

CYTOTOXIC, ANTIPROLIFERTIVE AND APOPTOTIC ACTIVITY OF SECONDARY METABOLITES FROM *ASPERGILLUS UNQUIS* CRI282-03 ON HUMAN COLON CANCER CELL LINE (COLO 205)

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Abstract: The purpose of this study is to study the antiproliferative and cytotoxic effect of three secondary metabolites which are nidulin, aspergillusidone D, and unguinol from marine-derived fungi *Aspergillus unguis* CRI282-03 on colon cancer cells (COLO 205). In the course of a microbial screening process, *A. unguis* CRI282-03 was isolated from soil samples collected at the Royal Navy Base in Tub-La-Mu bay, Pang-nga Province, Thailand. The isolated fungus of interest was cultivated in Chulabhorn Research Institute (CRI). Nidulin, aspergillusidone D and unguinol were tested on COLO 205 cell. In addition, the cytotoxic activity of those three pure constituents were determined using MTT assay, the antiproliferative as well as the anti-apoptotic activity were evaluated by flow cytometry method. The result from MTT assay showed that IC₅₀ value of nidulin, aspergillusidone D, and unguinol on COLO 205 cells were 15, 21 and 33 µM, respectively. Furthermore, nidulin provided anticancer activity through inducing apoptosis to COLO 205. From the performed assay nidulin showed greater antiproliferation activity than aspergillusidone D.

Keywords: *Aspergillus unguis* CRI282-03, nidulin, aspergillusidone D, unguinol, antiproliferative activity

บทคัดย่อ: งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ด้านการเจริญแบ่งตัวของเซลล์มะเร็งและทดสอบความเป็นพิษของสารทุติยภูมิสามชนิด ได้แก่ nidulin, aspergillusidone D และ unguinol ต่อเซลล์มะเร็งลำไส้ใหญ่มนุษย์ (COLO 205) ซึ่งสารดังกล่าวแยกได้จาก marine-derived fungi *Aspergillus unguis* CRI282-03 โดย *A. unguis* CRI282-03 ถูกเก็บจากดินที่ฐานทัพเรือทับละมุ จังหวัดพังงา ประเทศไทย เชื้อราถูกทำการแยกสารที่สถาบันวิจัยจุฬาภรณ์ สารทั้งสามชนิดได้นำมาทดสอบความเป็นพิษต่อเซลล์มะเร็งลำไส้ใหญ่ ด้วยวิธี MTT assay และทดสอบฤทธิ์ด้านการเจริญแบ่งตัวและทดสอบการตายแบบ apoptosis ของเซลล์มะเร็งโดย flow cytometry method ผลจากการทดสอบความเป็นพิษต่อเซลล์มะเร็งลำไส้ใหญ่ COLO 205 ด้วยวิธี MTT assay ของสาร nidulin, aspergillusidone D และ unguinol พบว่ามีค่า IC₅₀ เท่ากับ 15, 21 และ 33 ไมโครโมลาร์ ตามลำดับ นอกจากนี้ยังพบว่าสาร nidulin สามารถเหนี่ยวนำให้เซลล์มะเร็งลำไส้ใหญ่ COLO 205 เกิดการตายแบบ apoptosis และสาร nidulin มีฤทธิ์ด้านการเจริญแบ่งตัวของเซลล์มะเร็งที่ต่ำกว่า aspergillusidone D

คำสำคัญ: *Aspergillus unguis* CRI282-03, nidulin, aspergillusidone D, unguinol, antiproliferative activity

INTRODUCTION

Nowadays, the development of medicines are generally derived from chemical synthesis natural products and also chemical modification of natural products. In addition, 60% of drug used in treatment of infectious diseases were derived from natural products moreover, about 75% of anticancer drugs derived from natural products which was stated by Cancer Facts and statistics. The drug is widely known as aspirin is found in many plant species in the genus *Salix* and *Populus* and the discovery of penicillin drug, which is derived from the fungus *Penicillium notatum*. This is the noticeable starting point and draw attention to the study of biological active substances from fungi (Newman et al., 2003). The fungi are able to adapt and to prevent the invasion of the enemy by producing substances for their survive and many of its substances have shown several activities which can be beneficial for inventing new medicines. For example, antibiotics derived from the fungi include, streptomycin, erythromycin, rifamycin and amphotericin and drug used for dyslipdemia derived from fungi such as pravastatin and lovastatin.

Natural products are a source of compounds with diverse chemical and biological effects of current research in pharmaceutical companies have focused on finding compounds from natural products in order to use as a medicine directly or use as a chemotype for a structural change to a more favorable biological activity. There has been reported that several compounds from natural products and its derivatives can be modified the chemical structures and show interesting biological activity. Some substances currently in clinical trials, which tend to develop to be medicines, such as anticancer drugs.

The past study of bioactive compounds from fungi found that the extracts were useful in many medical issues. One of good example was the extract called terrein, which was extracted from the fungus *Aspergillus terreus* that illustrated to inhibit the formation of melanin in the melanocyte cell line by reducing the activity of tyrosinase enzyme, resulting in inhibition of melanin (Park, S.H., et al., 2004) and reduces inflammation of the dental pulp cells (Lee et al., 2008)(Yu et al., 2009). However, there were no reports of toxicity of the extracts terrein on a wild range of cancer cell types. The bioactive extracts from the fungus *Aspergillus terreus* is aspernolides A, aspernolides B and butenolides related to the inhibition of cyclin dependent kinase (cdk), which play an important role in the detection of abnormal cells within the cell cycle division (Parvatkar et al., 2009). Studies of terein on HeLa cell (human cervical carcinoma) found that terein inhibited the proliferation of HeLa cell by an IC_{50} of 0.29 mM (Porameesanaporn et al., 2013) and also had the inhibited effect on a wild range cancer cell types, including human cholangiocarcinoma cell line (HuCCA-1), human mouth epidermal carcinoma cells (KB), human mammary gland/breast cancer cell (MDA-MB-231), human mammary gland cancer cell (T47D) human epithelial lung cancer cell (H69AR) human promyelocytic leukemia cell (HL-60) and mouse lymphoblastic leukemia cell (P388) with IC_{50} values of 3.6, 3.0, 4.5, 2.5, 3.0, 6.27 and 6.22 $\mu\text{g} / \text{ml}$, respectively (Jongrungruangchok et al., 2008).

In this work, focus on substances derived from the fungus *Aspergillus unguis* CRI282-03 and also five to six known substances that their structures have already been reported. Aspergillusidone C (Sureram, S., et al., 2013) compound shows cytotoxic to breast cancer cells with the IC_{50} of 0.74 μM , which is close to the reference compound doxorubicine (IC_{50} 1.27 $\mu\text{g}/\text{ml}$) and show no toxicity to normal cells. Depsidones expressed the inhibition of aromatase enzyme activity with the IC_{50} 1.2-11.2 μM and also showed mild toxicity to human cholangiocarcinoma cell (HuC-CA-1), human hepatocellular carcinoma cell (HepG2), human alveolar basal epithelial carcinoma cell (A549) and human acute T lymphoblastic leukaemia cell (MOLT-3) (Sureram, S., et al., 2012).

