

**Artikel Asli/Original Article**

**Cytotoxicity of *Clinacanthus nutans* and Mechanism of Action of Its Active Fraction towards Human Cervical Cancer Cell Line, HeLA**  
(Sitotoksik dari *Clinacanthus nutans* dan Mekanisme Tindakan bagi Fraksi Aktif terhadap Titisian Sel Kanser Pangkal Rahim Manusia, HeLA)

SITI NUR FATIHAH MOHD ROSLAN, YUSMAZURA ZAKARIA & HASMAH ABDULLAH

ABSTRACT

Traditionally, *Clinacanthus nutans* (CN) or locally named as 'Belalai Gajah' is one of the herbal plant claimed to be able to treat cancer. The aim of this study are to extract, isolate and characterize the active anticancer compound from CN and to determine the mode of cell death induced by the compound. Bioassay guided fractionation was done on the CN extract by using column chromatography. The cytotoxicity activities of these fractions toward HeLA cells were examined by MTT assay. The nuclear morphology was examined by Hoechst 33258 staining and the cell cycle arrest was evaluated by propidium iodide staining using flow cytometry. The presence of active compound in the chosen fraction was determined by Liquid Chromatography Mass Spectrometry (LCMS). Out of 16 fractions collected, Fraction 11 (F11) showed the lowest  $IC_{50}$  value with  $27 \pm 2.6 \mu\text{g/mL}$ . The value of  $IC_{50}$  for F11 towards normal cell, NIH 3T3 cell and L929 cell, were  $70 \pm 4.0 \mu\text{g/mL}$  and  $45 \pm 1.5 \mu\text{g/mL}$  respectively. These values were higher than tamoxifen, therefore indicating that tamoxifen is more toxic towards normal cells compared to F11. Nuclear morphology of HeLA cell displayed DNA fragmentation, nuclear condensation and formation of apoptotic bodies upon treatment with F11 for 24 hours. The cell cycle distribution of HeLA cell treated with F11 was arrested at G1 phase. The active compound identified to potentially possess the anticancer property is 19-Oxo-all-trans-retinoic acid. In conclusion, 19-Oxo-all-trans-retinoic acids from F11 of the CN extract, is a potential anticancer agent for cervical cancer.

Keywords: *Clinacanthus nutans*; characterization; cervical cancer; apoptosis; cytotoxicity

ABSTRAK

Secara tradisi, *Clinacanthus nutans* (CN) atau dikenali sebagai 'belalai gajah' adalah salah satu tumbuhan herba yang dipercayai boleh merawat penyakit kanser. Tujuan kajian ini adalah untuk memencilkan dan mencirikan sebatian antikanser yang aktif dari CN dan menentukan cara kematian sel yang disebabkan oleh sebatian ini. Penfraksian berpandukan bioaktiviti telah dilakukan dengan menggunakan kromatografi turus. Aktiviti sitotoksiti untuk kesemua fraksi terhadap sel HeLA telah diuji oleh asai MTT. Morfologi nuklear telah ditentukan dengan menggunakan pewarna Hoechst 33258 dan penghalangan kitaran sel telah dinilai oleh analisis flowsitometri. Sebatian aktif yang terdapat dalam fraksi yang dikehendaki telah diuraikan dengan menggunakan Kromatografi Cecair Spektometri Jisim (LCMS). Fraksi 11 (F11) daripada 16 keseluruhan fraksi yang terkumpul merekodkan nilai  $IC_{50}$  paling rendah iaitu  $27 \pm 2.6 \mu\text{g/mL}$ . Apabila F11 diuji terhadap sel yang normal, nilai  $IC_{50}$  untuk F11 terhadap sel L929 adalah  $45 \pm 1.5 \mu\text{g/mL}$  manakala terhadap NIH 3T3 adalah  $70 \pm 4.0 \mu\text{g/mL}$ . Nilai-nilai ini adalah lebih tinggi berbanding sel yang dirawat dengan tamoxifen. Hal ini menunjukkan bahawa tamoxifen adalah lebih toksik terhadap sel normal berbanding dengan F11. Morfologi sel HeLA menunjukkan kondensasi DNA, fragmentasi nukleus dan pembentukan badan apoptotik apabila dirawat dengan F11 untuk tempoh 24 jam. Perkembangan kitaran sel HeLA yang dirawat dengan F11 menunjukkan kitaran sel terhenti di fasa G1. Sebatian antikanser aktif yang dipercayai mempunyai ciri-ciri antikanser adalah asid retinoik semua-trans-oxo-19. Kesimpulannya, F11 mengandungi sebatian antikanser, asid retinoik semua-trans-oxo-19, yang berpotensi untuk dibangunkan sebagai agen untuk anti kanser rahim.

Kata kunci: *Clinacanthus nutans*; pencirian; kanser pangkal rahim; apoptosis; sitotoksiti

INTRODUCTION

Throughout the ages, human have relied on nature to fulfill their basic needs especially for the production of

foods and medicines. The use of herbal as alternative medicines has become increasingly popular in Malaysia and also throughout the world. One of the reasons why people turn to alternative herbal therapies is because of

the failure of some conventional medical approaches to produce positive treatment results. In addition, herbal medicines are perceived by the public to have lesser side effects as compared to allopathic medicine. Combination of both traditional genetic approaches and the new wealth of genomic information have led to promising strategies in utilizing drugs profile to target tumor cells death in molecular context. Similarly, it may be possible to identify and validate new targets for drugs that would selectively kill tumor cells with a particular molecular context.

Cancer disease is a serious and major problem worldwide. The main cause of cancer is still to be determined. However, diet, chemicals, life style, family history, bacteria and viruses are important causes toward the formation of cancer (Alwahaibi & Mohamed 2008). Cancer is known to bring bad effects in human and in many cases, it would be fatal. In current world scenario, most patients diagnosed with cancer will depend on chemotherapy drugs to eradicate the cancer cells even though chemotherapy is known to have severe side effects on the patients. Hence, nowadays, people are focusing on producing herbal medicine since it is safer and is easily marketable in the society. *Clinacanthus nutans* (CN) was used in this study to examine the anticancer properties of its compound towards HeLA cell. In Malaysia, this plant is popular in Sabah and is known as snake plant or locally named as 'belalai gajah'. Traditionally, CN is used as an anti-inflammatory and also for skin rashes (Janwitayanuchit et al. 2003; Sangkitporn et al. 1995). *Clinacanthus nutans* (Burm. F.) Lindau and *Clinacanthus siamensis* Bremek are often being mistaken with each other since these two species have similar morphology in botanical viewpoint. However, these two species are actually different in term of their molecular aspects and pharmacological characteristics (Kunsorn et al. 2013). Apparently, these two plants are available in the market in different forms of preparation such as lotion, herbal tea, cream, capsule and concentrated extract. However, due to limited information on their pharmacological properties, most of their products do not gain much popularity among users (Alam et al. 2016). Currently, these plants have attracted many researchers to study their medical potential. According to the locals in Sabah, patients with stage three cancers that were not undergoing chemotherapy treatment, showed improvement after consuming CN. Previous study showed that CN extract inhibited cancer cell proliferation and is potent against cervical cancer cell line, HeLA. (Yong et al. 2013). However, the compound responsible for this anticancer effect is not yet elucidated. Hence, the aims of this study are to extract and isolate the active anticancer compound from CN and to examine the cytotoxic effects towards cancerous cell line, HeLA cell. Fraction that showed the most potent activity towards the cell line was selected for further study to determine the mode of cell death induced and for characterization of the active compound.

