ASM Sc. J., 13, Special Issue 6, 2020 for InCOMR2019, 7-13

Chemical Composition and Antiproliferative Activity of *Clinacanthus nutans* Extract on Human Cervical Cancer Cell Lines (HeLa)

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Cervical cancer is the third most common cancer (7.7%), after breast cancer (32.1%) and colorectal cancer (10.7%) among women in Malaysia. This study aimed to evaluate the antiproliferative property of *Clinachantus nutans* (CN) extract on cervical cancer and to identify the compound in CN extract that may has antiproliferative potential using human cervical adenocarcinoma (HeLa) cell lines. Results showed that the ethanol extract of CN effectively inhibit proliferation and induce cell death in HeLa cells. CN extract treatment on HeLa cells resulted in significant cell morphological changes and cell growth inhibition at half maximal inhibitory concentration (IC_{50}) of 500 µg/ml. Microscopic observations showed that CN extract treatment on HeLa cells induced apoptotic features. Gas chromatography-mass spectrometry (GC-MS) analysis of CN extract showed that lupeol, lup-20(29)-en-3-one, β- amyrin, linoleic acid ethyl ester and squalene were among the volatile components of CN. These results suggest that the extract of CN exhibits antiproliferative effect on HeLa cells and may serve as a potential candidate to be developed as a new anticancer therapy.

Keywords: *Clinacanthus nutans;* chemoprevention; phytochemical; antiproliferative; cervical cancer; lupeol

I. INTRODUCTION

Current treatments of cancer were still not efficient in reducing cancer cases and there are adverse effects too such as highly toxic and causing organ failure. The long-term carcinogenesis, together with complex molecular characteristics, makes cancer difficult to be cured completely (Meiyanto and Larasati, 2019). The adverse effects and long-term sequelae of anti-cancer chemotherapy remain a major source of concern for both patients and clinicians despite the improved efficacy and enhanced survival offered by modern treatments. Current drugs or other approaches to counteract chemotherapy-induced adverse effects are often incompletely effective, frequently do not address potential longer-term sequelae or may even induce other effects which only add to patient discomfort (Nurgali *et al.*, 2018).

A lot of sophisticated technologies such as surgery, radiotherapy, chemotherapy, hormone therapy or combination of these have been implicated to curb cervical cancer and vaccination is introduced to be the first step in preventing cervical cancer. However, mortality due to cervical cancer is still high in this world including Malaysia (World Health Organization, 2013).

The use of natural compounds for chemoprevention is highly compelling due to their safety, low toxicity, and

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general acceptance as dietary supplements (Crooker *et al.,* 2018). The plant of interest in this study is *Clinachantus nutans* (CN, Fig. 1), which was reported to show anticancer property by previous researches (Ng *et al.,* 2017, Yusmazura *et al.*, 2017). CN is also known as Sabah Snake Grass or *Daun Belalai Gajah* in Malaysia and is classified in the family of Acanthacae. CN is a native plant of South East Asia and mainly found in Thailand, Malaysia and Indonesia. In Thailand, CN is locally use by Thai folklore as medicinal plant to treat variety of symptoms such as inflammation, skin pruritis, poison bites and also herpes infections (Sangkitporn *et al.*, 1995; Janwitatayanchit *et al.*, 2003; Jew-Ming *et al.*, 2012). The leaves of CN have been used in Malaysia and was claimed to be effective to fight cancer (Yong *et al.*, 2013).

Figure 1. The leaves and stem of *Clinachantus nutans*

The aim of this study was to examine the antiproliferative activity of the whole plant extract of CN towards cervical cancer cells and to identify the bioactive components of the extract. HeLa cells will be cultured with ethanol extracts of CN (in vitro) and the IC_{50} value will be identified by using standard technique in cell culture. The phytoconstituents of the extract will be analysed using GCMS analysis.