This study has targeted to investigate the toxicity of aspergillusidone D, nidulin and unguinol, which are isolated from the fungus *A. unguis* CRI282-03 whether the substances can inhibit the proliferation of human colon adenocarcinoma cell line (COLO 205) and also normal fibroblast from mouse (L929) or not.

The apoptosis assay is determined by characterisation of cellular physiological changes such as externalization of phosphatidylserine (PS) to the cell surface, cleavage and degradation of specific cellular proteins, compaction and fragmentation of nuclear chromatin, and loss of membrane integrity (in late stages). The cell cycle assay are analysed by using flow cytometry describes the process of the replication and division of chromosomes within the nucleus, which occurs prior to cell division. By labeling cellular DNA with propidium iodide (PI) to discriminate cells in different stages of the cell cycle. Resting cells (G0/G1phase) contain two copies of each chromosome. As cells progress toward mitosis, they synthesise DNA (S phase), allowing more PI intercalation with a resulting increase in fluorescence intensity.

The aims of this study are firstly, to investigate the antiproliferation activity of substances such as depsidones and aspergillusidone D which are isolated from the fungus *A. unguis* CRI282-03 on COLO 205. Secondly, to study the deformation of nucleus (apoptosis assay) and to study cell cycle focusing at the DNA content in COLO 205 by flow cytometry method. Finally, to determine the cytotoxic effect of depsidones and aspergillusidone D on normal cell types (L929).

MATERIALS AND METHODS

Fungal material

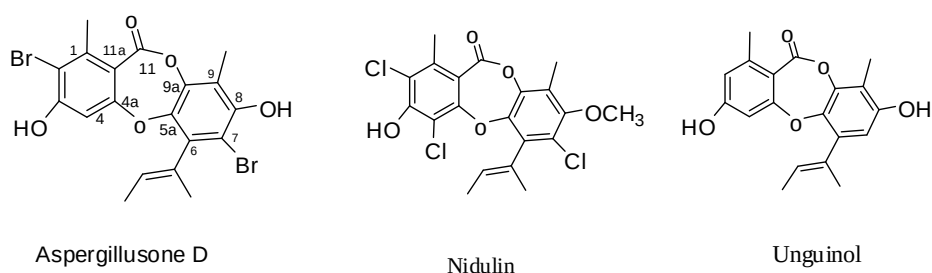
The marine-derived fungus *A. unguis* CRI282-03 was isolated unidentified marine sponge CRI282 and identified the fungus based on both the morphological characteristics and the analysis of DNA sequences of the ITS1–5.8S-ITS2 ribosomal RNA gene region. (Sureram et al., 2012)

Morphological characteristics

CRI282-03 fungus was cultured in on potato dextrose agar at 25 °C grew restrictedly 2.5 cm in 14 days.

Extraction and isolation of bioactive compounds

The fungus *A. unguis* CRI282-03 is cultured in potato dextrose broth (PDB) prepared in two solution which are KBr and KI solution with a salinity of 33 ppt. (Sureram et al., 2013)



COLO 205 cell line

Colon cancer cells COLO 205 which maintain characteristics of colon epithelial cell derived from ascites metastatic site in culture were routinely incubated with Roswell Park Memorial Institute (RPMI) 1640 medium containing L-glutamine and phenol red, supplemented with 10% (v/v) fetal bovine serum, 0.1% penicillin-streptomycin and hepes (complete RPMI). The cell cultures were incubated at a temperature of 37°C in humidified air containing 5% CO₂.

Preparation of COLO 205 cells

COLO 205 cells were cultured on 75-cm² Nunc flasks (Bang Trading 1992 Co.,Ltd., Thailand) with complete RPMI and incubated at 37°C in humidified air and 5% CO₂. Culture medium was changed every 48 hours to assure that essential nutrition was available to the cells. For further experiments COLO 205 were sub-cultured at 85-100% confluence by using Trypsin-EDTA mixture to detach cells from the coated plate surface and passage into 75-cm² Nunc flasks or onto 24 well Nunc plates for further *in vitro* study.

Investigation of the Cytotoxic activity of nidulin, aspergillusidone D and unguinol on COLO 205 cells

COLO 205 cells were dosed separately at concentrations of 5, 10, 20, 40, 80 and 160 µM with nidulin, aspergillusidone D or unguinol and incubated for 24 hours, with the intention to allow this substance enter to the cells. After incubations were complete, cell viability was determined. This investigation was set up to determine the cytotoxic activities of nidulin, aspergillusidone D and unguinol on COLO 205 cells at various concentrations by using MTT assay.

MTT solution was freshly prepared in incubation medium at a concentration of 0.2 mg/ml MTT solution of which 500 µL was added to each well of 24 well plates which were then incubated for 30-45 minutes at 37°C in an incubator. At the end of the incubation, the MTT solution was removed and then 125 µL of was added to each well and the plate was gently shaken to dissolve the formazan crystals. 2x50µL aliquots were then transferred to wells of a 96 well plate so that the absorbance (540 nm) could be measured in duplicate (Berridge *et al.*, 2005).

Investigation of anticancer activity

COLO 205 which maintain characteristics of colon epithelial cell derived from ascites metastatic site in culture is routinely incubated with Roswell Park Memorial Institute (RPMI) 1640 medium containing L-glutamine and phenol red, supplemented with 10% (v/v) fetal bovine serum, 0.1% penicillin-streptomycin and hepes (complete RPMI) and incubated at 37°C in humidified air and 5% CO₂.

COLO 205 cell is dosed separately on 24 well plates at various concentrations and incubated for 24 hours, with the intention to allow this substance enter to the cells. After incubations were complete, cell viability was determined by MTT assay and report as IC₅₀. Also the cytotoxic activity of tested substances are measures on L929 cells by using MTT assay (Berridge, M.V., et al., 2005).

Investigation of the apoptosis assay

The investigation of the apoptosis mechanism and cell cycle study are determined by performing flow cytometry to look at cells respond to specific induction signals by initiating intracellular processes that result in characteristic physiological changes. Among these are externalisation of phosphatidylserine (PS) to the cell surface, cleavage and degradation of specific cellular proteins, compaction and fragmentation of nuclear chromatin, and loss of membrane integrity (in late stages).