## MATERIALS AND METHODS

### PLANT SAMPLING AND EXTRACTION

Leaves of *Clinacanthus nutans* (CN) were collected from Pengkalan Chepa, Kelantan, Malaysia. The leaves (PIUM 0238-2) were authenticated by Herbarium, Kulliyah of Pharmacy, IUM. These collected leaves were separated from the stems, washed thoroughly and dried in an oven at 50°C for three days. The dried sample was grinded to powder. The dried powdered leaves (500 g) were sequentially extracted with hexane and chloroform (Merck, Germany), respectively. 50 g of powdered CN leaves were soaked in 300 ml of hexane and were incubated in the water bath at around 60°C for 8 hours. The mixture was then filtered. The leaves remnants were next extracted with chloroform by using Soxhlet extraction for two days. The chloroform extract was concentrated by using rotary evaporator at 60°C, which were later fractionated using column chromatography. The concentrated crude extract was dried in the fume hood for several days until semi-solid extract was obtained.

### BIOASSAY GUIDED FRACTIONATION

The CN extract was subjected to column chromatography on silica gel column (Merck, Germany). The column was eluted successively with hexane-ethyl acetate (1:1), ethyl acetate, chloroform-ethanol (1:1), and ethanol. Major fractions were collected by monitoring with TLC (Chloroform-methanol 1:1). These entire fractions were examined for cytotoxicity by MTT assay method.

Fraction with the lowest IC<sub>50</sub> value was selected for further isolation. Further isolation was conducted to identify major compound content in the most active fraction. This fraction was further chromatographed on a silica gel 60 and eluted with acetonitrile-methanol (20:80). Later, a portion of each fraction was separated by preparative thin-layer chromatography (chloroform-methanol 2:8).

### CELL CULTURE

One human cancer line and two normal human cell lines were purchased from the American Type Culture Collection (ATCC, USA). They were; 1) Human cervical cancer (HeLA cell); 2) normal subcutaneous connective tissue (L929 cell) 3) Normal embryo fibroblast cell (NIH 3T3). Each cells were cultured in Dulbecco's modified Eagle medium (Gibco, USA) supplemented with 5% fetal bovine serum (Gibco, USA) 100 units/ml penicillin and 100 µg/mL streptomycin (Gibco, USA) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Phosphate buffered saline (Merck, Germany) was used for washing and 0.025% trypsin-EDTA (Gibco, USA) was used to detach the adherent cell from the flask.

#### PREPARATION OF EXTRACT

CN extract was diluted in DMSO (Nacalai Tesque, Japan) to various concentrations (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.155625, 0.078125, 0.0390625 and 0.01953125 µg/mL). Two µL of each extract was introduced to the cell culture at 96 well plates (Eppendorf, Germany) after cells reached 80% confluency.

#### MTT ASSAY PROTOCOL

Culture flasks with 80-90% confluent cells were chosen to be plating into a 96-well plate. Before seeding, detached cells were counted by using cell counter (Invitrogen, USA). Cells were then seeded for 24 hours prior to treatment in a 96-well plate. The concentration of live cells was  $5 \times 10^4$  cell/well in 100 µl of medium. After overnight incubation, the old medium was discarded before the addition of 200 µl fresh culture medium. The CN fractions and tamoxifen (Sigma-Aldrich, USA) the positive control, was dissolved in DMSO. A range from 0.04 to 100 µg/ml was added to the cells by following two-fold serial dilution (Zakaria et al. 2009; Zazali et al. 2013). Each well received 2 µl from the serial dilution. Negative control cultures were supplied with only DMSO at the same concentration of solvent alone. The final concentration of DMSO was 1% (v/v) or less and this must be standardized throughout all wells. Each concentration of the extract was assayed in triplicate. Cells were incubated at 37°C and 5% CO<sub>2</sub>. The assay was terminated at 72 hours and the number of surviving cells was determined by MTT assay. At the end of the incubation, 50 µl of MTT reagent (Merck, Germany) (2 mg/ml MTT in serum free culture medium) was added to each well. The plate was then incubated for 4 hours. MTT solution was removed and purple formazan crystal formed at the bottom of the wells was dissolved with 200 µl DMSO and was gently mixed on a shaker (Heidolph, UK) for 20 minutes. The absorbance at 570 nm was recorded on a spectrophotometric plate reader (Biorad Model 680, USA). The proportion of surviving cells was calculated as (absorbance of extract)/ (absorbance of control)  $\times$  100%. Dose-response curves graph were plotted to obtain the IC<sub>50</sub> values.

#### HOECHST 33258 NUCLEAR STAINING ASSAY

Nuclear staining with Hoechst 33258 was performed as described by Zakaria et al. (2009) with minor modification. Hoechst 33258 is used to visualize the apoptosis event including apoptotic bodies and nuclear changes when the dye binds to the DNA producing fluorescence blue color under fluorescence microscope. Firstly, HeLA cells were seeded in petri dishes (Thermofisher, USA) at  $5 \times 10^4$  cells/ml that consist of sterilize microscope Poly-Lysine adhesion slide (Thermofisher, USA) and were incubated under humidified 5% CO<sub>2</sub> atmosphere at 37°C overnight. The next day, cells were treated with IC<sub>50</sub> of the most active fraction and IC<sub>50</sub> of tamoxifen as a positive control, for 24 hour, 48

hour and 72 hour. Negative control only received DMSO less than 1% of final concentration. At each incubation time, the slides were washed three times with PBS then left to dry in the Biological Safety Cabinet (ERLA, Malaysia). Then, the slides were fixed with cold ethanol for 2 hours at 4°C. After that, all slides were incubated with 30 µg/mL Hoechst 33258 (Invitrogen, USA) at room temperature in a dark condition. Nuclear morphology of each different time of treatment was examined under fluorescent microscope at 40X magnification (Imaging Source Europe GmbH, Bremen, Germany). Direct light was avoided when examining the slides. For long term storage, the slides were wrap with aluminum foil and kept in 4°C.

