extract for 72 h.



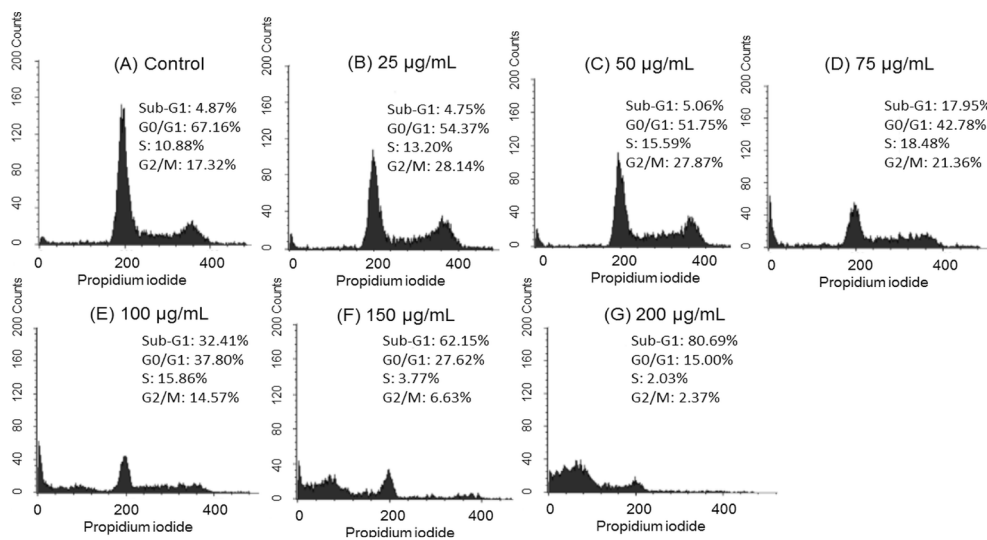


Figure 4. Flow Cytometric Cell Cycle Analysis of HepG2 Cells with Staining by Propidium Iodide. HepG2 cells were treated with different concentrations of *Clinacanthus nutans* hexane extract for 72 h.

were quantified using DCFH-DA assay. The cancer cells were treated with different concentrations of *C. nutans* hexane extracts (25, 50, 75, 100, 150 and 200 µg/mL) for 1 and 3 h. The results are shown in Figure 5. Basically, 25 µg/mL hexane extract induced significant increase of ROS levels in A549 and HepG2 cells after 1 h incubation, with 2.7-fold and 1.78-fold higher than those of untreated cells respectively. CNE1 cells treated with 50 µg/mL hexane extract only experienced moderate ROS increase (1.34 fold).

For 1 h incubation of treated A549 cells, ROS level induced by increasing hexane extract concentration

dropped from 2.7 fold increase (at 25 µg/mL) to about 1 fold (at 100 µg/mL) and remained constant thereafter. The A549 cancer cells treated with 25 µg/mL extract were about 100% viable while those treated with 100 µg/mL or more experienced drastic drop in viability, i.e. percentage of viable cells presence decreased continuously to about 25%.

For treated HepG2 cells, the elevated ROS levels (1.78 fold increases) remained almost constant over the range 25-100 µg/mL of hexane extract used. Interestingly, viability of HepG2 cells treated with increasing concentration of hexane extracts (25-100 µg/mL) decreased slowly

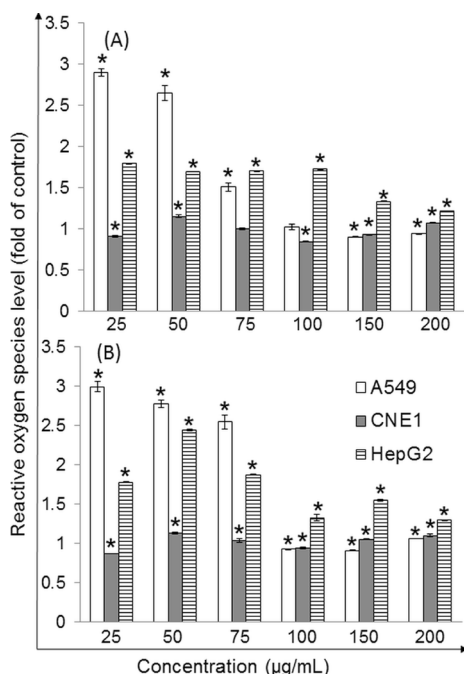


Figure 5. Determination of Induced Reactive Oxygen Species in A549, CNE1, and HepG2 Cells Treated with Different Concentrations of *Clinacanthus Nutans* Hexane Extract for (A) One and (B) three h using DCFH-DA assay. All given values were the means±SDs of 3 independent tests. \* denotes statistical significance at  $P < 0.05$  when compared to the untreated control (fold change value = 1).

