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## Anti-Cancer Effects of *Clinacanthus nutans* Extract Towards Human Cervical Cancer Cell Line, HeLa

**Abstract**— *Clinacanthus nutans* or locally known as Belalai Gajah in Malaysia has been used in China in various manners to treat inflammatory conditions like hematoma, contusion, strains and rheumatism. Recently, *C. nutans* has become popular for the treatment of cancer among Malaysian. Thus, the aim of this present study is to prove the anti cancer activity of *C. nutans* extracts in a treatment of cervical cancer as claimed by local people. Aqueous and methanol extracts were extracted from the leaves of *C. nutans* and phytochemical screening was performed for determination of secondary metabolites. The cytotoxic activities of both aqueous and methanol extracts were investigated against HeLa cell by using MTT assay. The mode of cell death was examined by Hoechst 33258 nuclear staining. The secondary metabolite constituents detected in *C. nutans* aqueous extract are terpenoids and flavonoids, whereas methanol extract contains terpenoids, alkaloids and flavonoids. Results also showed that *C. nutans* aqueous extract exerted a significant cytotoxic effect on HeLa cells ( $IC_{50}=13\pm 0.82$   $\mu\text{g/ml}$ ) but no  $IC_{50}$  was detected by methanol extract. No significant cytotoxic activities ( $IC_{50}$  = not detected) were observed in normal kidney cell line, Vero, treated with both aqueous and methanol extracts of *C. nutans* which showed the cytoselective property of the extracts. However, HeLa and Vero cells treated with control drug, tamoxifen showed a significant cytotoxicity effects with  $IC_{50}$  values of  $3.8\pm 0.19$   $\mu\text{g/ml}$  and  $2.2\pm 0.029$   $\mu\text{g/ml}$  respectively. Hoechst 33258 stained showed the aqueous extract of *C. nutans* induced cell death on HeLa cells via apoptosis. Thus, suggesting *C. nutans* aqueous extract as a potential promising alternate therapeutic substance for cancer prevention and treatment especially for cervical cancer treatment.

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### 1 INTRODUCTION

Cervical cancer has become the second most common cancer in Malaysia and fourth in the world [1]. The cancer starts in the cervix, the lower, narrow part of the uterus and mostly the cases of cervical cancer are caused by a high-risk type of Human Papilloma virus (HPV). Basically the patients were from the reproductive age group. In 2006, it was estimated that almost 0.5 million cases of cervical cancer were reported worldwide [2]. World Health Organization (WHO) also reported that every year, about 300 000 women died from cervical cancer. Nowadays, cervical cancer remains one of the major cancer burdens worldwide especially in under-developed and developing countries. The cervical cancer causes not only physical impact on women but also social and sexual impacts. Compared to the other developed countries, Malaysia has the highest prevalence of cervical cancer cases [3].

The Malaysian National Cancer Registry reported that cervical cancer is the most frequently occurring cancers among Malaysian

women beside breast cancer, colon cancer, ovarian cancer, lung cancer and leukaemia. The rate of cervical cancer incidence of all women cancers in Malaysia is 12.9 %. This was higher compared to other Asian and West Countries, and even globally [3]. In spite of the deaths from cervical cancer among young women are rare, but the incidence of cervical cancer increased from the age of 30 and highest at age 60-69 years. It was reported that 54.7% or half of the cases were among the women age 40-58 years. The highest incidence rates were in Chinese (28.8%), followed by Indian (22.4%) and Malay (10.5%). The Ministry of Health Malaysia reported that every year, about 2000 to 3000 cervical cancer patients were admitted to the hospitals [4].

Even though cervical cancer is preventable, and in Malaysia, the pap smear screening was started since 1960s, there is a failure to reduce the incidence and mortality of cervical cancer patients. The vaccination program against cervical cancer also did not decrease the mortality rate [5]. It was reported that about RM

312 million (USD 76 million) was spent every year to prevent and manage the cervical cancer cases in Malaysia [5]. Thus, there is an urgent need to look into the prevention and treatment strategy in order to reduce the incidence of cervical cancer. Currently, people have turn to alternative treatments by using natural products or herbal medicines. By definition, natural product is a chemical compound or any substance, found in nature, and produced by a living organism. Herbal medicine is a plant or mixtures of plant extracts which is used to treat illness and also to promote health. In Malaysia, most of traditional practitioners believe in herbal medicine to treat diseases including cancer. Beside the low cost, the lower risk of side effect also becomes one of the major factors why they prefer to use natural products. According to the report by Cameron and Bell, 2004, the development and search of novel and effective anticancer agents have become very important issues to counter against the side effects of non-specific cytotoxic drugs. The resistance to treatment also becomes a great problem in cervical cancer management [6].

Natural products serve as vital source of drugs since ancient times. Large fractions of the current pharmacopeia, comes from plant origin [7]. Currently, natural compounds have provided many effective anticancer agents. Approximately over 50% of drugs isolated from natural sources or are related to them were used in clinical trials for anticancer activities [8]. With the rise of synthetic molecules and pharmaceuticals in the past century, natural products were touted as alternative medicines. Proponents of nutritional supplements find greater purpose for natural products besides as adjunctive agents and supportive care. In recent years, the use of nutritional supplements has showed an upward trend [9]. The surveys have shown approximately 80% of cancer patients reported utilizing nutritional supplements worldwide in 2002 [10,11].