#### CELL CYCLE ANALYSIS

Determination of cell cycle was performed as reported by Zakaria et al. (2009) with minor modification. Cells were cultured in 25 cm<sup>2</sup> culture flasks under humidified 5% CO<sub>2</sub> atmosphere at 37°C. The seeding density was  $5 \times 10^4$  cell/ml. Cells were incubated overnight before the treatment with the most active CN fraction. Tamoxifen and DMSO were used as a positive and negative control respectively. When HeLA cell was about 70-80% confluent, the cells were treated with IC<sub>50</sub> of the most active CN fraction and IC<sub>50</sub> of tamoxifen. After incubation at 24 hours, 48 hours and 72 hours, the medium was decanted into 15mL falcon tube (BD, USA) in order to collect apoptotic floating cells. Cells were harvested by trypsinization with trypsin EDTA solution, and centrifuged at 1500 rpm for 5 minutes. The pelleted cells were then fixed with ice-cold 70% ethanol via gentle vortexing (ERLA, Malaysia) for 1 hour at 4°C. After that, the cells were centrifuged (2000 rpm, 4°C) and the pellet was re-suspended with 1 ml 1% Triton X-100 (Thermofisher, USA) in PBS and 100 µl of 1 mg/ml RNase A (Amresco, USA) was added. The solution was incubated for 30 minutes at 37°C. After that, cells were stained with 100 µl of 10 µg/ml Propidium Iodide solution (Merck, Germany) in staining buffer (1% Triton-X 100 in PBS) in dark. Stained cells were incubated at room temperature for 30 minutes. Then, the samples were put on ice and 300 µl of staining buffer was added prior to analysis with Flow Cytometry (FCM) (Beckman-Coulter, USA). Samples were kept on ice and protected against light until the FCM analysis (within 24 hour).

#### LCMS ANALYSIS

Characteristic of the compounds present in the most active fraction was further investigated. This fraction for analysis was prepared at concentration of 10 mg/ml with methanol (Merck, Germany). The sample was sent to Monash University for LCMS analysis. The compound detection was done by Agilent-1290-Infinity-system coupled to Agilent-6520 Accurate-Mass Q-TOF mass-spectrometer with dual ESI. The MS parameter was set to MS mode with negative and positive polarity programmed.



## STATISTICAL ANALYSIS

The data was presented as mean  $\pm$  S.D. of three independent experiments. The statistical significances of data obtained were calculated and evaluated using two ways analysis of variance (two-way ANOVA) with Tukey's multiple comparison tests or Student's paired t test. Null hypothesis rejection was set for significance level of 0.05 ( $p < 0.05$ ). The results were considered significance, if \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

## RESULTS

### FRACTIONATION OF CN BY COLUMN CHROMATOGRAPHY

A total of 132 fractions were collected. These fractions were combined with similar TLC profile pattern resulting in total of 16 fractions; ethyl acetate: hexane (12 fractions), ethyl acetate (2), ethanol: chloroform (2 fractions) and ethanol (no fractions).

### CYTOTOXICITY OF CN FRACTIONS

The  $IC_{50}$  is defined as a reduction of 50% in absorbance compared to the control values (Noriaki et al. 1988). The lowest value of  $IC_{50}$  from the 16 fractions showed that it is the most active fractions towards cancer cells. Preliminary study showed that CN extract inhibited cancer cell proliferation and was potent against cervical cancer cell line, HeLA. HeLA cell were then selected for further research studies.

Sixteen different fractions were subjected to cytotoxicity assay to obtain the  $IC_{50}$  value. Throughout this study, F11 from the elution of ethyl acetate: hexane has the lowest value of  $IC_{50}$  compared to the other fractions at  $27 \pm 2.6$   $\mu\text{g}/\text{mL}$ .  $IC_{50}$  values of the other fractions were, F1 ( $70 \pm 4.5$ )  $\mu\text{g}/\text{mL}$ , F2 ( $89 \pm 3.1$ )  $\mu\text{g}/\text{mL}$ , F3 ( $80 \pm 7.1$ )  $\mu\text{g}/\text{mL}$ , F6 ( $60 \pm 7.6$ )  $\mu\text{g}/\text{mL}$ , F12 ( $70 \pm 5.1$ )  $\mu\text{g}/\text{mL}$ , F14 ( $76 \pm 4.6$ )  $\mu\text{g}/\text{mL}$ , and F16 ( $34 \pm 2.6$ )  $\mu\text{g}/\text{mL}$ . While as for the other fractions, F4, F5, F7, F8, F9, F10, F13 and F15, could not be extrapolated since none of the concentration of these fraction was able to reduce the proliferation activity to 50%.  $IC_{50}$  values exceeding 100  $\mu\text{g}/\text{mL}$  were considered not detected. The  $IC_{50}$  values of all fractions are shown in Table 1. F11 has the lowest  $IC_{50}$  value; therefore this fraction was used for treatment in the subsequent experiment towards normal cell lines, NIH 3T3 and L929.  $IC_{50}$  values for F11 were of  $70 \pm 4.0$   $\mu\text{g}/\text{mL}$  against NIH 3T3 and  $45 \pm 1.5$   $\mu\text{g}/\text{mL}$  against L929.

The positive control, tamoxifen gave  $IC_{50}$  value of 1  $\mu\text{g}/\text{mL}$  against L929 cell lines and 1.3  $\mu\text{g}/\text{mL}$  against NIH T3T. This showed that tamoxifen is toxic for both cancerous and non-cancerous cells. Table 2 shows the  $IC_{50}$  value of F11 and tamoxifen against cancer and normal cell line.

### MORPHOLOGY OF APOPTOSIS BY HOECHST STAINING

When cells undergo apoptosis, Hoesct 33258 dye will stain the nuclei of the cell and produce bright blue color.

Nuclei of the living cells, will only produce homogeneous and weak fluorescence of blue color (Mingxin et al. 2006). Based on Figure 1, untreated HeLA cell remained unstained at 24 and 48 hours, but started to produce fluorescence blue color at 72 hours, indicating it was beginning to undergo apoptosis. At 72 hours, treated cell with F11 exhibited brighter blue emission light in than the control untreated cell. In untreated cell, no condensation of chromatin was detected all time point indicating that the cell was not undergoing apoptosis. As early as 24 hours, cell treated with F11 displayed condensation of chromatin in the nuclei. At 48 and 72 hours after treatment, the condensation of the chromatin and the structure of the apoptotic bodies formed can be seen clearly. These are the characteristics of apoptotic cell.

Tamoxifen treated cell showed apoptosis morphology and presence of DNA fragmentation at 24 hours of treatment. At 48 and 72 hours, the apoptotic bodies formed and the nuclear fragmentation and condensation were more evident. By comparing cell treated with F11 and tamoxifen, both treated cells displayed the same characteristics of apoptotic events, indicating that the observations obtained from F11 treated cell are acceptable. It clearly showed that F11 induced apoptosis in HeLA cell. The apoptosis event of HeLA cell treated with F11 was further investigate to analyze the cell cycle arrest.