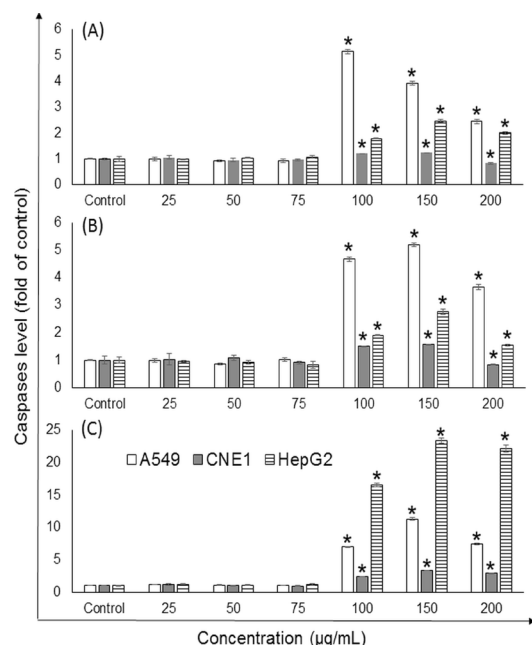


Figure 6. Determination of Induced Caspases in A549, CNE1, and HepG2 Cells Treated with Different Concentrations of *Clinacanthus Nutans* Hexane Extract for 24 H Using (A) caspase 8, (B) caspase 9, and (C) caspase 3/7 kits. All given values were the means±SDs of 3 independent tests. \* denotes statistical significance at  $P < 0.05$  when compared to the untreated control.

Table 2. Phyto-Constituents Identified in the Hexane Extract of *Clinacanthus Nutans* by GC-MS

Peak no.	Identified/Similar compounds	Molecular mass (g mol <sup>-1</sup> )	Retention time	Area %
1	Vanillin	152.15	9.392	0.02
2	Phenol,2,4-bis(1,1-dimethylethyl)-	206.32	10.7	0.37
3	2(4H)-Benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	180.24	11.031	0.41
4	Dodecanoic acid	200.32	11.319	0.18
5	Methyl tetradecanoate	242.4	12.958	0.3
6	Tetradecanoic acid	228.37	13.621	1.05
7	2-Pentadecanone, 6,10,14-trimethyl-	268.48	14.29	1.92
8	Hexadecanoic acid, methyl ester	270.45	15.097	2.31
9	Octadecanoic acid	284.48	15.773	4.68
10	n-Hexadecanoic acid	256.42	16.054	8.95
11	9,12-Octadecadienoic acid, methyl ester	294.47	16.792	5.37
12	Phytol	296.53	17.049	6.01
13	9,12-Octadecadienoic acid (Z,Z)-	280.45	17.643	14.24
14	9,12-Octadecadienoic acid (Z,Z)-	280.45	17.906	4.9
15	Octadecanoic acid	284.48	18.162	1.66
16	2-Methyl-Z,Z-3,13-octadecadienol	280.49	18.581	1.36
17	Z-2-Octadecen-1-ol acetate	310.51	19.176	3.28
18	12-Methyl-E,E-2,13-octadecadien-1-ol	280.49	19.351	3.13
19	11,13-Dimethyl-12-tetradecen-1-ol acetate	282.46	21.19	0.14
20	Ethanol, 2-(octadecyloxy)-	314.55	21.678	0.36
21	2-Methyl-Z,Z-3,13-octadecadienol	280.49	21.947	0.09
22	Squalene	410.72	22.672	2.83
23	Disulfide, didodecyl	402.78	23.116	2.52
24	Tetracosane	338.65	23.83	0.67
25	Hentriacontane	436.85	24.687	3.4
26	Vitamin E	430.71	25.331	2.09
27	Oxirane, hexadecyl-	344.57	26.25	0.07
28	Ergost-5-en-3-ol, (3.beta.)-	400.68	26.607	1.23
29	Stigmasterol	412.69	27.014	5.14
30	beta-Sitosterol	414.71	27.871	5.47
31	beta-Amyrin	426.72	28.415	0.92

from 100 – 80%. As such, there was correlation between constant small ROS elevation and the mostly viable cells for HepG2 cells treated with 25-100 µg/mL of hexane extract. Increase in ROS level of HepG2 cells treated with the maximum concentration of hexane extract (200 µg/mL) was relatively minimal although their viability had decreased by about 80%. This implies that ROS was not a main cause of the observed antiproliferative effect towards HepG2 cells.