II. MATERIALS AND METHOD

A. Plant Material and Preparation of Extracts

C. nutans (the whole plant that already dried and grinded) was purchased from Herbagus Sdn. Bhd., Bertam, Penang, Malaysia. The fresh plant samples obtained from the company containing the leaves, stem and roots were identified by comparing with the deposited voucher at the School of Biological Sciences, Universiti Sains Malaysia. The plant material (1.0 Kg) was defatted with petroleum ether, followed by extraction with ethanol for three days. The extracts were concentrated by using rotary evaporator until all the solvent were removed. The crude extracts were stored in -20°C.

B. Cell Culture

Cell culture techniques such as resuscitation of frozen cells, maintenance of cell culture, subculture of cell line and treatment of cell were conducted in Biological Safety Cabinet to maintain a sterile environment. The cells were cultured in supplemented DMEM (supplemented with FBS and Pen-strep). Cells were maintained in a humidified incubator at 37°C, 5% CO_2 and 95°C air and were regularly observed using an inverted microscope to monitor the confluence of cells and any contamination of the cells.

C. IC_{50} Determination

The HeLa cells (seeding number 1 x 10^4) seeded in 6-well plates were treated with a series of different CN extracts concentration. After the treatment, the culture was grown for three days (37°C, 5% CO_2, 95% air). Cell culture were routinely observed for any contamination and any morphological changes. Cell counting were done after 72 hours (Day 3 of treatment). After collecting the old medium, the cells were washed with PBS, treated with trypsin, incubated for 5-10 minutes, and then all cells were harvested. After centrifuged at 1000 rpm for 5-minute, supernatant was sucked out and the pellet were re-suspended with 1 ml complete DMEM growth medium. Cell viability were counted by using TBE Assay. IC_{50} value were determined from this cell viability value.

The cell number within a defined area of four square is counted, and the cell concentration in 1 ml of cell suspension was derived using the formula below:

$$\text{Number of cells} = Y \times 2500 \times 2^* \quad (1)$$

Y=Total cells in four square

2*=dilution factor

Cell concentration = 1000/total number of cells x 1 x 10^4 (2)

1 x 10^4 =number of cells seeded in 6 well plates

C. Cell Proliferation Assay

HeLa cells were seeded in four of 6-well plates (number of cells : 1x10^4). After 24 hours, the cells were treated with CN extract at the IC_{50} concentration. For comparison of cell growth inhibition, control was exposed to culture medium without CN extract. The cells were incubated in incubator at 37°C, 5% CO_2 and 95% air and were extended for 7 days of incubation. The total live cells numbers were counted by using TBE method on Day 1, Day 3, Day 5 and Day 6. In the meantime, the medium was changed every 3 days and the cells were observed for any contamination. From the cell viability counting, the cell proliferation graph was plotted.

D. Determination of Compounds by Using GC-MS

The ethanol extract was chromatographed on a silica gel 60 column. The group fractions were then separated by preparative Thin-Layer Chromatography (TLC). After that, volatile compounds will be determined by using Gas Chromatography equipped with Mass Spectrometry (GC-MS).

GC-MS analysis was carried out on a gas chromatograph from Agilent Technologies 6890 Series equipped with a 5973 Mass Selective Detector (70 eV direct inlet). The column HP-5MS (20mx0.25mm ID, 0.25µm film thickness) initially set at 600°C, then increased to 3000°C at rate 100°C/min, with a hold t at 3 min; and finally increased to 3200°C at rate 200°C/min, with a hold t of 1 min. The flow velocity of He was 1ml/min. The total ion chromatogram obtained was auto integrated by ChemStation (Agilent) and the components were identified by comparison with an accompanying mass spectral database of the National Institute of Standards and Technology (NIST). The relative quantification of the components of each sample was obtained through the relative area of the peaks in the chromatograms.

E. Statistical Analysis

Statistical analysis was conducted using SPSS software for each experiment, three independent experiments (n=3) will be conducted and all data will be expressed in mean and SD manner.