TABLE 1. The  $IC_{50}$  values of CN fractions on HeLA cell

Fractions of CN extract	$IC_{50}/\mu\text{g}/\text{mL}^{-1}$
F1	$70 \pm 4.5$
F2	$89 \pm 3.1^*$
F3	$80 \pm 7.1^*$
F4	>100
F5	>100
F6	$60 \pm 7.6^*$
F7	>100
F8	>100
F9	>100
F10	>100
F11	$27 \pm 2.6^{***}$
F12	$70 \pm 5.1^{**}$
F13	>100
F14	$76 \pm 4.6^*$
F15	>100
F16	$34 \pm 2.6^*$
Positive control, Tamoxifen	$3.3 \pm 1.51$

### EFFECT OF F11 ON CELL CYCLE PROGRESSION

Since F11 induced cell death towards HeLA cells happened via apoptosis, the effect of F11 towards DNA content in the cell cycle was also examined. The relative untreated and F11-treated HeLA cell was presented at three distinct stages that are  $G_0/G_1$ , S and  $G_2/M$  phases. These phases are

TABLE 2. The  $IC_{50}$  values of F11 and tamoxifen against cancer and normal cell line. The value are represented as mean  $\pm$  S.D. with  $**p < 0.001$  and  $***p < 0.0001$  were taken as significantly different from tamoxifen, positive control

Cell line	$IC_{50}/\mu\text{g mL}^{-1}$		
	F11	Tamoxifen	
Cancer cell	HeLA Cell	$27 \pm 2.6^{***}$	$3.3 \pm 1.5$
Non-cancerous cell	L929	$45 \pm 1.5^{**}$	$1.0 \pm 0.006$
	NIH 3T3	$70 \pm 4.0^{**}$	$1.3 \pm 0.1$

represented in the interphase stage of cell cycle. Based on the results (Figure 2), there were no significance changes in DNA content in each phase of the cell under normal conditions (untreated). The expected pattern showed for continuing cell growth as for region at  $G_1$  phase is the highest peak and the lowest peak is at  $G_2/M$  phase. At  $G_1$  phase, the DNA content were  $41.54 \pm 0.32\%$ ,  $54.83 \pm 1.14\%$  and  $53.35 \pm 1.35\%$  at 24, 48 and 72 hours respectively. During S phase, the DNA content were  $41.27 \pm 1.14\%$  at 24 hours,  $28.39 \pm 1.81\%$  at 48 hours and  $31.26 \pm 0.61\%$  at 72 hours. For  $G_2/M$  phase, throughout 24, 48 and 72 hours, it

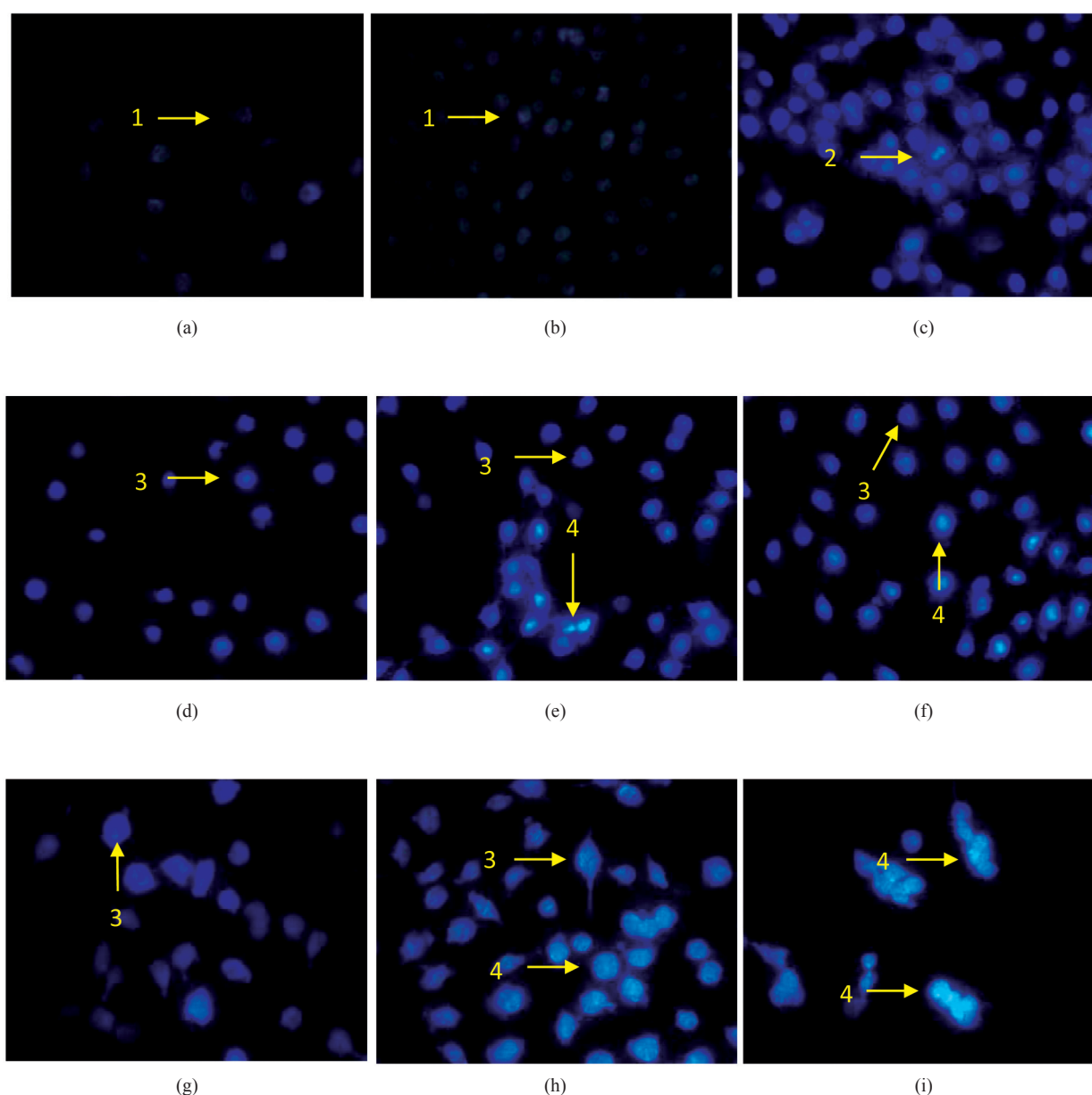


FIGURE 1. Nuclear staining of HeLA cells with Hoescht stain 33258. Cell were left untreated (a, b and c), treated with  $27 \mu\text{g/mL}$  of F11 (d, e, f) and  $3.3 \mu\text{g/mL}$  with tamoxifen for 24, 48 and 72 hours (g, h, i) respectively. Arrow indicates (1) unstained (2) bright fluorescence (3) apoptotic body (4) nuclear condensation. Magnification:  $40\times$

remains the lowest peak, which were  $17.19 \pm 1.35\%$ ,  $16.78 \pm 0.86\%$  and  $15.48 \pm 1.22\%$  respectively. Since the pattern of cell cycle distribution was almost the same throughout all time point, there is no cell cycle arrest happened for untreated HeLA cell.

In F11-treated HeLA cells, the profile of the cell cycle changed upon treatment. At 24 hours after treatment, DNA content at  $G_1$  phase increased up to  $49.47 \pm 7.71\%$ , decreased to  $34.43 \pm 8.9\%$  in S phase and declined to  $15.6 \pm 3.8\%$  in the  $G_2/M$  phase. After 48 hours of treatment, the DNA content at  $G_1$  phase was  $48.42 \pm 8.05\%$ , which was similar with the DNA content at  $G_1$  phase of the 24 hours of treatment. The DNA content was slightly decreased to  $38.92 \pm 5.14\%$  at S phase, and further declining to  $12.64 \pm 3.14\%$  at  $G_2/M$  phase. At 72 hours of treatment, the DNA content at  $G_1$  phase continued to increase up to  $74.91 \pm 6.22\%$  while in S phase, its dramatically decreased to  $15.48 \pm 6.48\%$  and at  $G_2/M$  phase, the DNA content declined to  $9.25 \pm 0.53\%$ . These results showed that the proliferation of HeLA cells treated with F11 was inhibited during  $G_1$  phase. This is because there was increment of DNA content at this phase and decrement during S phase and  $G_2/M$  phase.