Cells are treated with nidulin and aspergillusidone D at 5 μ M, and unguinol at 20 μ M and incubated for 24 hours in 24-well plates. After incubation with tested substances culture medium is removed from flask and place in a 50-mL conical screw cap tube. This is to retain any detached apoptotic or dead cells present in the flask. Wash cells with 10 mL of 1X PBS then remove PBS and place in 50-mL conical screw cap tube. (Passaging adherent cells then preparing of cell suspension at the concentrations between 2×10^4 and 1×10^5 cells/well (or 1×10^5 to 5×10^5 cells/mL). Allow Guava Nexin Reagent to warm to room temperature. Adding 100 μ L of cells in suspension to each well or tube. Subsequently, adding 100 μ L of Guava Nexin Reagent to each well or tube. Then measuring by Guava Nexin Software Module performs which will then calculations automatically. The results are displayed on the computer screen after each sample is acquired. Acquired data are displayed in dot-plot format with a user-controlled quadrant marker for instantaneous on-screen presentation of the results (Daigle, S.R., et al., 2011).

Investigation of the cell cycle assay

Cells are treated with nidulin and aspergillusidone D at 5 μ M, and unguinol at 20 μ M and incubated for 24 hours in 12-well plates after completed incubation with tested substances culture medium was collected. Cells are then added 1mL of 1X PBS to rinse any remaining cells. Transfer the 1X PBS from each well to the appropriate 15-mL conical tube. Centrifuge the tubes at 300 x g for 5 minutes then aspirate and discard the supernatant. Add 500 μ L of medium or PBS to each 15-mL conical tube. Mix the harvested cell sample by vortexing or pipetting repeatedly to ensure a homogenous suspension. (Adjusting cell samples to between 5×10^5 and 1×10^6 cells/mL using medium or PBS. Proceed to cell fixation using ice-cold 70% ethanol while vortexing on medium speed. After cell fixation is finished, add 1×10^5 to 2×10^5 cells in 200 μ L to 1 mL volume of ethanol-fixed cells to a 12 x 75 mm polystyrene test tube. Centrifuge ethanol-fixed cells at 450 x g for 5 minutes and remove supernatant. Adding 1X PBS and centrifuge at 450 x g for 5 minutes and Remove and discard the supernatant. Resuspend the cells in 200 μ L Guava Cell Cycle Reagent and incubate at room temperature for 30 minutes, shielded from light. Transfer all samples to 1.5-mL microcentrifuge tubes. Acquire the data on the Guava instrument (Daigle, S.R., et al., 2011).

Data and Statistical Analysis

Results are represented as mean and standard deviation. Data was analyzed and graphed with Microsoft Excel 2007 and GraphPad Prism, version 6.0. Significant differences between the mean values within the group were determined by using a Student's t test or one-way analysis of variance (ANOVA) followed by a Tukey test for further comparison. The level of significant was set up at $P \leq 0.05$. All graphs were drawn using GraphPad Prism.

RESULTS AND DISCUSSION

Effects of nidulin, aspergillusidone D and unguinol on cellular viability of L929 cells

L929 cells were incubated with nidulin, aspergillusidone D and unguinol at concentrations 0.5, 1.0, 1.5, 2.5, 5.0 and 10.0 μM for 24 hours. The result showed that there was not significant change in mean percentage of cell viability of all three constituents at the concentration of 0.5 μM to 5.0 μM . However, at the concentration of 10 μM all three constituents showed cytotoxic effects on L929 cells with a significantly different compare to untreated cells (Figure 2).

Effects of nidulin on cellular viability of COLO 205

COLO 205 was incubated with nidulin at concentrations 0, 5, 10, 20, 40, 80 and 160 μM for 24 hours. The result from this experiment found that there was a notable decrease in mean percentage of cell viability from 100% (0 μM) to 6% (40, 80 and 160 μM nidulin) (Figure 3). Additionally, nidulin at greater than 40 μM showed the percentage inhibition about 95% which all three concentrations, including 40, 80 and 160 μM were not significantly different from each other. This experiment demonstrated that nidulin is toxic to COLO 205 cells at increasing concentrations and the IC_{50} of nidulin was 15 μM (Figure 4).

Effects of aspergillusidone D on cellular viability of COLO 205

COLO 205 cells were exposed to aspergillusidone D at concentrations of 0, 5, 10, 20, 40, 80 and 160 μM for 24 hours. The result from this experiment demonstrated that there was a gradual reduction in mean percentage of cell viability from 100% (0 μM aspergillusidone D) to 55% (20 μM aspergillusidone D) and declined dramatically from 55% (20 μM aspergillusidone D) to 6% (40, 80 and 160 aspergillusidone D) (Figure 5). Although, there was a very slightly rise in mean percentage of cell viability observed at higher concentration, from 6% (40 μM) to 8% (80 μM), there was not significantly different from each other. In addition, this experiment demonstrated that aspergillusidone D is toxic to COLO 205 cells at increasing concentrations and the IC_{50} of aspergillusidone D was 21 μM (Figure 6).

Effects of unguinol on cellular viability of COLO 205

COLO 205 cells were treated with unguinol at concentrations 0, 5, 10, 20, 40, 80 and 160 μM for 24 hours. The result from this experiment found there was a very slightly decline in mean percentage of cell viability from 100% (0 μM) to 6% (160 μM unguinol) (Figure 7). Unguinol at concentration of 5 and 10 μM were about the same mean percentage cell viability at 89%. Interestingly, there was a gradually decline in mean percentage cell viability from 70% (20 μM unguinol) to 40% (40 μM unguinol) after which mean percentage cell viability significantly decreased again to 6% (80 μM) respectively, compared to control (unguinol 0 μM). This experiment demonstrated that unguinol has cytotoxic activity to COLO 205 cells at increasing concentrations and the IC_{50} of unguinol was 33 μM (Figure 8).

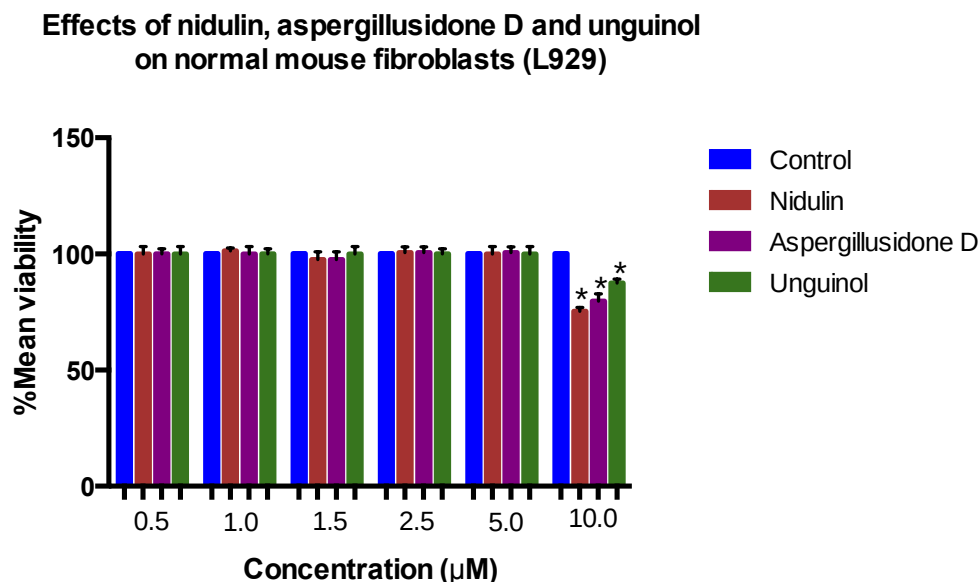


Figure 2. Bar chart illustrates mean percentage of cell viability and standard deviation of L929 cells against nidulin, aspergillusidone D and unguinol at increasing concentrations after 24 hours incubation. N = 3, * $P \leq 0.05$ vs. untreated cells.