III. RESULT AND DISCUSSION

A. Determination of IC_{50}

The potentiality of CN extracts to inhibit the growth of HeLa cells were investigated using TBE method. Table 1 showed the effect of different concentration of the ethanol extracts on cell viability of HeLa cells. HeLa cells treated with CN extract showed reduced cell viability with increasing dose when compared with untreated cells. After 72 hours of incubation, at 0 µg/ml the mean cell viability counted was 100 %. At the concentration of 300 µg/ml, the mean viable cells counted was reduced to 95.55% and 83.30% at 400µg/ml. At 500µg/ml the viable cell counted was 54.47 % out of total cells.

Table 1. The effect of different concentration of CN extract on cell viability of HeLa cells

Concentration (µg/ml)	Replicate 1	Replicate 2	Replicate 3	Total Live cells	SD	Cell number average (x10⁵cells/ml)	Cell viability (%)
0	415	358	356	376.33	33.50	18.82	100.00
300	327	294	358	326.33	32.01	16.32	95.55
400	347	346	247	313.33	57.45	15.67	83.30
500	258	181	176	205.00	45.97	10.25	54.47
600	60	105	89	84.67	22.81	4.23	22.50
700	7	4	11	7.33	3.51	0.37	1.95

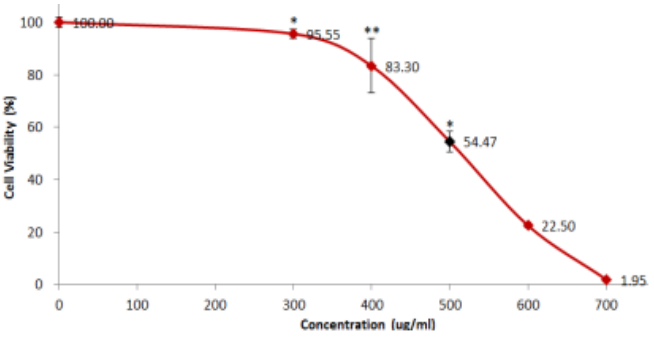

Figure 2. Cell growth inhibition effects on HeLa cells treated with CN ethanol extract. Values represent means ± SD of the triplicates

Figure 2 showed that CN extract inhibited the growth of cancer cells with IC_{50} value of 500 µg/ml. The concentration of IC_{50} value in this study was higher compared to the concentration estimated by other researchers (Yong *et al.*, 2013; Yusmazura *et al.*, 2017; Haron *et al.*, 2019). This may be due to the factor that the whole plants of CN were used in this research to prepare the extract whilst previous researchers used only fresh or dried leaves for their extract preparation. Yusmazura *et al.* (2017) reported that the aqueous extract of CN leaves induced cell death on HeLa cells with IC_{50} 13±0.82 µg/ml but no IC_{50} was detected by the methanol extract. Thus, more study should be conducted to estimate the amount of active compound in every parts of CN to determine the different IC_{50} value from various parts of CN and solvents used for extraction. Although the IC_{50} value was high and may be considered as inactive as anticancer agent, microscopic observations showed that ethanol extract of CN treatment induced cell death on HeLa cells.

B. Cell Morphology Changes at Various Concentrations

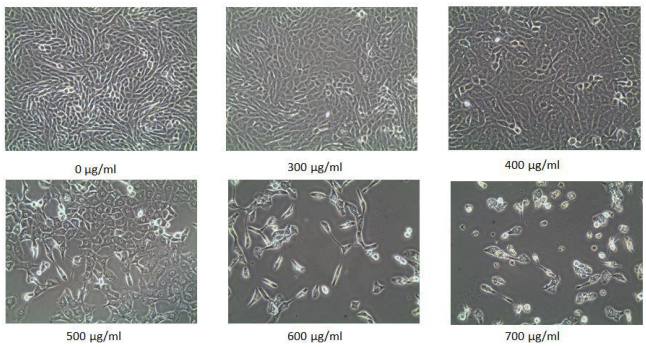

Figure 3. Morphology of HeLa cells treated with CN ethanol extract (Mag: 20x10)