Cells treated with tamoxifen treatment for positive control, the growth peaks were dramatically increased at  $G_1$  phase throughout 24 to 72 hours with the DNA content percentage were  $44.36 \pm 1.23\%$  for 24 hours,  $60.28 \pm 0.66\%$  at 48 hours and  $82.44 \pm 5.01\%$  at 72 hours. The DNA content during S phase decreased to  $41.53 \pm 0.84\%$  at 24 hours,  $25.98 \pm 0.71\%$  at 48 hours and  $11.72 \pm 4.9\%$  at 72 hours. During  $G_2/M$  phase, the DNA content declined to  $14.11 \pm 1.61\%$  at 24 hours and  $13.75 \pm 0.16\%$  at 48 hours. The major declined on  $G_2/M$  occurred at 72 hours with the

percentage of  $6.28 \pm 1.78\%$ . This indicate that the cycle arrest for tamoxifen-treated HeLA cell cycle arrest at the  $G_1$  phase. Figure 2 showed the results of cell cycle distribution by flow cytometry displayed in DNA histograms.

#### CHARACTERIZATION OF F11

F11 was subjected to LCMS analysis in order to characterize the active anticancer compound present. Four major peaks from negative polarity and five major peaks from positive polarity in chromatogram were selected. These major peaks are believed responsible for the anticancer effect. Major known constituents in F11 at the negative polarity are 19-Oxo-all-trans-retinoic acid (3.76%), (2S)-2-( {2-[1-(propan-2-yl)-1H-1,2,4-triazol-5-yl]-5,6-dihydroimidazo[1,2-d][1,4]benzoxazepin-9-yl} oxy)propanamide (2.19%), pregnenolone sulfate (1.83%) and 9,13-di-cis-retinoic acid (1.72%). There was one unknown compound,  $C_{21}H_{30}O_6S$  (1.61%), among the major peaks that did not match with the METLIN database (Table 3). As for positive polarity, the known constituents for major peaks are tetradecaethyleneglycol (4.59%), Elaidamide (9.63%) and 19-oxo-all-trans-retinoic acid (2.45%). There were three unknown major compounds that did not have any match with the library, METLIN database. The compounds are  $C_{23}H_{42}N_4O_8$  (4.71%),  $C_{25}H_{46}N_4O_9$  (4.95%) and  $C_{27}H_{50}N_4O_{10}$  (5.26%) (Table 4). The LCMS chromatograms of the negative and positive mode are as shown in the Figure 3 and Figure 4 respectively. These major compounds were selected with db score higher than 90%, which indicated that they have the highest similarity with the compounds on the database.

TABLE 3. List of constituents (major peaks) identified by LCMS scan on F11 for negative polarity

No.	Formula	Compound	Retention time	Mass	% of volume
1	$C_{20}H_{26}O_3$	19-Oxo-all-trans-retinoic acid	16.864	314.1894	3.76
2	$C_{19}H_{22}N_6O_3$	(2S)-2-( {2-[1-(propan-2-yl)-1H-1,2,4-triazol-5-yl]-5,6-dihydroimidazo[1,2-d][1,4]benzoxazepin-9-yl} oxy)propanamide	16.88	382.176	2.19
3	$C_{21}H_{30}O_6$	Unknown	16.88	410.1769	1.61
4	$C_{21}H_{32}O_5$	pregnenolone sulfate	18.972	396.1972	1.83
5	$C_{20}H_{28}O_2$	9,13-di-cis-retinoic acid	18.972	300.209	1.72

TABLE 4. List of constituents (major peaks) identified by LCMS scan on F11 for positive polarity

No.	Formula	Compound	Retention time	Mass	% of volume
1	$C_{23}H_{42}N_4O_8$	Unknown	9.231	502.3009	4.71
2	$C_{25}H_{46}N_4O_9$	Unknown	9.359	546.3266	4.95
3	$C_{27}H_{50}N_4O_{10}$	Unknown	9.488	590.3528	5.26
4	$C_{28}H_{58}O_{15}$	tetradecaethyleneglycol	9.585	634.3784	4.59
5	$C_{20}H_{26}O_3$	19-oxo-all-trans-retinoic acid	16.954	314.1968	2.45
6	$C_{18}H_{35}NO$	Elaidamide	21.058	281.2731	9.63