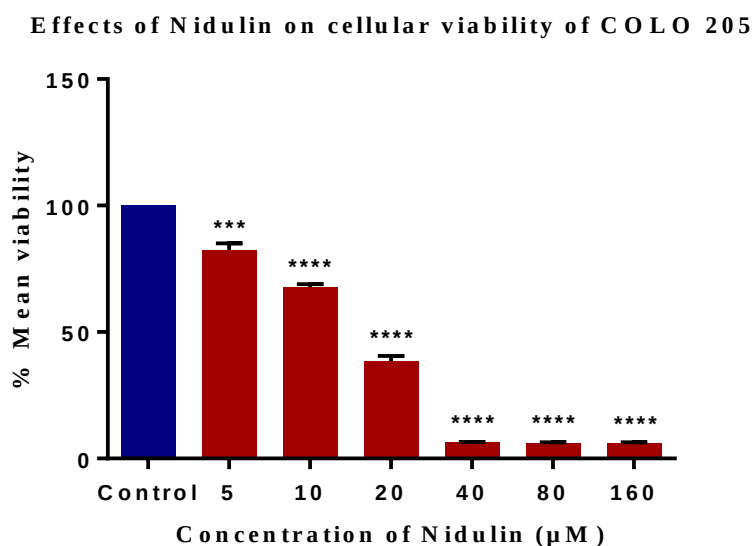


Figure 3. Bar chart illustrating mean percentage of cell viability and standard deviation of COLO 205 cells against nidulin at increasing concentrations after 24 hours incubation. N=6, **** $P \leq 0.0001$ vs. untreated cells (0 µM nidulin).

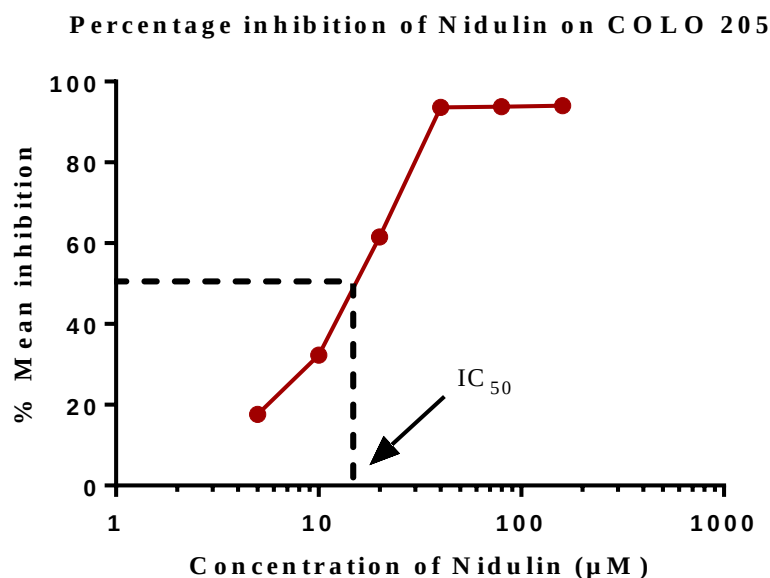


Figure 4. Dose-inhibition curve showing 50% inhibition concentration of nidulin on COLO 205 at 15 µM.

Effects of Aspergillusidone D on cellular viability of COLO 205

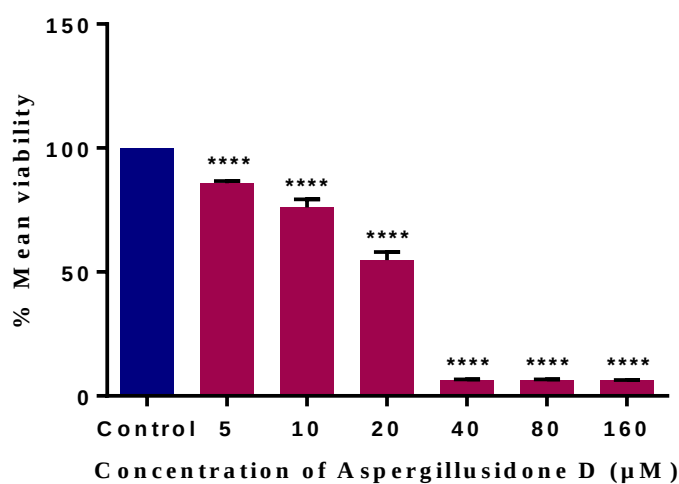


Figure 5. Bar chart illustrating mean percentage of cell viability and standard deviation of COLO 205 cells against aspergillusidone D at increasing concentrations after 24 hours incubation. N=6, ****P≤0.0001 vs. untreated cells(0 µM aspergillusidone D).

Percentage inhibition of Aspergillusidone D on COLO 205

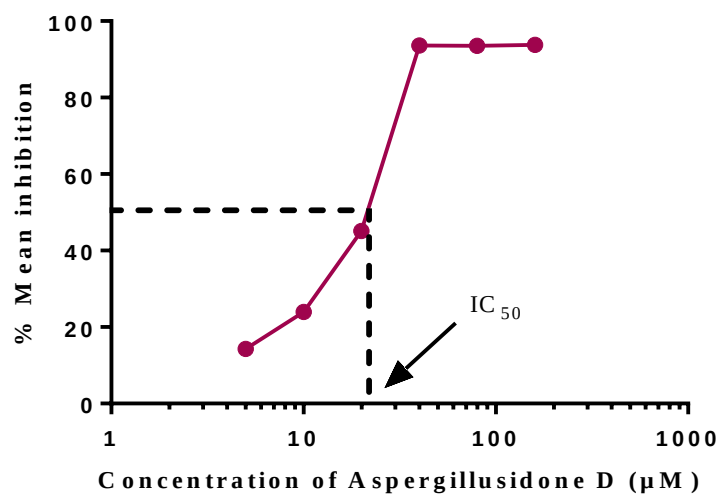


Figure 6. Dose-inhibition curve showing 50% inhibition concentration of aspergillusidone D on COLO 205 at 21 μM.