The CNE1 cells did not experience much changes in ROS level compared with untreated cells for 1 h treatment with hexane extracts in the 25-200 µg/mL range. As the CNE1 cells treated with 200 µg/mL experienced a drop in more than 80% viability, ROS as a cause of antiproliferation and apoptosis induction was most unlikely. Further mechanistic study into the detailed mode of action of the hexane extract towards CNE1 and HepG2 cells are in progress. The results of the three types of cancer cells treated for 3h with increasing concentration of hexane extract were similar to those similarly treated

for 1 h. Preliminary test on normal cells showed that the 200 µg/mL hexane extract is non-toxic, causing loss of less than 20% (data not shown).

#### Assay for Caspase Activity

At high concentrations (>100 µg/mL), it was observed that hexane extract generally upregulate caspase 3/7, 8, and 9 across all three cell lines (Figure 6). In A549 cells, hexane extracts at 100, 150, and 200 µg/mL caused 5.1, 3.9, and 2.4 fold change of caspase 8 activities, respectively. Similarly, 4.7, 5.2, and 3.7 fold change of caspase 9 activity and 7, 11.3, and 7.4 fold change of caspase 3/7 activity were observed at the three same concentrations. In CNE1 cells, the induction of caspase activity was minimal as compared to what observed in A549 cells, with a slight decrease in caspase activity seen at 200 µg/mL. Basically, the significant change in caspase 8 activity was only observed in cells treated with 100, 150, and 200 µg/mL of hexane extracts, with 1.2, 1.2, and 0.8 fold change of caspase 8 activity, respectively.

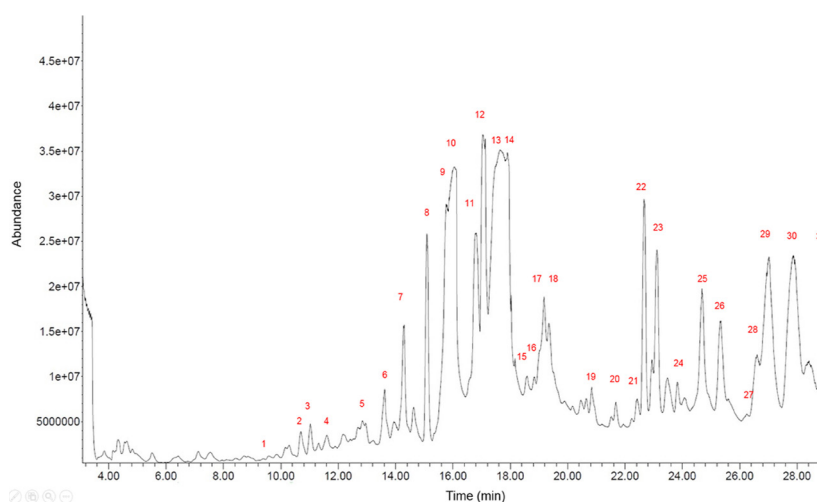


Figure 7. Gas Chromatography-Mass Spectra Analysis of *Clinacanthus Nutans* Hexane Extracts

Similarly, hexane extracts at 100, 150, and 200  $\mu\text{g/mL}$  changed caspase 9 activities to 1.5, 1.6, and 0.8 fold, respectively. Activities of caspase 3/7 were 2.4, 3.3, and 2.9 fold for the same range of hexane extracts. In HepG2 cells, hexane extracts at 100, 150, and 200  $\mu\text{g/mL}$  caused 1.8, 2.5, and 2.0 fold change of caspase 8 activities, respectively. Caspase 9 activity and caspase 3/7 activity were changed to 1.9, 2.8, 1.5 fold, and 16.6, 23.4 and 22.2 fold, respectively.