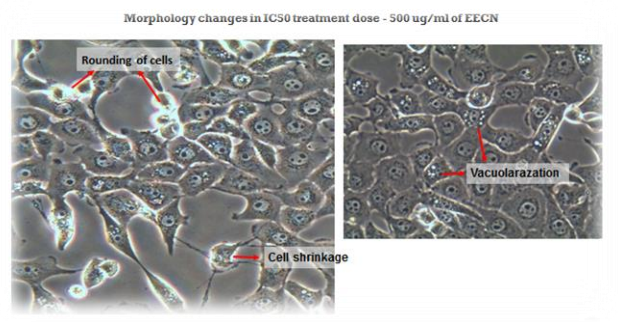

Figure 4. The effect of CN extract on HeLa cells morphology (Magnificent of 20X)

Figure 3 and Figure 4 represent the morphological changes of HeLa cells when treated with various concentrations of CN extracts. With 0 µg/ml, the cells adhere to each other and occupying the space and did not show any obvious cell death. When the extract concentration was increased to 300 µg/ml, a few cell deaths were observed. At the concentration of 400 µg/ml, there were more dead cells observed whereby more empty spaces were visualized. At 500 µg/ml, cell death was more distinct, whereby the cytoplasm of cells almost disappeared. At concentrations of more than 500 µg/ml, most of the cells were dead. Microscopic observations on morphological changes of treated HeLa cells showed that CN extract treatment induced apoptotic features.

C. Cell Proliferation Assay

In order to investigate the growth inhibitory effect of CN extract on HeLa cells, a time-course cell proliferation assay was done. The IC_{50} value of 500µg/ml was treated in HeLa cells and measured at 2 days intervals for 6 days. As shown in Table 2, CN extract effectively inhibited the proliferation of HeLa cells in a time-dependent manner. These results indicate that CN extracts were able to induce growth inhibition of cervical cancer cells. Further studies are needed to delineate the possible mechanism underlying the antiproliferative activity of CN extracts against HeLa cell lines.

Table 2. Cell growth inhibitory effect of CN extracts in cell proliferation of HeLa cells

Day (d)	Untreated					Cell Viability (%)	Cell number (x10⁶ cells/ml)	Treated					Cell Viability (%)	Cell number (x10⁶ cells/ml)
	Replicates			Mean	SD			Replicates			Mean	SD		
	R1	R2	R3					R1	R2	R3				
1	29	23	12	21.33	8.62	1.07	100.00	13	13	18	14.67	2.89	0.73	68.78
3	93	91	118	100.67	15.04	5.03	100.00	23	121	73	72.33	49.00	3.62	71.85
5	256	244	335	278.33	49.44	13.92	100.00	128	167	117	137.33	26.27	6.87	49.34
6	210	206	269	228.33	35.28	11.42	100.00	24	13	3	13.33	10.50	0.67	5.84

D. GCMS Analysis

Figure 5 shows the phytochemical contents of the ethanol extract of CN. The constituents that showed relative larger areas (%) from the extracts of CN were lupeol (77.4%), 9,12,25-octadecatrienoic acid, ethyl ester, (Z,Z,Z)-(7.4%), β-amyrin (4.2%), linoleic acid ethyl ester (4.3%) and lup-

20(29)-en-3-one (2.3%). The major compound, lupeol (Retention time, Rt 24.43 min, $C_{30}H_{50}O$), is a dietary triterpene which is found in vegetables, fruits and in medicinal plants such as American ginseng and *Tamarindus indica* (Beveridge *et al.*, 2002; Imam *et al.*, 2007). Triterpenoids (C_{30} compounds) have been reported to hold great potential in the therapy and prevention of a wide variety of cancer (Bishayee *et al.*, 2011)