*C. nutans* is a well known medicinal folk plant especially in Thailand (Thai name: phaya yaw). Fresh leaves of *C. nutans* have long been traditionally used in Thailand to treat skin rashes, insect and snake bites, herpes simplex virus (HSV), and varicella-zoster virus (VZV) lesions [12]. Extracts from the leaves were reported to possess analgesic and anti-inflammatory activities [13], anti-viral activities against varicella-zoster virus [14], herpes simplex virus type-2 [15] and anti-oxidant activity [16]. In Malaysia, *C. nutans* leaves were used traditionally to cure sore

throat, kidney problems, gout, prostate inflammation and skin problems like shingles. Currently, the suggestion of treatment of various cancers using *C. nutans* has spread throughout the country. However, the anticancer property of this plant is poorly defined. In this study, we examined aqueous and methanol crude extracts from leaves of *C. nutans* for presence of anti cancer activity against human cervical cancer cells, HeLa. Based on our previous study, among breast cancer cell line (MCF-7), cervical cancer cell lines (HeLa), and bladder cancer cell lines (T24), HeLa cells treated with *C. nutans* extract shows the most significant cytotoxic effect. The mode of cell death was then measured by Hoechst 33258 staining. Standard phytochemical screening was carried out in order to evaluate the major chemical constituents in *C. nutans* leaves which might be responsible for the anti-cancer property.

## 2 MATERIALS AND METHODS

### 2.1 Sample Preparation

Dried Leaves of *C. nutans* were obtained from YPL Tropical Herbal Farm. The dried leaves were then crushed into powder form. Aqueous extract was prepared by soaking 25 g of crushed leaves in 250 ml of distilled water at  $60^{\circ}\text{C}\pm 1^{\circ}\text{C}$  for 72 hours. Similar with methanol extract, 25 g of crushed leaves of *C. nutans* was soaked in 250 ml of methanol and extracted using soxhlet system. The heating power was set at  $60^{\circ}\text{C}$  for 48 hours. Both aqueous and methanol extracts then filtered and concentrated using vacuum rotary evaporator. The dried extracts were weighed and stored in refrigerator with sealed tube upon use. The percentage of yield (% w/w) for the dried extracts were calculated as:  $\text{Yield (\%)} = (W1 \times 100)/W2$ , where W1 is the weight of the extract after lyophilization of solvent, and W2 is the weight of the plant powder.

### 2.2 Qualitative Phytochemical Analysis

Qualitative phytochemical test was carried out according to the methods by Ayoola et al. 2008, Khan et al. 2011, Manjamalai et al. 2010 and Siddiqui et al. 2009, with slight modifications [17,18,19,20]. The plant extract was screened for terpenoids, alkaloids, saponins, tannins and flavonoids. All the samples except for the test of saponins and alkaloids were prepared by stirring 0.5 g of *C. nutans* extract in 5 ml of distilled water and then filtered using Whatman filter paper. The sample for saponins was boiled before filtration

while for alkaloids, it was stirred with 5 ml of 1% aqueous HCl on a steam bath and then filtered. The terpenoids test was done by mixing the filtrate with 2 ml of chloroform. Then, 3 ml of concentrated H<sub>2</sub>SO<sub>4</sub> was added carefully to form a layer. The presence of terpenoids was detected by a reddish brown coloration at the interface. Alkaloids were detected by adding 1 ml of the filtrate with a few drops of Dragendorff's reagent (solution of Potassium Bismuth Iodide). The formation of brown or reddish precipitates upon addition of Dragendorff's reagent indicates the presence of alkaloids. The saponins tests were done by mixing the filtrate with 5 ml of distilled water and vigorously shaken for a stable persistent froth. The frothing was added with 3 drops of olive oil with vigorous shake. The presence of saponins was indicated with the formation of emulsion. The test for tannins was carried out with addition of 2 ml of ferric chloride, FeCl<sub>3</sub>, into filtrate. A blue-black precipitate indicates the presence of tannins. Flavonoids test was done by dividing the filtrate into two portions. One portion was added with 5 ml of diluted ammonia solution and followed by addition of concentrated H<sub>2</sub>SO<sub>4</sub>. The presence of flavonoids was indicated by a yellow colour formation. Another portion of the filtrate was added with a few drops of sodium hydroxide. The formation of intense yellow color, which becomes colorless on addition of dilute acetic acid indicate the presence of flavonoids.

### 2.3 Cell Culture

Human cervical carcinoma cell, HeLa (ATCC<sup>®</sup> CCL-2<sup>™</sup>) and non carcinoma kidney epithelial cell, Vero (ATCC<sup>®</sup> CCL-81<sup>™</sup>) were obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA). Cells were maintained in Dulbecco's modified eagle's medium (DMEM; Gibco Thermo Fisher Scientific, USA) supplemented with 5% heat-inactivated fetal calf serum (Invitrogen Co.), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco Thermo Fisher Scientific, USA) in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C. Cells were kept in the logarithmic growth phase by routine passage every 2–3 days using 0.025% trypsin-EDTA treatment.

### 2.4 Cell Proliferation Assay

The anti-proliferative activity was evaluated by using the MTT assay method with some modifications [21]. Briefly, cells were seeded 24 hours prior to treatment in a 96-well plate at 5 ×

10<sup>4</sup> cells/well in order to obtain 75% to 85% of confluent cultures. Before addition to the culture medium, 5 mg of the *C. nutans* aqueous extract and methanol extract were dissolved in distilled water and DMSO (Sigma Chemical Co., St. Louis, Missouri, USA) respectively. All the samples undergo a 2× serial dilution for 10 points which ranged from 5 mg/ml to 0.0195 mg/ml. The final concentration of DMSO used in the corresponding wells did not exceed 1% (v/v). According to Jiwajinda, 2002, this concentration of DMSO does not effects cell viability [22]. Tamoxifen (Sigma Chemical Co.) was used as a positive control and negative control cultures received the same concentration of solvent alone. The cells were then incubated for 72 hours at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. At the end of incubation, 50 µl of MTT solution (2 mg/ml MTT in plain culture medium) was added to each well and incubated for 4 hours. MTT solution was discarded. The purple formazan crystal formed at the bottom of the wells was dissolved with 200 µl of DMSO for 20 minutes. The absorbance at 570 nm was read on a spectrophotometer plate reader. The proportion of surviving cells was calculated as Absorbance of treated sample/ Absorbance of control x 100. Dose-response curves were constructed to obtain the IC<sub>50</sub> values. All experimental data were derived from at least 3 independent experiments.