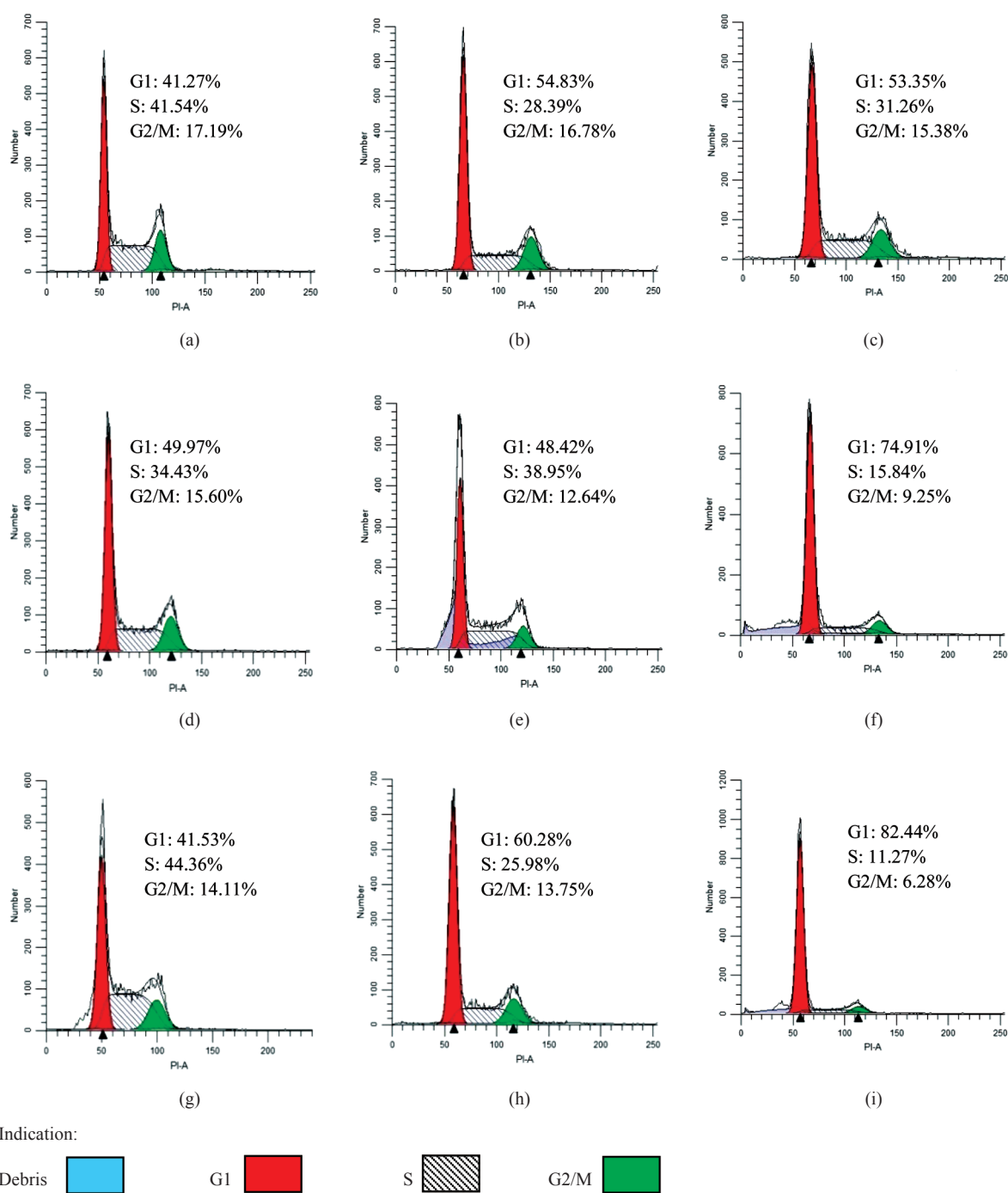


FIGURE 2. The histogram profile of cell cycle progression detected by flowcytometry analysis in untreated (a – c) HeLA cell (negative control), F11-treated (d – f) HeLA cell and Tamo-treated (g – i) HeLA cells (positive control) for 24 hours (column 1), 48 hours (column 2) and 72 hours (column 3)

#### CHARACTERIZATION OF ISOLATED F11

F11 was further isolated by column chromatography. There were four major fractions collected. These four fractions were analyzed using LCMS for characterization and determination of major compound. These four fractions were namely F11-a, F11-b, F11-c and F11-d.

The characterization was conducted to confirm whether the active anticancer compound in F11 could be selected as the major compound in CN. The major compound for all isolated fraction, F11-a, F11-b, F11-c and F11-d were summarized in Table 5 to Table 12. Each of the fractions shows the compound for negative and positive polarity.

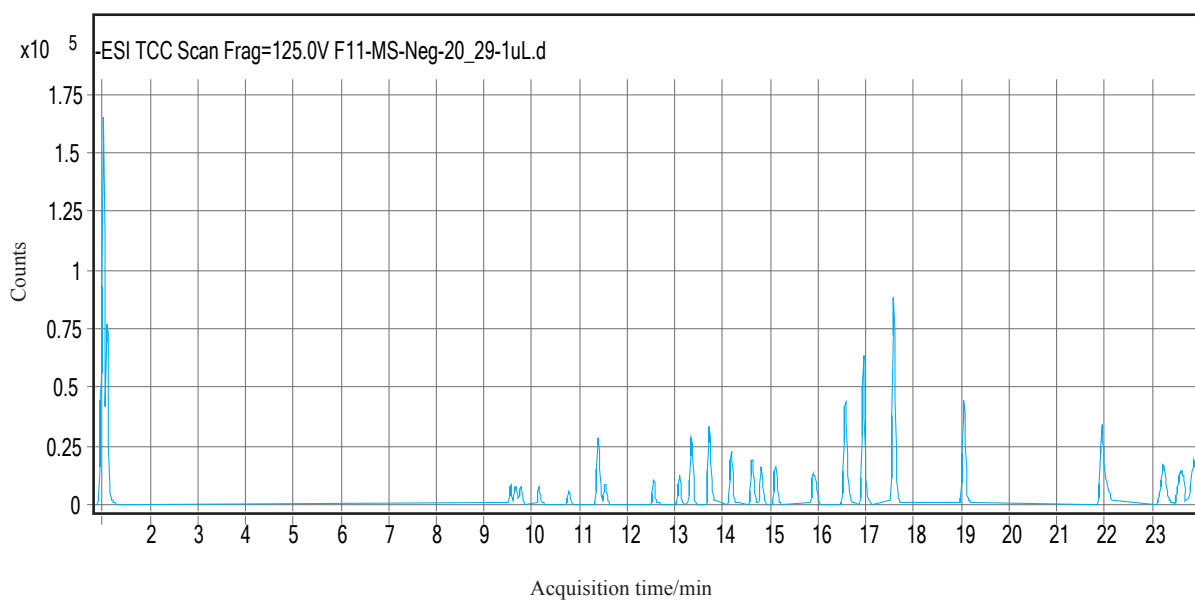


FIGURE 3. LCMS chromatogram of negative mode for F11

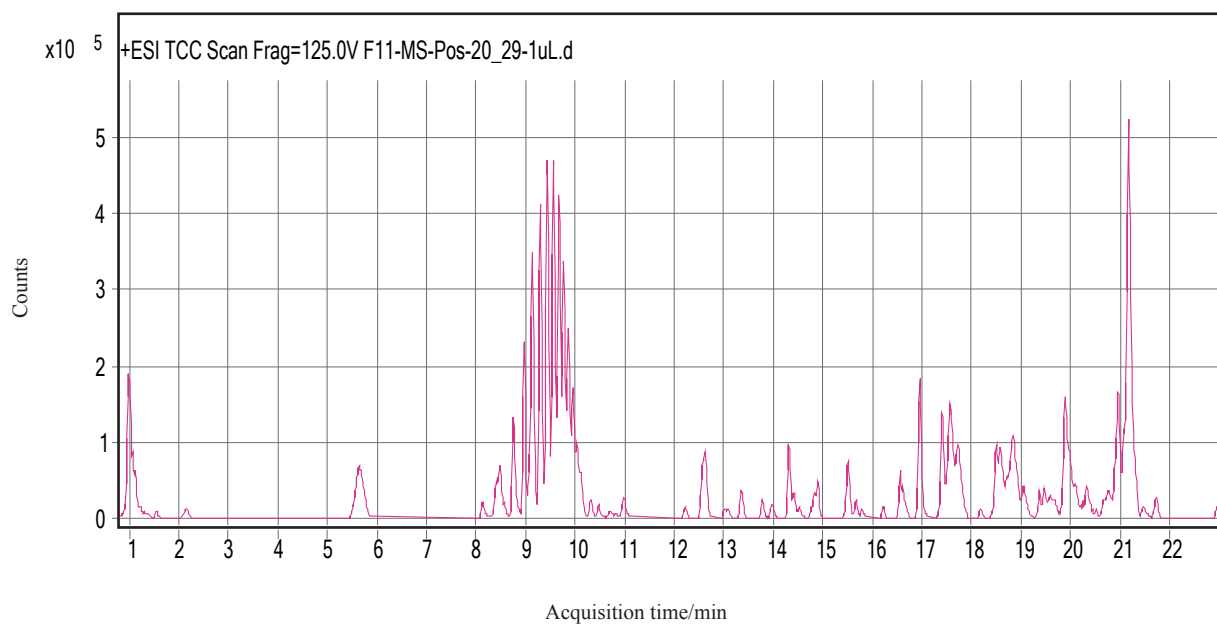


FIGURE 4. LCMS chromatogram of positive mode for F11

TABLE 5. List of constituents (major peaks) identified by LCMS scan on F1 for negative polarity