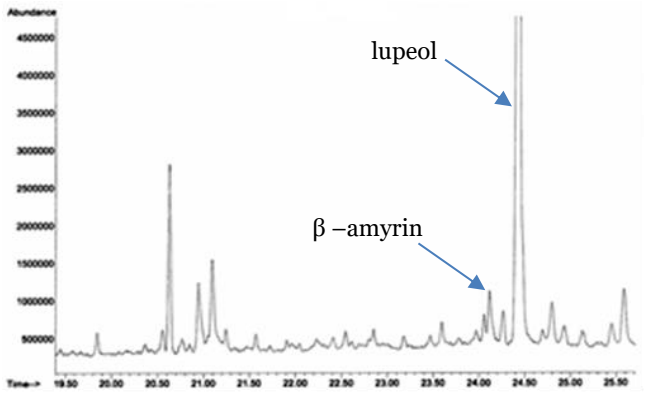

Figure 5. GCMS chromatogram of ethanol extract of CN

The isolation of lupeol (Fig. 6a) from *C. nutans* was first reported by Dampawan *et al.* (1977). Few studies have been carried out to investigate the structure–activity relationships of lupeol in various human cancer cell lines. Studies by Wang *et al.* (2018) and Babu *et al.* (2019) indicated that lupeol inhibited proliferation and induces apoptosis in colorector and lung cancer cells.

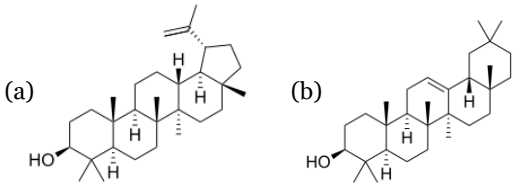

Figure 6. Chemical structure of lupeol (a) and β –amyrin (b)

β-amyrin (Rt 24.12 min, Fig. 6b) has been reported to exhibit various pharmacological activities against various health-related conditions such as inflammation, microbial, fungal, and viral infections and cancer cells (Santos *et al.*, 2012).

The finding of lupeol and other triterpenoids (Table 3) as bioactive compounds exist in *C. nutans* may complement the existing herbs that contain the same compounds with various medicinal activities. However, further studies need to be done to identify specific bioactive compounds that are responsible for the anticancer activity. Other extracts of CN which do not contain lupeol were also found to possess antiproliferative activities against HeLa cells (Haron *et al.*, 2019). Thus, further analysis needed to be conducted to isolate, purified and standardize the compounds from *C. nutans* to ensure purity and efficacy.

Table 3. List of volatile components in the ethanol extract of CN by GCMS

Retention time (min)	Bioactive compound	Match quality	Peak area (%)	Molecular formula	Molecular Weight (g/mol)	Reported studies for cytotoxic effect on cancer cells
15.57	Linoleic acid ethyl ester	99%	4.3	$C_{20}H_{36}O_2$	308.50	Natalia *et al.*, 2017
15.62	9,12,25-octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	99%	7.4	$C_{20}H_{34}O_2$	306.49	Tulika & Mala, 2017
17.20	4,8,12,16-tetramethylheptadecan-4-olide	96%	1.9	$C_{21}H_{40}O_2$	324.50	Abay *et al.*, 2015
20.76	Squalene	90%	0.9	$C_{30}H_{50}$	410.71	De Los Reyes *et al.*, 2015
24.12	β –amyrin	95%	4.2	$C_{30}H_{50}O$	426.71	Lee *et al.* 2014
24.27	lup-20(29)-en-3-one (Lupenone)	94%	2.3	$C_{30}H_{48}O$	424.74	Bastos *et al.*, 2017
24.44	Lupeol	90%	77.4	$C_{30}H_{50}O$	426.71	Pitchai *et al.*,2014,
24.93	fern-7-en-3b-ol	76%	1.5	$C_{30}H_{50}O$	426.40	-

IV. CONCLUSION

The results suggest that phytochemical constituents present in the ethanol extract of CN could be used as a chemopreventive agent for those at risk of cervical cancer. The results also suggest that the ethanol extract of *C. nutans* exhibits antiproliferative effect on HeLa cells and has the potential to be a chemopreventive agent especially to cervical cancer. The cancer inhibitory properties support the use of *C. nutans* as anticancer agents.

V. ACKNOWLEDGEMENTS

This research was funded by the Research Acculturation Grant Scheme (RAGS) awarded by the Ministry of Higher Education (MOHE), Malaysia. The authors are thankful to Usains Biomics Sdn. Bhd. for their kind help to run GC-MS analysis of the samples and Research Management Centre, UiTM for the support to complete this research.

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