### 2.5 Hoechst 33258 Nuclear Staining Assay

Nuclear staining with Hoechst 33258 (Sigma Aldrich) was performed according to previous researcher with slight modifications [23,24]. Briefly, HeLa cells were seeded in 25 cm<sup>2</sup> culture flasks at 5×10<sup>4</sup> cells/ml with frosty slide for fluorescence microscopy. The cells were incubated overnight under a humidified 5% CO<sub>2</sub> at 37°C to obtain 75% to 85% of confluent cultures. The old medium was changed with fresh medium before the treatment. The cells were treated with IC<sub>50</sub> of tested *C. nutans* extract and tamoxifen for 24 hours, 48 hours and 72 hours. Negative control only received sterile deionized water (dH<sub>2</sub>O). After the treatments, the medium was discarded and the 4% paraformaldehyde (Sigma Aldrich) was added to fix the cells for 30 minutes at 4°C. After discarding the 4% paraformaldehyde, the slide was washed with PBS (phosphate buffer saline) for three times. A sufficient Hoechst 33258 staining solution (30 µg/ml) was added to cover the cells followed by incubation for 30 minutes, at room temperature and protected from light. The staining solution

was then removed and the slide was washed again three times with PBS. The slide was dried before viewing under a fluorescent microscope (Imaging Source Europe GmbH).

### 2.6 Statistical Analysis

All statistical analysis was performed using Graph Pad PRISM Version 6.0 by GraphPad Software Inc, California. All measurements were carried out in triplicates from three independent experiments and expressed as the mean ± S.D. The data were first tested for their normality using Kolmogorov-Smirnov test. The statistical significances of data obtained were calculated and determined using Students' paired t test or two ways analysis of variance (ANOVA) with Tukey multiple comparison test. The significant level of 0.05 (p<0.05) was set for null hypothesis rejection.

## 3 RESULTS

### 3.1 Yield of Extraction

From 25 g of dried powder of *C. nutans*, 3 g of dried aqueous extract was obtained which gave the extraction yield of 12%. Meanwhile, for the methanol extract, 25 g of dried powder yielded 5.5 g of dried methanol extract. The yield percentage of methanol extract calculated was 22% as shown in table 1.

### 3.2 Phytochemical Analysis

Table II shows the phytochemical contents of aqueous and methanol extracts observed from the standard qualitative phytochemical analysis. Results showed that both aqueous and methanol extract contained both terpenoids and flavonoids. Moreover, both extracts did not contain saponins and tannins. Alkaloids was observed in methanol extract but not in aqueous extract of *C. nutans* leaves.

### 3.3 Cell Proliferation Assay

In the present study, the aqueous extract showed cytotoxic effect towards HeLa cell with IC<sub>50</sub> value of 13±0.82 µg/ml while no IC<sub>50</sub> value was observed against Vero cell (Figure 1). However, for methanol extract, the cytotoxic test displayed no significant cytotoxic activity for both HeLa and Vero cell lines (Figure 2). Tamoxifen, as a positive control drug used in this study

**Table I:** The Percentage Yields of Aqueous And Methanol Extracts

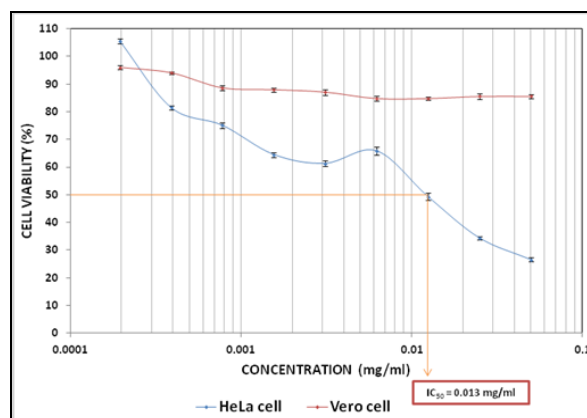
Plant	Percentage of yield %/(w/w)	
	Aqueous extract	Methanol extract
<i>Clinacanthus nutans</i> (dried powder leaves) 25 g	12	22

**Table II:** Phytochemical Constituents of Aqueous And Methanol Extract Of *C. Nutans*

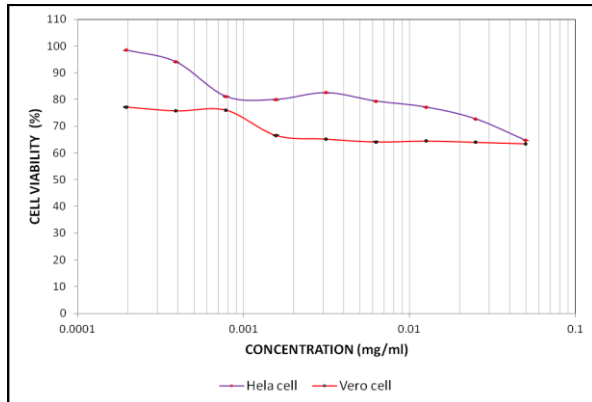
Secondary metabolite	Aqueous extract	Methanol crude extract
Terpenoids	+	+
Alkaloids	-	+
Saponins	-	-
Tannis	-	-
Flavonoids	+	+

+ Indicate the presence of phytochemicals  
- Indicate the absence of phytochemicals