No.	Formula	Compound	Retention time	Mass
1	$C_{33}H_{22}N_6O_9S_3$	Unknown	16.279	742.0603
2	$C_{21}H_{26}O_3$	2-Methoxy-17a-ethynylestradiol	17.852	326.1894
3	$C_{21}H_{26}O_3$	Unknown	18.286	326.1913
4	$C_{14}H_{30}O_4S$	Tetradecyl sulfate	18.608	294.1851
5	$C_{20}H_{30}Cl_3N_7O$	Unknown	20.227	489.1582



TABLE 6. List of constituents (major peaks) identified by LCMS scan on F1 for positive polarity

No	Formula	Compound	Retention Time	Mass
1	C <sub>28</sub> H <sub>8</sub> N <sub>6</sub> O <sub>3</sub>	Unknown	8.239	476.0671
2	C <sub>25</sub> H <sub>20</sub> N <sub>12</sub> S	Unknown	8.407	520.1654
3	C <sub>20</sub> H <sub>32</sub> N <sub>14</sub> O <sub>6</sub>	Unknown	8.554	564.2633
4	C <sub>20</sub> H <sub>40</sub> N <sub>20</sub> O <sub>3</sub>	Unknown	8.685	608.3599

TABLE 7. List of constituents (major peaks) identified by LCMS scan on F2 for negative polarity

No	Formula	Compound	Retention Time	Mass
1	C <sub>20</sub> H <sub>26</sub> O <sub>3</sub>	19-Oxo-all-retinoic acid	15.421	14.1876
2	C <sub>17</sub> H <sub>28</sub> O <sub>3</sub> S	Unknown	17.038	312.176
3	C <sub>14</sub> H <sub>21</sub> NO <sub>3</sub>	Unknown	17.124	251.1518
4	C <sub>18</sub> H <sub>30</sub> O <sub>3</sub> S	Unknown	18.279	326.192
5	C <sub>14</sub> H <sub>30</sub> O <sub>4</sub> S	Tetradecyl sulfate	18.528	294.1865

TABLE 8. List of constituents (major peaks) identified by LCMS scan on F2 for positive polarity

No	Formula	Compound	Retention Time	Mass
1	C <sub>10</sub> H <sub>18</sub> N <sub>2</sub> O <sub>2</sub>	Slaframine	1.962	198.1368
2	C <sub>5</sub> H <sub>9</sub> NO	δ-Valerolactam	1.962	99.0686
3	C <sub>25</sub> H <sub>30</sub> N <sub>10</sub> O <sub>9</sub>	Unknown	8.827	634.3766
4	C <sub>20</sub> H <sub>26</sub> O <sub>3</sub>	19-oxo-all-trans-retinoic acid	15.422	314.1881
5	C <sub>38</sub> H <sub>30</sub> ClN <sub>3</sub> O <sub>4</sub>	Unknown	20.685	627.1929

TABLE 9. List of constituents (major peaks) identified by LCMS scan on F3 for negative polarity

No	Formula	Compound	Retention Time	Mass
1	C <sub>12</sub> H <sub>26</sub> O <sub>4</sub> S	Lauryl hydrogen sulfate	16.009	266.1544
2	C <sub>18</sub> H <sub>30</sub> O <sub>3</sub> S	Unknown	18.317	326.1914
3	C <sub>14</sub> H <sub>30</sub> O <sub>4</sub> S	Tetradecyl sulfate	18.557	294.186
4	C <sub>19</sub> H <sub>32</sub> O <sub>3</sub> S	Unknown	19.649	340.207
5	C <sub>20</sub> H <sub>26</sub> N <sub>4</sub> O	Lisuride	19.846	338.2123

TABLE 10. List of constituents (major peaks) identified by LCMS scan on F3 for positive polarity

No	Formula	Compound	Retention time	Mass
1	C <sub>6</sub> H <sub>4</sub> Cl <sub>2</sub> N <sub>2</sub> O	Unknown	0.585	189.9714
2	C <sub>5</sub> H <sub>9</sub> NO	δ-Valerolactam	1.958	99.0678
3	C <sub>24</sub> H <sub>50</sub> O <sub>13</sub>	Unknown	8.557	502.2983
4	C <sub>12</sub> H <sub>10</sub> O <sub>2</sub> S	Cis-1,2-Dihydroxy-1,2- dihydrodibenzothiophene	12.843	218.0405
5	C <sub>38</sub> H <sub>30</sub> ClN <sub>3</sub> O <sub>4</sub>	Unknown	20.684	627.1924

TABLE 11. List of constituents (major peaks) identified by LCMS scan on F4 for negative polarity

No	Formula	Compound	Retention time	Mass
1	C <sub>13</sub> H <sub>18</sub> O <sub>3</sub>	Unknown	12.426	222.1271
2	C <sub>16</sub> H <sub>20</sub> N <sub>6</sub> O	Unknown	17.522	312.1709
3	C <sub>21</sub> H <sub>26</sub> O <sub>3</sub>	2-Methoxy-17a-ethynylestradiol	18.309	326.1863
4	C <sub>21</sub> H <sub>24</sub> O <sub>5</sub>	8-Prenylafzelechin 5-methyl ether	20.268	356.162

TABLE 12. List of constituents (major peaks) identified by LCMS scan on F4 for positive polarity

No	Formula	Compound	Retention time	Mass
1	C <sub>5</sub> H <sub>9</sub> NO	δ-Valerolactam	1.959	99.0685
2	C <sub>24</sub> H <sub>50</sub> O <sub>13</sub>	Unknown	8.556	546.3252
3	C <sub>12</sub> H <sub>10</sub> O <sub>2</sub> S	Cis-1,2-Dihydroxy-1,2- dihydrodibenzothiophene	12.834	180.1146
4	C <sub>20</sub> H <sub>26</sub> O <sub>3</sub>	19-oxo-all-trans-retinoic acid	15.421	314.1885
5	C <sub>38</sub> H <sub>30</sub> ClN <sub>3</sub> O <sub>4</sub>	Unknown	20.684	627.1924

## DISCUSSION

Normally, chemotherapy or neoadjuvant chemotherapy is always the first chosen strategy to treat cancer patients. However, the use of chemotherapy is limited due to the lack of selectivity and too many side effects (Sultana et al. 2003). Therefore, the researchers need to explore more potent antitumor drugs with lesser or minimal side effect as new sources of pharmacologically active compounds especially from natural sources (Shi et al. 2006). According to the study by Yong Keong Yok (2013), the value of  $IC_{50}$  obtained from the CN chloroform extract was 36.31  $\mu\text{l}/\text{mg}$  against HeLA cell. However, fraction of CN F11, exhibited lower value of  $IC_{50}$  against HeLA cell which is 27  $\mu\text{g}/\text{mL}$ . This is because of the pure substance existed in the isolated compound as compared to the crude extract, which consists of various amounts of active constituents (Colegate & Molyneux 2007). A study from Rodrigues et al. (2011) also showed that the isolated compound exhibited stronger cytotoxicity compared to the crude extract due to the presence of other compound involved in antiproliferative activity.