Effects of Unguinol on cellular viability of COLO 205

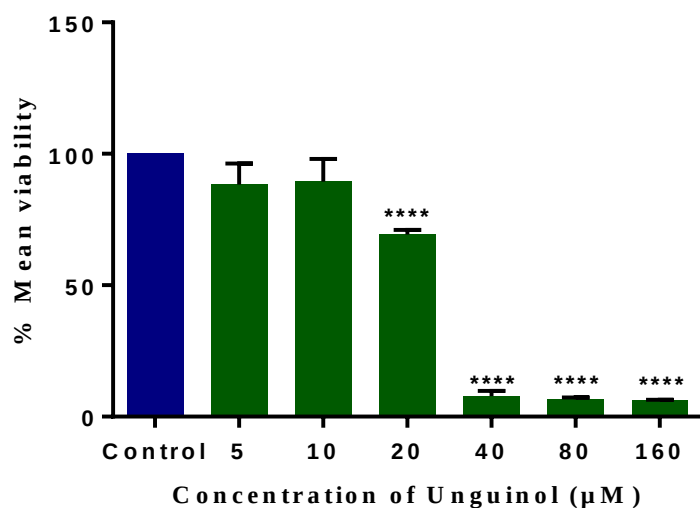


Figure 7. Bar chart illustrating mean percentage of cell viability and standard deviation of COLO 205 cells against unguinol at increasing concentrations after 24 hours incubation. N=6, ****P≤0.0001 vs. untreated cells (0 μM unguinol).

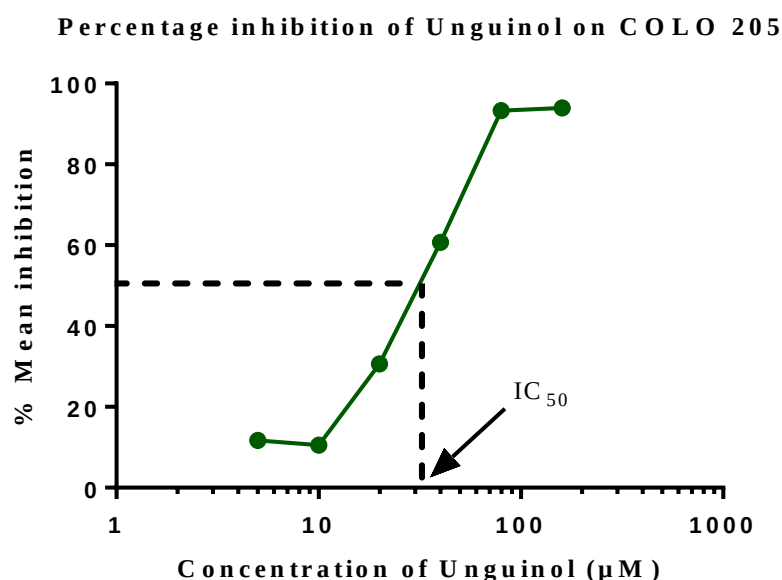


Figure 8. Dose-inhibition curve showing 50% inhibition concentration of unguinol on COLO 205 at 33 µM.

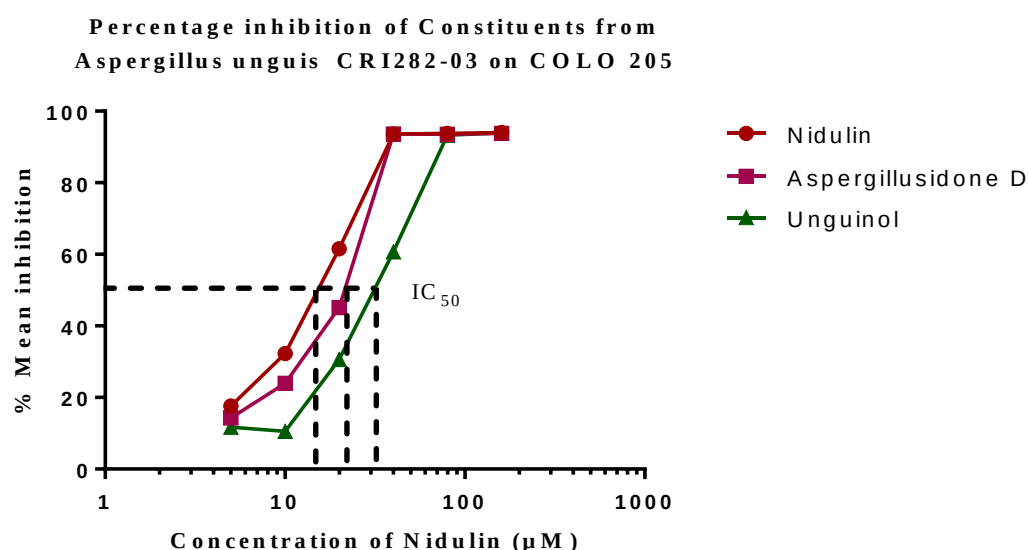


Figure 9. Dose-inhibition curve illustrates the comparison of IC_{50} of three constituents extracted from *A. unguis* CRI282-03 on COLO 205. Nidulin shows greater potency than aspergillusidone D and unguinol.

Apoptosis assay of nidulin, aspergillusidone D and unguinol on COLO 205

Cells are treated with nidulin and aspergillusidone D at 5 µM, and unguinol at 20 µM and incubated for 24 hours in 24-well plates. The concentration used was based on the concentration tested in MTT assay that provided cytotoxic activity without causing extremely cell deaths. After finish incubation with tested substances culture medium, staining, and adding assay reagent, the measurement was performed by Guava Nexin Software Module performs which will then calculations automatically. The results are

displayed on the computer screen after each sample is acquired. Acquired data are displayed in dot-plot format with a user-controlled quadrant marker for instantaneous on-screen presentation of the results. Assay utilizes Annexin V-PE to detect PS on the external membrane of apoptotic cells. The cell impermeant dye, 7-AAD, is also used in the Guava Nexin Assay as an indicator of cell membrane structural integrity. 7-AAD dye is excluded from live, healthy cells as well as early apoptotic cells. Three populations of cells can be distinguished in this assay. Firstly, Non-apoptotic cells are showed by Annexin V (negative) and 7-AAD (negative). Secondly, early apoptotic cells are Annexin V (positive) and 7-AAD (negative). Finally, late stage apoptotic and dead cells are showed as Annexin V (positive) and 7-AAD (positive). The result showed that nidulin provided substantial apoptotic activity whereas aspergillusidone D and unguinol conducted no significant apoptotic activity.

Nidulin provided notable efficacy causing cell death via apoptotic process or programme cell death as shown by the increase in the percentage of cells in lower right quadrant to reach 88.7% as these was occurred due to the positive staining of Annexin V and 7-AAD was negative. On the other, aspergillusidone D and unguinol showed no apoptotic activity on COLO 205 as both of them illustrated no significantly different in the percentage of cells in lower right quadrant compared to control.