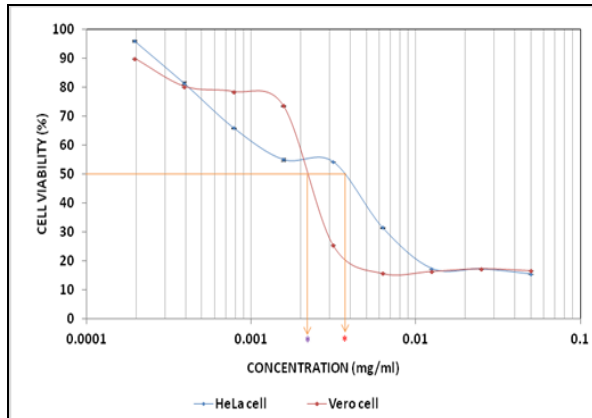
showed high cytotoxic effect against HeLa and Vero cells with IC<sub>50</sub> values of 3.8 µg/ml and 2.2 µg/ml (Figure 3) respectively. The Table III summarized of IC<sub>50</sub> values of HeLa and Vero cells treated with both aqueous and methanol extract of *C. nutans* and tamoxifen.



**Figure 1:** Dose-response curve of *C. nutans* aqueous extract against malignant cells, HeLa and non-malignant cell line, Vero. Aqueous extract of *C. nutans* showed a significant cytotoxic effect towards HeLa cell with IC<sub>50</sub> value of 13µg/ml±0.82. No IC<sub>50</sub> detected on Vero cell. Each value represents means ± SD from three independent experiment (n=3).



**Figure 2:** Dose-response curve of *C. nutans* methanol extract against malignant cells, HeLa and non-malignant cells, Vero. Both cell lines showed no significant cytotoxic effect with no IC<sub>50</sub> detected. Each value represents means ± SD from three independent experiment (n=3).



**Figure 3:** Dose-response curve of tamoxifen against malignant cell, HeLa and non-malignant cell, Vero. Significant cytotoxic effects were showed on both HeLa and Vero cells. Each value represents means ± SD from three independent experiment (n=3). \* = IC<sub>50</sub> value of Vero cell; \* = IC<sub>50</sub> value of HeLa cell.

3.4 Hoechst 33258 nuclear staining

Based on our findings as shown in Figure 4, at all treatment durations of *C. nutans* aqueous extract, untreated HeLa cells remained unstained. There was no bright fluorescence or fragmentation detected in the nucleus. The cells morphology remains intact and no cells shrinkage was demonstrated for 24 and 48 hours. At 72 hours, some cells seem to undergo apoptosis processes which is a normal process of the cell life cycle.

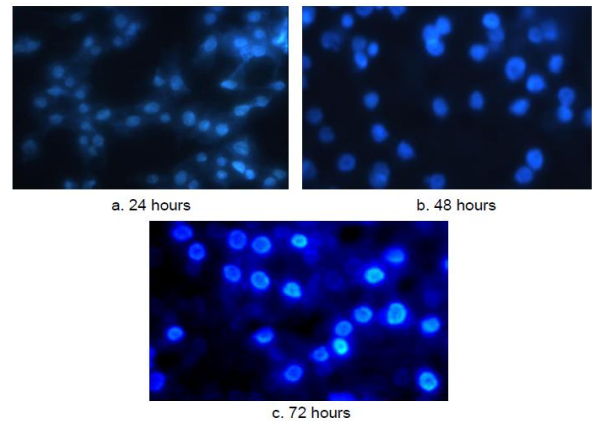
However, as early as 24 hours of treatment with *C. nutans* aqueous extract, HeLa cells displayed apoptotic morphology which was detected by the presence of fluorescence in nuclear area (Figure 5). The presence of chromatin condensation was observed. After 48 hours and 72 hours of treatment, nuclear fragmentation and apoptotic bodies were clearly

seen. A positive control, tamoxifen also showed the apoptotic morphology as early as 24 hours of treatment (Figure 6).

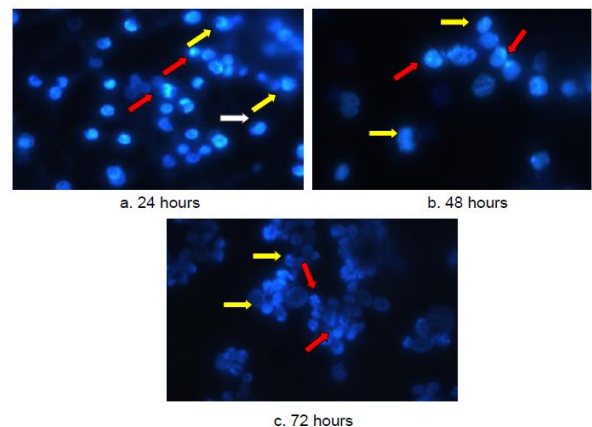
**Table III:** Summary of IC<sub>50</sub> value for HeLa and Vero cells treated with both aqueous and methanol extract of *C. nutans* and tamoxifen

Cell lines	IC <sub>50</sub> value (ug/ml ±SD)		
	Aqueous extract of <i>C. nutans</i>	Methanol extract of <i>C. nutans</i>	Tamoxifen
HeLa (n=3)	13±0.82	ND	3.8±0.19
Vero (n=3)	ND	ND	2.2±0.029

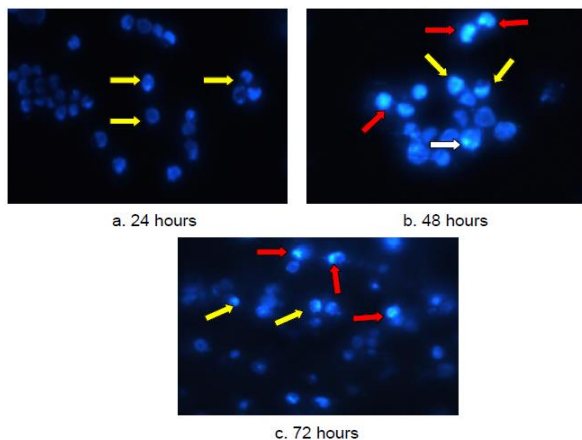
ND= Not Detected



**Figure 4:** Untreated nuclear staining of HeLa cells with Hoechst 33258. HeLa cell remained uniformly unstained with round and unpunctuated nucleus at all experimental durations, 24 hours (a), 48 Hours (b) and 72 hours (c). Magnification: 40x.