Meanwhile, the anti-proliferative activity for normal cell lines, normal fibroblast NIH 3T3 and L929 normal connective cells, were also tested to see whether F11 is toxic towards them. There were no effects of F11 towards these cells as compared to HeLA cell. This was supported with the finding by Yong et al. (2013) which showed that the normal cell used for this study was human umbilical veins endothelial cell (HUVECs) exhibited lowest percentage of inhibition as compared to other cancer cells when treated with CN extract. If the value of  $IC_{50}$  is more than 99  $\mu\text{g}/\text{ml}$  against the normal cell, it is considered as no inhibitory effect on cells (Zazali et al. 2013). According to the US NCI plant-screening program,  $IC_{50}$  of a pure compound less than 4  $\mu\text{g}/\text{ml}$  is considered to have *in vitro* cytotoxic activity (Zakaria et al. 2009). Since the  $IC_{50}$  value of F11 towards normal cell is more than 4  $\mu\text{g}/\text{mL}$ , it does not affect the growth of normal cell. The selective cytotoxicity is may be due to the difference of the genetics, biochemical and molecular of the mitochondria in cancer and normal cell line (Mclanchan et al. 2005). The metabolic rates of cancer cells are higher compared to non-malignant cells, which may be related to the changes in the mitochondrial TCA cycle (Piva & McEvoy-Bowe 1998). This will make the tumor mitochondria to be unstable (Liu et al. 2007) and thus, making these cells more sensitive to F11.

One of the ways to determine the potential anticancer of drugs or extracts is the ability to induce apoptosis in cancer cells. It is important to ensure that the drugs or extract induce apoptosis in cancer cells because apoptosis eliminates cancer cells while not affecting normal cells and its surrounding tissues (Ashkezanki 2008). In this study, the morphology of the cells was determined by fluorescent microscope. The AT rich regions of double stranded DNA showed strong fluorescence because of the binding to Hoechst dye (Zakaria et al. 2009). The blue fluorescence

Hoechst 333248 was able to diffuse into the membrane of HeLA cells and stained their membrane. In this study, treatment with  $IC_{50}$  value of F11 induced apoptosis in HeLA cells. Cell treated with F11 at 24 hours, started to show nuclear chromatin condensation, cytoplasmic vacuolation, nuclear fragmentation and presence of apoptotic bodies which in line with findings of previous studies. (Zakaria et al. 2009; Rahman et al. 2013; Beedessee et al. 2012). The DNA fragmentation showed at 24 hours of treatment is the early event of apoptosis that represent irreversible process to cell death (Allen et al. 1997). Increase in the nuclear membrane permeability of the apoptotic cell makes the fluorescence dye more intense which indicates chromatin condensation and nuclear fragmentation (Abdullah et al. 2010). This observation was also observed in tamoxifen-treated HeLA cells at all time point. This observation proved that the HeLA cells treated with F11 underwent apoptotic pathway.

Determination of cell cycle distribution in cells treated with F11 was analyzed using flow cytometer via the measurement of DNA content. Upon treatment with F11, the population at  $G_1$  phase increased but the cell growth was inhibited when entering S phase because of the stress or DNA damage that was triggered by F11. Thus, there is no growth occurred in  $G_2/M$  phase. The checkpoint arrest at  $G_1/S$  phase is important to avoid the replication of the cell and to allow damaged DNA to be repaired (Vermeulen et al. 2003). As for this study, the growth of the cell arrest at  $G_1$  checkpoint after 72 hours showed that cell division has stopped completely since DNA synthesis in HeLA cell cannot be occur. The cell cycle distribution for Tamoxifen-treated HeLA cells showed similar pattern to the F11-treated HeLA cells.

Identification of active compound present in F11 was analyzed by using LCMS. LCMS is one of the powerful analytical tools for identification of organic compounds as it provides molecular weight and chemical structure information. The major peaks showed in the chromatogram are believed to contribute to the anticancer effect. The constituents in F11 that has been detected by LCMS as negative polarity are 19-Oxo-all-trans-retinoic acid, (2S)-2-(2-[1-(propan-2-yl)-1H-1,2,4-triazol-5-yl]-5,6-dihydroimidazo[1,2-d][1,4]benzoxazepin-9-yl)oxy) propanamide, pregnenolone sulfate and 9,13-di-cis-retinoic acid and at for positive polarity, the major constituent are tetradecaethyleneglycol and Elaidamide. Out of all major constituents present in F11, only 19-oxo-all-trans-retinoic acid and 9,13-di-cis-retinoic acid exhibit anticancer effect based on previous studies (Adamson 1996; Uchida et al. 2001; Akira et al. 1993). So, it is assumed that 19-oxo-all-retinoic acid is the active anticancer compound and to confirm that this is the major compound, double column chromatography was conducted on F11. The results showed that 19-oxo-all-trans-retinoic acid was present in F11-b (in negative and positive mode) and F11-d (positive mode) and it was detected at the same retention time as F11. Thus, 19-oxo-tran-retinoic acid is confirmed to be as one of the

major compounds. In this study, the active compound that is responsible for the anticancer effect is 19-oxo-all-trans-retinoic acid since the volume of all-trans-retinoic acid in F11 is higher than 9,13-di-cis-retinoic acid and this compound was also detected in the isolated fraction, which prove this is the major compound.

For the unknown compound, this new compound has not been established and known yet since it cannot be identified in the database. However, it is assumed that  $C_{21}H_3O_6S$  is a conjugate acid since the compound is similar with prenenolone acid. As for  $C_{23}H_{42}N_4O_8$ ,  $C_{25}H_{46}N_4O_9$  and  $C_{27}H_{50}N_4O_{10}$ , it is assumed to be alkaloid since the chemical compound contain basic nitrogen atom. These compounds need to be isolated from F11 in order to obtain pure compound and then analyze by NMR to identify the name and structure of the compound.

### CONCLUSION

Fraction 11 (F11) from the elution of ethyl acetate: hexane inhibited cells growth at G1 phase checkpoint indicating that the cell division has stop at this point. The mechanism of cell death induce by F11 was via apoptosis. The active compound believed to contribute to the anticancer properties is 19-oxo-all-trans-retinoic acid. This can be concluded that the active compound of CN, 19-oxo-all-trans-retinoic acid, demonstrates promising anticancer effect by inducing apoptosis in HeLA cells.

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- Siti Nur Fatimah Mohd Roslan  
Yusmazura Zakaria  
Hasmah Abdullah  
School of Health Science  
Universiti Sains Malaysia  
16150 Kubang Kerian  
Kelantan, Malaysia
- Corresponding author: Yusmazura Zakaria  
E-mail: yusmazura@usm.my
- Tel: +609 767 7781  
Fax: +609 767 7515
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