#### Gas chromatography-mass spectrometry analysis

A GC-MS analysis was performed on hexane extract of *C. nutans* (Figure 7). Thirty one known compounds were identified as summarized in Table 2. Out of the 31 identified constituents, vanillin, phytol, squalene, tetracosane, vitamin E, stigmaterol, and beta-sitosterol had existing anticancer evidence.

## Discussion

In Malaysia, numerous anecdotal reports have claimed that *C.nutans* confers anticancer effects by consuming the boiled water extracts. In this study, we performed extractions using solvents of different polarities (solvent, relative polarity: hexane, 0.009; chloroform, 0.259; ethyl acetate, 0.228). These extractions cover a large range of polarity. It is anticipated that most of the bioactive compounds from *C.nutans* plant can be harvested and tested. Shall the plant possess any anticancer effect it should be reflected in the antiproliferative assay.

In this probe, we first screened the antiproliferative effects of *C.nutans* extracts in A549, CNE1, and HepG2 cells. Out of the four organic solvent extracts that we tested, only those of hexane and chloroform showed significant antiproliferative activity ( $25 < \text{IC}_{50} < 200 \mu\text{g/mL}$ ) on the three types of cancer cells. The chloroform extract was selective towards HepG2 liver cancer cells and revealed the highest potency among the solvent extracts, with 50  $\mu\text{g/mL}$  of this extract inducing loss of more than 80% viability. In fact, Yong et al., (2013) also reported similar antiproliferative effects of the chloroform extract of *C.nutans* leaves on K-562 and Raji cell lines

at 100  $\mu\text{g/mL}$ , and the other five cancer cell lines in a concentration-dependent manner, but not on IMR-32 cells. As far as we know, no anticancer property of stem extract from *C.nutans* has been reported. The antiproliferative effect of the methanol and water extracts was reportedly poor (Yong et al., 2013), in agreement with our results. Similarly, Rathanasamy et al., (2013) found that the methanolic and aqueous extracts from *C.nutans* leaves were not antiproliferative towards *Allium cepa* cells. On top of that, methanolic extract of *C.nutans* leaves was not toxic to human Saos-2 osteosarcoma cells in another study (Liew et al., 2012). Therefore, the active anticancer compounds from *C.nutans* leaves and stem are not likely to be found in the polar fraction solvents, like methanol (relative polarity, 0.762) and water (polarity, 1.000). Intriguingly, a recent study reported that 30% ethanol extract of *C.nutans* aerial parts has anticancer effects against hepatoma cells in HepA-bearing mice. This anticancer effect is mediated via apoptosis with promotion of immune response as evident by enhancement of immune cytokine levels noticeable in the serum of the HepA-bearing mice. (Huang et al., 2015). Nevertheless, our group did not test the ethanol extract and it would be interesting to confirm the findings from another group.

Upon selecting hexane extracts for further analysis, we investigated the effects of hexane extracts on apoptosis in A549, CNE1, and HepG2 cells. Our results showed that at concentrations higher than 150  $\mu\text{g/mL}$ , hexane extract greatly induced apoptosis in all CNE1 and HepG2 cells; whereas, at 200  $\mu\text{g/mL}$ , hexane extract greatly induced apoptosis in A549. Thus, the antiproliferative effect of the hexane extract was mainly due to apoptosis as percentage of cell in sub-G1 phase increased in a dose dependent manner (Kretschmer et al., 2012; Rajput et al., 2013).

As it is known that intracellular build-up of oxidative stress can induce apoptosis (Simon et al., 2000), we quantified the intracellular levels of ROS. There were minimal changes in ROS level in CNE1 cells. In HepG2 cells, there was a moderate increase of ROS level (up to 2.4 fold) at all doses tested in different incubation times. To our surprise, high ROS levels (up to 3 folds) were observed in A549 cells treated with low doses (25 and 50  $\mu\text{g/mL}$ );

however, at high dose, ROS levels dropped. There is strong evidence supporting the capability of ROS elevation for triggering apoptosis (Thayyullathil et al., 2008; Sinha et al., 2013). However, our cell cycle analysis showed that apoptosis occurs at high dose and not at low dose. Along with our finding of low ROS levels observed at high dose groups in A549 cells, it is postulated that ROS build-up might not be the main event triggering apoptosis in A549. On top of that, how high ROS levels in low dose groups not leading to apoptosis in A549 cells would require further experiments to investigate.