**Figure 5:** Nuclear staining of *C. nutans* –treated HeLa cells with Hoechst 33258. Apoptotic morphology was shown as early as 24 hours of treatment (a). Cell shrinkage (white arrow), DNA condensation (red arrow) and nuclear fragmentation (yellow arrow) clearly seen following treatment of 48 hours (b) and 72 hours (c). Magnification: 40x.



**Figure 6:** Nuclear staining of positive control, Tamoxifen-treated HeLa cells with Hoechst 33258. Similar with the *C. nutans* treatment, apoptotic morphology was shown as early as 24 hours of treatment (a). Cell shrinkage (white arrow), DNA condensation (red arrow) and nuclear fragmentation (yellow arrow) clearly seen following treatment of 48 hours (b) and 72 hours (c). Magnification: 40x.

#### 4 DISCUSSIONS

Extraction is the term used pharmaceutically, which involves the use of selective solvents for the separation of medicinally active part of plant or animal tissues from the inactive or inert components by standard extraction procedures. The products attained from plants are relatively impure liquids, semisolids or powders which are intended for external or oral uses only. The extraction procedure includes infusions, decoctions, tinctures, fluid extracts, semisolid extracts and powdered extracts. In this present study, two polar solvents were used, water and methanol. Water has higher polarity with polarity index of 10.2 as compared to the methanol with polarity index of 5.1. The percentage yield of aqueous extract of *C. nutans* was much lower compared to the methanol extract. This may indicate that the *C. nutans* extracted compounds are mostly non polar or semi polar. Secondary metabolites are the most important bioactive constituents of plants which contribute to the medicinal value of the plants. Basically, the medicinal values were due to their potential in the treatment and prevention of human disease and to meet the needs of society. Therefore, standard qualitative phytochemical screening was carried out to evaluate the bioactive constituent present in aqueous and methanol extracts of *C. nutans* leaves. In this study, the only difference between aqueous and methanol extracts was the presence of alkaloids. Aqueous extract was showed a negative result against alkaloids test but not methanol extract. The differences of chemical

constituents between this two extract of *C. nutans* might explain why methanol extract does not show toxicity effect towards HeLa cells as compared to aqueous extract.

Terpenoids or isoprenoids are the functional modified terpenes [25]. Terpenes represent one of the largest and most diverse classes of secondary metabolites among the various secondary metabolites [26]. Different terpenoid molecules have anti inflammatory, anti microbial, anti fungal, anti parasitic, anti viral, anti allergenic, anti spasmodic, anti hyperglycemic, chemotherapeutic, and immunomodulatory properties [27,28,29,30,31]. According to Food and Drug Administration (FDA), terpenoids exhibit most effective anti-cancer agents [25].

Flavonoids are found to be present in practically all dietary plants such as fruits and vegetables. All around the world, especially in China, flavonoids containing medical plants, and herbal remedies have been used as folk medicine [32]. Some study found that most of the flavonoids inhibit proliferation in many kinds of cultured human cancer cell lines, whereas they are less or no toxic to human normal cells [33,34]. The *in vitro* and *in vivo* studies have shown that some flavonoids can prevent carcinogen metabolic activation [35,36,37]. Additionally, flavonoids also have demonstrated induction of apoptosis [34,38,39], cell cycle arrest [40, 41], and anti-oxidative activity [42].

Alkaloids have been traditionally isolated from plants, but an increasing number are also found in animals, insects, and marine invertebrate and microorganism. Alkaloids have many pharmacological activities including anti hypertensive, anti arrhythmic, anti malarial activity, and anti cancer actions. Additionally, alkaloids also used as antiseptics and antibiotic in medicine [43]. These three secondary metabolites, terpenoids, flavonoids and alkaloids are proved by many researchers to have anti inflammatory or anti cancer activity. Thus, based on this study, anti cancer activity of *C. nutans* aqueous extract might due to the complimentary of those three compounds since the phytochemical test showed the positive results towards them.

In order to screen the plants for anti cancer agents, one of the useful tools is *in vitro* cytotoxicity test. Cytotoxicity is the ability of plants derived compound or natural product to arrest the proliferation of cells. MTT assay is one of the mostly used cytotoxicity test or cell proliferation assays, which is a quantitative colorimetric assay

[44]. MTT is a water-soluble tetrazolium dye that is reduced by live cells to water-insoluble, purple formazan. The amount of formazan can be determined by solubilizing in DMSO and measuring spectrophotometrically. Comparisons between the spectra of treated and untreated cells will give a relative estimation of cytotoxicity [45]. The inhibitory concentration ( $IC_{50}$ ) obtained from the dose-response curve of cell viability was used to study the cytotoxic effect of tested compound. The value of  $IC_{50}$  is used in the early stage of the discovery process to evaluate the suitability and the performance of drugs [46]. According to the FDA,  $IC_{50}$  represents the concentration of a drug that is required for 50% inhibition *in vitro*.

In this present study, the aqueous extract of *C. nutans* has showed a cytotoxic effect towards HeLa cell ( $IC_{50}$  value of  $13 \pm 0.82 \mu\text{g/ml}$ ) without affecting normal cell, Vero (no  $IC_{50}$ ). Therefore, this may suggest that the *C. nutans* aqueous extract has the cytoselective activity which is the most important property for a cancer drug.