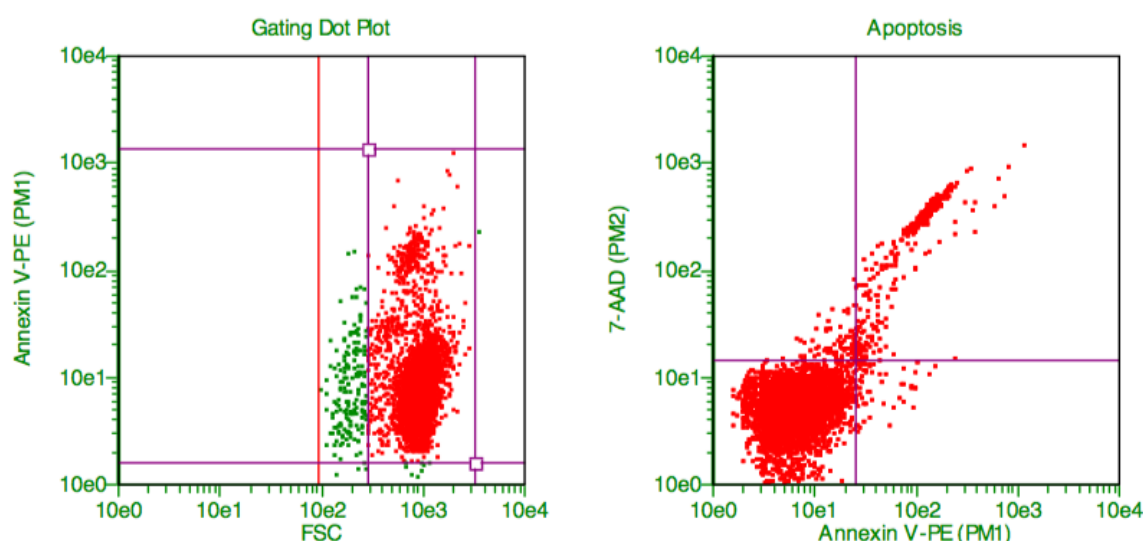


Figure 10. Apoptosis analysis of control (untreated cells) showed viable cells on lower left quadrant at the percentage of 90.6, early apoptotic stage of cells indicated in lower right quadrant was 1.3%. Late apoptotic stage and death cells showed in upper right quadrant were 6.6%.

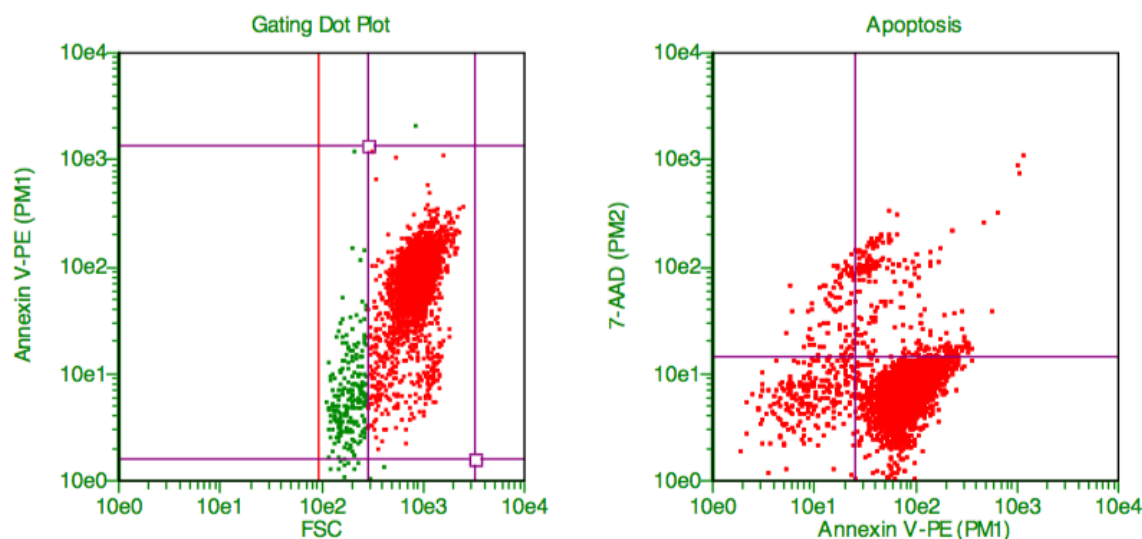


Figure 11. Apoptosis analysis of nidulin at the concentration of 5 μ M illustrated notably early apoptosis stage as shown in lower right quadrant as the percentage of 88.7, and showed significantly declined in viable cells to 4.9% compared to control. Late apoptotic stage and death cells showed in upper right quadrant were 4.7%.

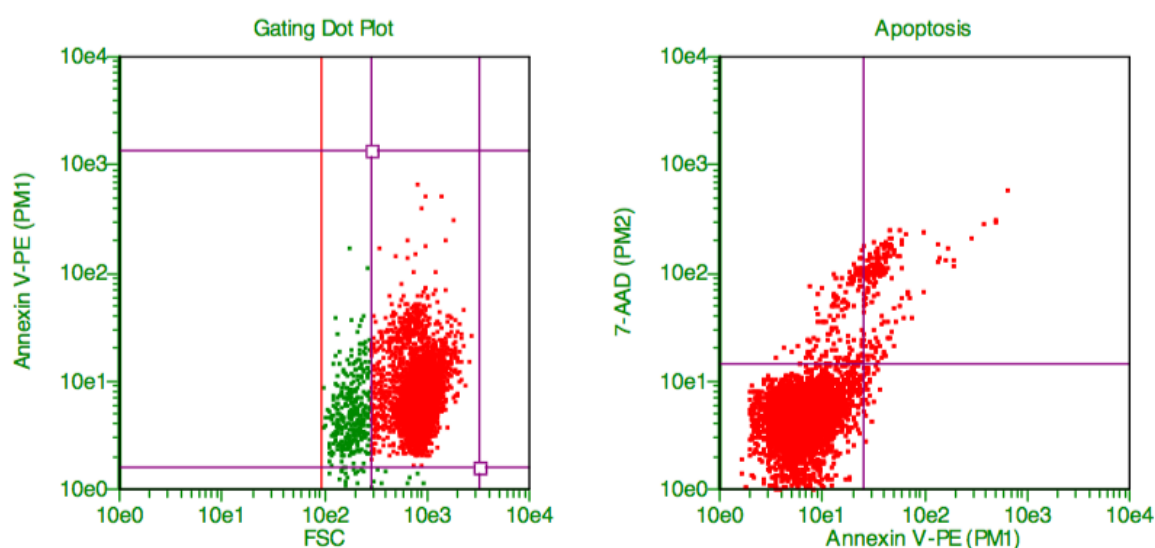


Figure 12. Apoptosis analysis of aspergillusidone D at the concentration of 5 μ M showed no apoptosis as shown in lower right quadrant as the percentage of 0.6, and showed the percentage of viable cells changed slightly to 92.5% compared to control