Caspase-3 can be activated through two machineries, namely the mitochondria-related apoptosis protease-activating factor-1/caspase-9/caspase-3 cascade, and Fas-associated adapter protein/caspase-8/caspase-3 cascade which is the extrinsic apoptotic pathway (MacKenzie and Clark, 2012). In order to elucidate whether *C. nutans* hexane extract promoted apoptosis via intrinsic caspase pathway or extrinsic caspase pathway, we investigated caspase 3/7, 8, and 9. At high concentrations (>100 µg/mL), it was observed that hexane extracts upregulate activities of caspase 3/7, 8, and 9 across all three cell lines. In A549 cells, activities of caspase 8, caspase 9, and caspase 3/7 were promoted to an average of 4, 4.5, and 8.6 fold upon addition of hexane extracts higher than 100 µg/mL. Hence, it is likely that antiproliferative effects of *C. nutans* hexane extracts were mediated through induction of both intrinsic and extrinsic caspase pathways in A549 cells. In CNE1 cells; however, the upregulation of caspase 8, 9, and 3/7 activities were minimal comparing to that in A549 cells. Firstly, the addition of hexane extracts higher than 100 µg/mL seems to trigger only less than two fold change of activities both of caspase 8 and 9. Given that the caspase 3/7 activity is only around three fold of control cells upon treatment by hexane extracts higher than 100 µg/mL, it is concluded that the activations of both intrinsic and extrinsic caspase pathways in CNE1 cells were diminished, resulting in less apoptotic events, as reflected by lower levels of caspase 3/7 activities. In HepG2 cells, activities of caspase 8 and 9 are quite low compared to A549 cells, around average of 2 fold each. However, the response in caspase 3/7 activity was huge, with around 20 fold change upon treatment with hexane extract higher than 100 µg/mL. This indicates that apoptosis might be a more prominent events in HepG2 cells compared to the other two cell lines, although the activation of both intrinsic and extrinsic caspase pathways were not as pronounced.

The preliminary phytochemical study identified 31 known compounds using GC-MS analysis. Out of the 31 identified phytochemicals, vanillin (Lirdprapamongkol et al., 2005; Ho et al., 2009; Lirdprapamongkol et al., 2009), phytol (Kim et al., 2015), squalene (Murakoshi et al., 1992; Rao et al., 1998; Smith et al., 1998; Warleta et al., 2010), tetracosane (Uddin et al., 2012), vitamin E (Prasad and Edwards-Prasad, 1982; Jiang et al., 2004; Birringer et al., 2010; Torricelli et al., 2013; Wang et al., 2015), stigmasterol (Kasahara et al., 1994; Kim et al., 2014; Ali et al., 2015) and beta-sitosterol (Awad et al., 1996; Awad et al., 1998; von Holtz et al., 1998; Awad et al., 2000; Awad et al., 2003; Ju et al., 2004; Jourdain et

al., 2006; Awad et al., 2007; Koschutnig et al., 2009; Zhao et al., 2009; Baskar et al., 2010) had existing anticancer evidence. However, as this study only detected and not tested the isolated phytoconstituents for their respective anticancer properties, further study is required to identify the exact bioactive compound responsible for the observed anticancer effect and elucidate the involved mechanism.

The hexane and chloroform extracts of *C. nutans* stem and leaves are most antiproliferative towards A549, CNE1, and HepG2 cancer cells. Ethyl acetate extract (25-300 µg/mL) had little or no effect on the viability of CNE1 and HepG2 cells but 300 µg/mL of this extract could induce more than 30% viability loss in A549 cells. Hexane extract could induce apoptosis in all cell types. It was also found that high percentage of cells treated with 300 µg/mL of this extract was situated at sub-G1 phase, indicating high population of apoptotic cells. Increased ROS level in these cells indicated oxidative stress-induced apoptosis. Study of the apoptotic mechanism demonstrated activations of both intrinsic and extrinsic caspase pathways. In conclusion, hexane extracts of *C. nutans* might contain potential phytoconstituents that are useful for the development of novel anticancer therapy.

#### Conflict of interests

The authors report no conflicts of interest in this work.

#### Abbreviations

TCM, traditional Chinese medicine; ROS, reactive oxygen species; GC-MS, gas chromatography-mass spectra; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffer saline; DMSO, dimethyl sulfoxide; MTT, tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DCFH-DA, dichlorodihydrofluorescein diacetate; NIST, National Institute Standard and Technology.

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