On the other hand, tamoxifen gave the lowest  $IC_{50}$  against HeLa and Vero cells, indicated that tamoxifen was toxic towards both cancerous and non-cancerous cells. Therefore, it was explained why tamoxifen has been reported having certain side effects towards cancer patients.

According to US National Cancer Institute (NCI), the extracts possessing an  $IC_{50}$  value against the tested cancer cells less than  $20 \mu\text{g/ml}$  are considered to be cytotoxic [47,48]. Thus, it is become a benchmark for significant anti proliferative of crude extract. Wong et al. 2013, reported that  $IC_{50}$  values are widely used to assess the potency of a compound [49]. The compound is considered more potent if its achieved lower than  $IC_{50}$  value. Based on these criteria, aqueous extract of *C. nutans* was selected for the further study on mode of cell death instead of methanol extract.

In order to confirm the mechanism of cell death induced by the *C. nutans* aqueous extract, the nuclear morphology of HeLa cell treated with the extract was measured by Hoechst 33258 nuclear staining. The morphology features of apoptosis cell stained by Hoechst can be easily distinguished from the normal cells as the apoptosis cells will show condensed or fragmented nuclei [50]. Other morphology characteristics of apoptosis are cytoplasmic blebbing and cell shrinkage [60]. According to our

findings, untreated HeLa cells remain intact until 72 hours of treatment, indicating that the cells were alive. The live cells basically maintained their original morphology and were adherent to the flask compared to the apoptotic cells where they were shown up as round cells and starting to lose the grip within the surrounding cells [51].

Therefore, after 24 hours of treatment with *C. nutans* aqueous extract, apoptotic morphology was observed in HeLa cells with the presence of chromatin condensation. Nuclear fragmentation and apoptotic bodies also detected following the 48 hours duration of treatment. By comparing *C. nutans* aqueous extract with tamoxifen, it was significantly shown that *C. nutans* aqueous extract triggered cell death in HeLa cells via apoptosis. Tamoxifen treatment displayed similar apoptotic events and nuclear fluorescence throughout all the treatment durations. This indicates that the data obtained for *C. nutans* aqueous extract are valid and acceptable.

In the treatment of cancer, the therapeutic goal is to trigger tumor-selective cell death. Thus, the mechanisms responsible for cancer cell death are obviously important in determination of the efficacy of treatment [52]. Some studies found that, cancer development can progress by defects in apoptotic pathways or repression of apoptosis [53,54]. Thus for abnormal cells, instead of slowing down their proliferation, agents that can induce apoptosis to eliminate abnormal cells may have chemopreventive potential [55]. Moreover, the key killing mechanism for most anti tumor therapies including immunotherapy, chemotherapy, gamma-irradiation, or cytokines is by induction of apoptosis in cancer cells [56, 57]. In our present study, it was revealed that the mode of cell death induced by *C. nutans* aqueous extract against HeLa cell is via induction of apoptosis. However, to further confirm the mechanism of cell death induces by *C. nutans* aqueous extract, the determination of apoptosis event by Annexin V-FITC and detection of proteins expression (p53, Bax, Bcl-2 and caspases) are currently in progress. This further study also included the effect of *C. nutans* aqueous extract on regulation of cell cycle arrest.

## 5 CONCLUSION

Aqueous extract from leaves of *C. nutans* possessed cytotoxic effects against human cervical cancer cell lines, HeLa ( $IC_{50}=13 \pm 0.82 \mu\text{g/ml}$ ) as compared to methanol extract ( $IC_{50}=\text{---}$ )

not detected) with no cytotoxic effects towards non-malignant cell line, Vero. Hence, the cytotoxic activity induced by the extract is only selective towards cancerous cells compared to the tamoxifen. Further investigation by Hoechst 33258 nuclear staining demonstrated that aqueous extract of *C. nutans* induced cell death by apoptosis. Therefore, it provides an important lead for development of anti cancer therapeutics for management of cervical cancer. The potential of *C. nutans* as an anti cancer drug can be further studied by the characterization and purification of responsible active compound and followed by *in vivo* study.

#### CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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#### REFERENCES