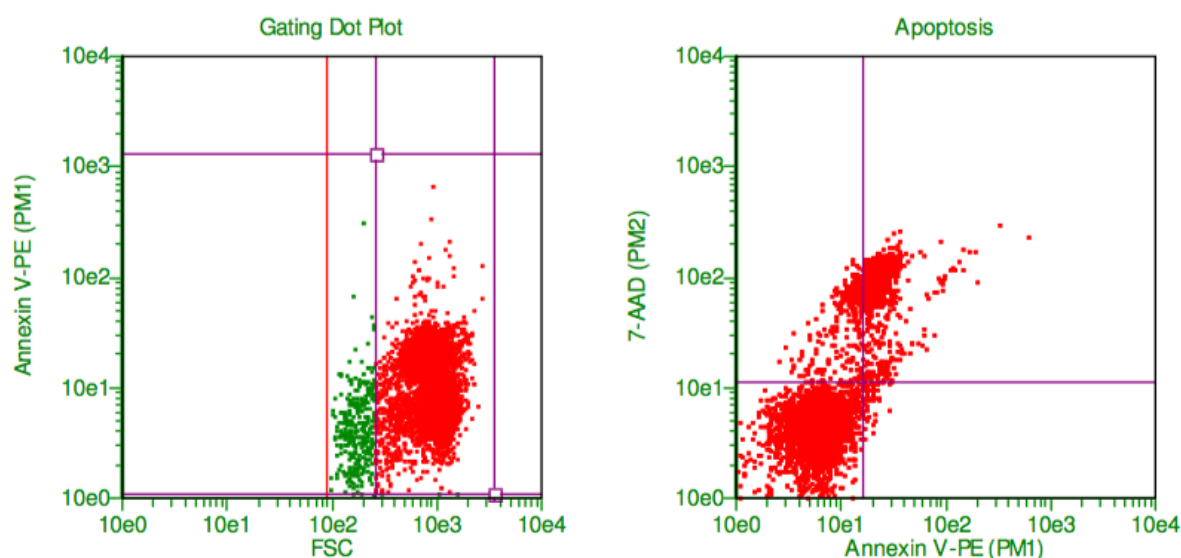


Figure 13. Apoptosis analysis of unguinol at the concentration of 20 μ M showed early apoptosis stage as shown in lower right quadrant as the percentage of 1.2, and showed significantly declined in viable cells to 64.6% compare to control.

Cell cycle assay of nidulin, aspergillusidone D and unguinol on COLO 205

The cell cycle describes the process of the replication and division of chromosomes within the nucleus, which occurs prior to cell division. Cancer cells develop when the normal mechanisms for regulating cell cycle are disrupted. Therefore, for cells to divide they must first duplicate their nuclear DNA. By labeling cellular DNA with propidium iodide (PI) to discriminate cells in different stages of the cell cycle. Resting cells (G0/G1phase) contain two copies of each chromosome. As cells progress toward mitosis, they synthesise DNA (S phase), allowing more PI intercalation with a resulting increase in fluorescence intensity. When all chromosomes have replicated and the DNA content has doubled (G2/M phase), the cells fluoresce with twice the intensity of the G0/G1 population. The G2/M cells eventually divide into two cells. Cells can be fixed, permeabilised and stained with PI.

Among all three constituents, aspergillusidone D showed significantly reduced in DNA count in G0/G1phase (pink) (Fig. 16). Although nidulin and unguinol seems to have no effects on cell cycle phases, those two constituents clearly showed its potential to cause DNA damage on COLO 205 proven by the reduction of DNA counts in particular cell cycle phases (Figure 15, 17).

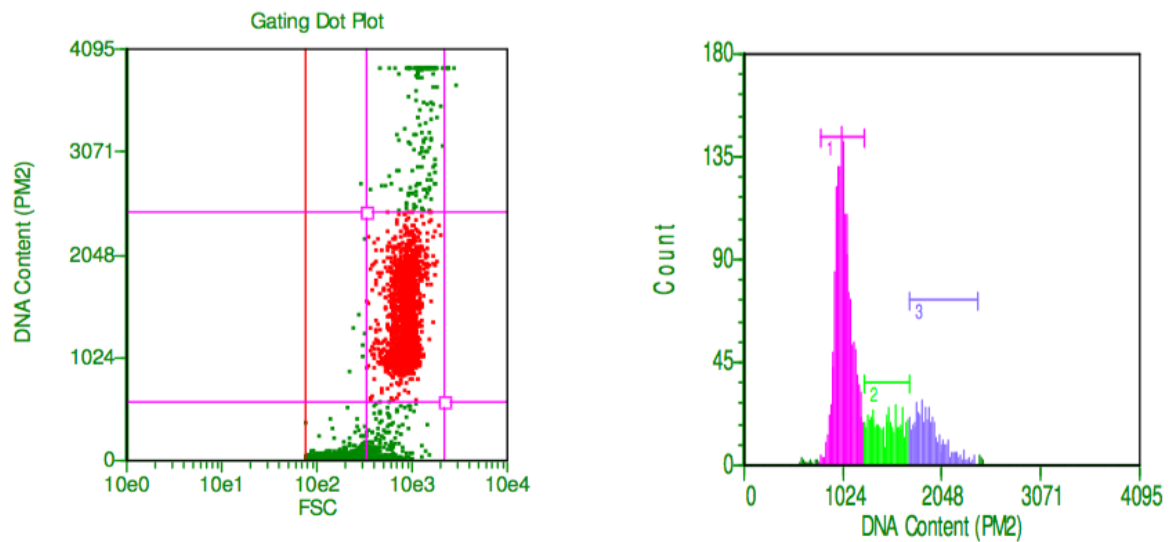


Figure 14. Cell cycle assay of control (untreated cells) on COLO 205 which showed DNA contents and cell count of cells in the G0/G1phase (pink), S phase (green), and G2/M phase (blue).