- [1] Bruni L, Barrionuevo-Rosas L, Albero G, Serrano B, Mena M, Gómez D, Muñoz J, Bosch FX, de Sanjosé S. ICO Information Centre on HPV and Cancer (HPV Information Centre). Human Papillomavirus and Related Diseases in Malaysia. Summary Report. [Accessed 7 October 2016]
- [2] Mohd Rushdan MN. Current status and future direction of cervical cancer prevention program in Malaysia. 2008. [ONLINE] Available: [http://login.totalweblite.com/clients/obgynanrush/Downloads/Cervical\\_Cancer\\_Prevention\\_in\\_Malaysia\\_by\\_Rushdan\\_for\\_OGSM\\_web1120200812\\_2411PM.pdf](http://login.totalweblite.com/clients/obgynanrush/Downloads/Cervical_Cancer_Prevention_in_Malaysia_by_Rushdan_for_OGSM_web1120200812_2411PM.pdf).
- [3] Malaysian National Cancer Registry Report. Ministry of Health, Malaysia. 2003.
- [4] Ministry of Health. Cervical Cancer Incidence and Mortality Rates. [ONLINE] Available: <http://appliedresearch.cancer.gov/icsn/cervical/mortality.html>.
- [5] Sharifa Ezat WP, Syed Aljunid. Cost-effectiveness of HPV vaccination in the prevention of cervical cancer in Malaysia. *Asian Pac J Cancer Prev* 2010; 11(1): pp 79-90.
- [6] Cameron D, Bell R. Optimizing treatment for patients with breast cancer. *Seminars Oncology* 2004; 31: pp 4-5.
- [7] Kingston DGI. Modern natural products drug discovery and its relevance to biodiversity conservation. *Journal of Natural Product* 2011; 74: pp 496-511.
- [8] Newman DJ, Gragg GM. Natural products as sources of new drugs over the last 25 years. *Journal of Natural Product* 2007; 70: pp 461-477.
- [9] Dennis T, Fanous M, Mousa S. Natural products for chemopreventive and adjunctive therapy in oncologic disease. *Nutrition and Cancer* 2009; 61(5): pp 587-597.
- [10] Cassileth BR, Deng G. Complementary and alternative therapies for cancer. *Oncologist* 2004; 9: pp 80-89.
- [11] Hyodo H, Eguchi K, Nishina T, Endo H, Tanimizu M. Perceptions and attitudes of clinical oncologists on complementary and alternative medicine: a nationwide survey in Japan. *Cancer* 2003; 97: pp 2861-2868.
- [12] Santi Sakdarat, Aussavashai Shuyprom, Thaweephol Dechatiwongse Na Ayudhya, Peter GW, Gloria Karagianis. Chemical composition investigation of *Clinacanthus nutans* Lindau leaves. *Thai Journal of Phytopharmacay* 2008; 13(2): pp 13-24.
- [13] Satayavivad J, Bunyapraphatsara N, Kittisiripornkul S, Tanasomwong W. Analgesic and anti-inflammatory activities of extract of *Clinacanthus nutans* (Burm. f.) Lindau. *Thai Journal of Phytopharmaceutical Sciences* 1996; 3: pp 7-17.
- [14] Thawaranantha D, Balachandra K, Jongtrakulsiri S, Chavalittumrong P, Bhumiswasdi J, Janyavasud C. In vitro antiviral activity of *Clinacanthus nutans* on varicella-zoster virus. *Siriraj Hosp Ga* 1992; 44: pp 285-91.
- [15] Jayavasud C, Dechatiwongse T, Balachandra K. The virucidal activity of *Clinacanthus nutans* Lindau extract against Herpes simplex virus type-2: an in vitro study. *Bulletin Department Medical Sciences* 1992; 34: pp 153-158.
- [16] Pannangpetch P, Laupattarakasem P, Kukongviriyapan V, Kukongviriyapan U, Kongyingyoes B, Aromdee C. Antioxidant activity and protective effect against oxidative hemolysis of *Clinacanthus nutans* (Burm.f) Lindau. *Songklanakarin Journal of Sciences and Technology* 2007; 29(1): pp 1-9.
- [17] Ayoola G, Coker H, Adesegu, S, Adepoju-Bello A, Obaweya K, Ezennia E. Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in south western Nigeria. *Tropical Journal of Pharmaceutical Research* 2008; 7(3): pp 1019-1024.
- [18] Khan A, Qureshi R, Ullah F, Gilani S, Norsheen A, Sahreen S, Laghari M, Rehman S, Hussain I, Murad W. Phytochemical analysis of selected medicinal plants of Margala Hills and surroundings. *Journal of Medicinal Plants Research* 2011; 5(25): pp 6017-6023.
- [19] Manjamalai A, Singh R, Guruvayoorapam C, Grace V. Analysis of phytochemical constituents and antimicrobial activity of some medicinal plants in Tamilnadu, India. *Global Journal of Biotechnology & Biochemistry* 2010; 5(2): pp 120-128.
- [20] Siddiqui S, Verma A, Rather A, Jabeen F, Meghvasani M. Preliminary phytochemicals analysis of some important medicinal and aromatic plants. *Advanced in Biological Research* 2009; 3(5-6): pp188-195.
- [21] Berridge MV, Herst PM, Tan AS. Tetrazolium dye as a tool in cell biology: new insight into their cellular reduction. *Biotechnology Annual Review* 2005; 11: pp127-152.
- [22] Jiwajinda S, Santisopasri V, Murakami A. In vitro anti-tumor promoting and anti-parasitic activities of the quassinoids from *Eurycoma longifolia*, a medicinal plant in Southeast Asia. *J Ethnopharmacology* 2002; 82(1): pp 55-58.
- [23] Ping-Chung K, Amooru GD, Kuo-Hsiung L, Thian-Shung W. Cytotoxic and antimalarial constituents from the roots of *Eurycoma longifolia*. *Bioorganic & Medical Chemistry* 2003; 12: pp 537-544.



- [24] Hishikawa K, Oemar BS, Tanner FC, Nakaki T, Luscher TF, Fujii T. Connective tissue growth factor induces apoptosis in human breast cancer cell line MCF-7. *J Biol Chem* 1999; 274: pp 37461-37466.
- [25] Ajikumar PK, Tyo K, Carlsen S, Mucha O, Too HP, Stephanopoulos G. Terpenoids: opportunities for biosynthesis of natural product drugs using engineered microorganisms. *Molecular pharmaceutics* 2008; 5(2): pp 167-190.
- [26] Friedman M, Henika PR, Mandrell RE. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *Journal of Food Protection* 2002; 65: pp 1545-1560.
- [27] Frank T, Bieri K, Speiser B. Feeding deterrent effect of carvone, a compound from caraway seeds, on the slug *Arion lusitanicus*. *Annals of Applied Biology* 2002; 141: pp 93-100.
- [28] Hammer K A, Carson CF, Riley TV. Antifungal activity of the components of *Melaleuca alternifolia* (tea tree) oil. *Journal of Applied Microbiology* 2003; pp 95: 853-860.
- [29] Wagner KH, Elmadafa I. Biological relevance of terpenoids. verview focusing on mono-, di-, and tetraterpenes. *Annals Nutritional and Metabolism* 2003; 47: pp 95-106.
- [30] Paduch R, Kandefor-Szerszen' M, Trytek M, Fiedurek J. Terpenes: substances useful in human healthcare. *Archivum Immunologiae Therapy* 2007; 55: pp 315-327.
- [31] Ren WY, Qiao ZH, Wang HW, Zhu L, Zhang L. Flavonoids: Promising anticancer agents. *Medicinal*
- [32] Wenzel U, Kuntz S, Brendel MD, Daniel H. Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. *Cancer Research* 2000; 60: pp 3823-3831.
- [33] Pouget C, Lauthier F, Simon A, Fagnere C, Basly JP, Delage C, Chulia AJ. Flavonoids: Structural requirements for anti-proliferative activity on breast cancer cells. *Bioorganic and Medicinal Chemistry Letters* 2001; 11: pp 3095-3097.
- [34] Calomme M, Pieters L, Vlietinck A, Vanden Berghe D. Inhibition of bacterial mutagenesis by Citrus flavonoids. *Planta Medica* 1996; 62: pp 222-226.
- [35] Carroll KK, Guthrie N, So FV, Chambers AF. Anticancer properties of flavonoids, with emphasis on citrus flavonoids. In: Rice-Evans CA, Packer L, editors. *Flavonoids in health and disease*. New York: Marcel Dekker Inc. 1998; pp 437-446.
- [36] Dashwood RH, Xu M, Hernaez JF, Hasaniya N, Youn K, Razzuk A. Cancer chemopreventive mechanisms of tea against heterocyclic amine mutagens from cooked meat. *Proceeding of the Society for Experimental Biology and Medicine* 1999; 220: pp 239-243.
- [37] Sakagami H, Jiang Y, Kusama K, Atsumi T, Ueha T, Toguchi M, Iwakura, I, Satoh K, Fukai T, Nomura T. Induction of apoptosis by flavones, flavonols (3-hydroxyflavones) and isoprenoid-substituted flavonoids in human oral tumor cell lines. *Anticancer Research* 2000; 20: pp 271-277.
- [38] Iwashita K, Kobori M, Yamaki K, Tsushida T. Flavonoids inhibit cell growth and induce apoptosis in B16 melanoma 4A5 cells. *Biosciences Biotechnology and Biochemistry* 2000; 64: pp 1813-1820.
- [39] Choi JA, Kim JY, Lee JY, Kang CM, Kwon HJ, Yoo YD, Kim TW, Lee, YS. Lee SJ. Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. *International Journal of Oncology* 2001; 19: pp 837-844.
- [40] Casagrande F, Darbon JM. Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: Regulation of cyclin-dependent kinases CDK2 and CDK1. *Biochemical Pharmacology* 2001; 6: pp 1205-1215.
- [41] Kandaswami C, Middleton E Jr. Free radical scavenging and antioxidant activity of plant flavonoids. *Advances in Experimental Medicine and Biology* 1994; 366: pp 351-376.
- [42] Roberts M, Wink M. *Alkaloids: Biochemistry, Ecology and Medicinal Application*. Plenum press. New York and London. 1998. 6<sup>th</sup> eds. pp 5-6. 1998.
- [43] Bopp SK, Lettieri T. Comparison of four different colorimetric and fluorometric cytotoxicity assays in a zebrafish liver cell line. *BMC Pharmacol* 2008; 8: pp 8.
- [44] Stern ST, Adiseshaiah PP, Potter TM. LLC-PK1 Kidney Cytotoxicity Assay. NCL Method GTA- Nanotechnology Characterization Laboratory Frederick National Laboratory for Cancer Research Leidos Biomedical Research, Inc. 2015. Frederick, MD 21702.
- [45] Sebaugh JL. Guidelines for accurate EC50/IC50 estimation. *Pharmaceutical Statistic* 2011; 10: pp 128-134.
- [46] Geran RI, Greenberg NH, Macdonald MM, Schumacher AM, Abbott BJ. Protocols for screening chemical agents and natural products against animal tumors and other biological systems. *Cancer Chemotherapy Reports* 1972; 3(2): pp 1-17.
- [47] Chen CF, Hwang JM, Lee W, Chiang HC, Lin JC, Chen HY. Search for antitumor agent for Chinese herbs. Antitumor screening method. *Chinese Medical Journal (Taipei)* 1988; 41: pp 177-184.
- [48] Wong F, Woo C, Hsu A, Tan B. The anticancer activities of *Vernonia amygdalina* extract in human breast cancer cell line are mediated through caspase-dependent and p53-independent pathways. *PLoS ONE* 2013; 8(10): pp 1-5.
- [49] Hunter AL, Choy JC, Granville DJ. Detection of apoptosis in cardiovascular disease. *Methods in Molecular Medicine* 2005; 112: pp 227-289.
- [50] Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. *International Review Cytology* 1980; 68: pp 251-305.
- [51] Reddy A, Kae S, Nurestri AS, Halijah I. Cytotoxic effect of *Alpinia scabra* (Blume) Naves extracts on human breast and ovarian cancer cells. *BMC Complimentary and Alternative Medicine* 2013; 13: pp 314.
- [52] Seller WR, Fisher DE. Apoptosis and cancer drug targeting. *J Clin Invest*. 1999; 104(12): pp 1655-1661.
- [53] Korsmeyer SJ. Bcl-2 initiates a new category of oncogenes: regulators of cell death. *Blood* 1992; 80(4): pp 879-886.
- [54] Miyashita T, Reed JC. Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. *Blood* 1993; 81: pp 151-157.
- [55] Sun SY. Apoptosis induction by chemopreventive agents. *Drug News Perspect* 2001; 14: pp 75.
- [56] Herr I, Debatin KM. Cellular stress response and apoptosis in cancer therapy. *Blood* 2001; 98: pp 2603-2614.
- [57] Lowe SW, Lin AW. Apoptosis in cancer. *Carcinogenesis* 2000; 21: pp 485 - 495.