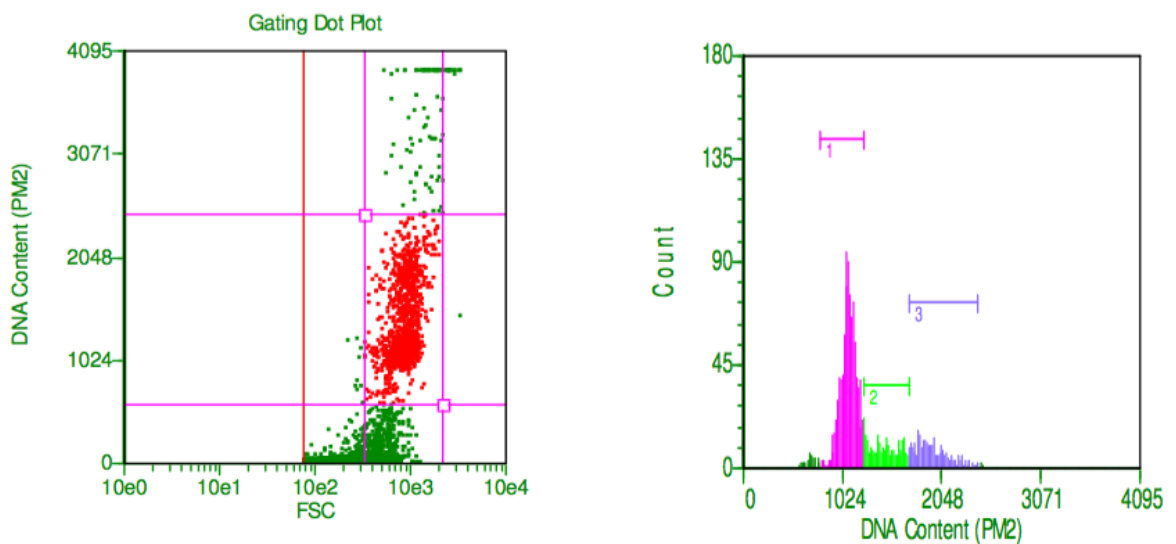


Figure 15. Cell cycle assay of nidulin on COLO 205 at 5 μ M which showed slightly reduction of DNA counts in the G0/G1phase (pink), and slightly declined in S phase (green), and G2/M phase (blue) compared with control.

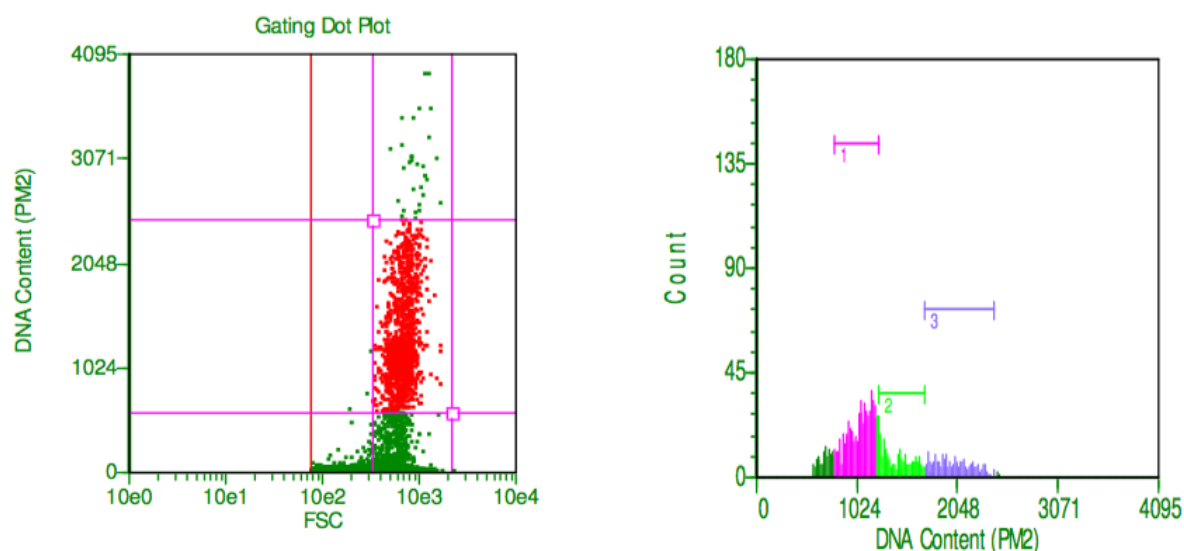


Figure 16. Cell cycle assay of aspergillusidone D on COLO 205 at 5 μ M which showed significant reduction of DNA counts in G0/G1 phase (pink) with slightly declined in S phase (green) and G2/M phase (blue) compared with control.

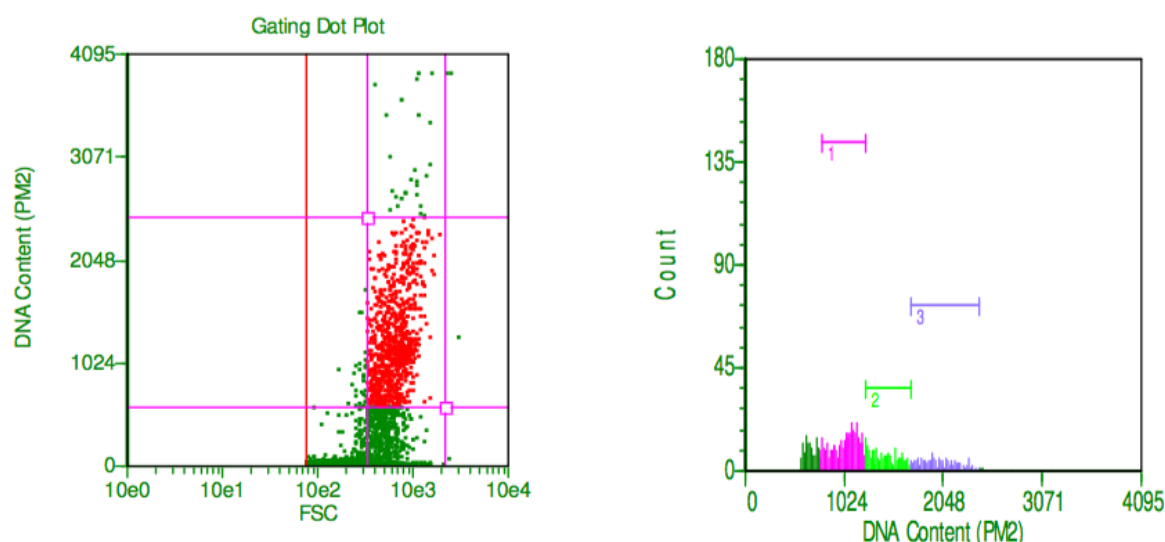


Figure 17. Cell cycle assay of unguinol on COLO 205 at 20 μ M which showed the reduction of DNA counts at all three phases the G0/G1phase (pink), S phase (green), and G2/M phase (blue) compared with control.

CONCLUSION

Among all three constituents tested, nidulin showed the highest potency of cytotoxic effects on COLO 205 cells. Unguinol seemed to show the least potency, however the maximum percentage inhibition of all constituents were about the same at 95%. This investigation has proved that constituents extracted from *A. unguis* CRI282-03, nidulin, aspergillusidone D and unguinol had a significant cytotoxic activity on COLO 205 cells which had IC_{50} at 15, 21 and 33 μ M respectively (Fig. 8.). Additionally, nidulin demonstrated the apoptotic activity significantly as shown in figure 10 whereas aspergillusidone D and

unguinol have not composed of apoptotic activity. As in the cell cycle assay all three constituents performed non cell-cycle specific anticancer activity but all of them showed its potential to damage DNA of COLO 205 as indicated in the reduction of DNA contents.

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