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Cytotoxic Activity, Proliferation Inhibition and Apoptosis Induction of *Rhaphidophora Pinnata (L.F.)* Schott Chloroform Fraction to MCF-7 Cell Line

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Abstract: Breast cancer is a type of cancer that has an ability to survive by continuously proliferate. Herbal plants are used for treatment various diseases. *Ekor Naga* plant (*Rhaphidophora pinnata* (Lf) Schott), Araceae family, has been used for antibacteria, anticancer, rheumatism and cough. Separation was done by column chromatography, and grouped based on thin layer chromatography profiles. There were nine isolates (A–I isolates) from the separation. The fractions were tested for their cytotoxicity, proliferation inhabition by using MTT method (3-(4,5-*dimetiltiazol-2-yl)-diphenyl tetrazolium* bromide-2.5, and apoptosis observation by using *acridine orange-ethidium bromide* reagents to MCF -7 cancer cells.
The cytotoxic activity results of the nine isolates (A - I) of cytotoxicity activity had IC_{50} score in the range of 190.550; 134.014; 204.474; 87.831; 96.016; 34.161; 9.621; 758.572; 245.446 ug/ml, four isolates (D-G) had IC_{50} score ≤ 100 mg / ml which function as anticancer. The result of proliferative test for isolate D it showed the inhibition of cell proliferation cell and spur apoptosis of MCF-7 cells with a red-orange fluorescence, blebing nucleus also had been . Characterization result using infrared spectrophotometer showed the functional groups of O-H; C = O; C-O, and C = C which have function as anticancer.
Keywords: *Rhaphidophora pinnata*, "ekor naga", proliferation, apoptosis, MCF-7 cells.

Introduction

Chemotherapeutic agent for cancer treatment is still an option in cancer medication. Several studies had been done on the extraction testing of natural products potency as potential chemoprevention agents which has function as escorting agent of chemotherapy. So it could increase the sensitivity of cancer cells and reduced the side effects caused by chemotherapy agents[1].

An effort of seeking chemotherapeutic agents from natural products is continuously developed at the target, the growth regulatory genes or cells with a minimum side effects, it is really necessary in cancer treatment. The extracts of several herbs have been used in a long time in cancer medication[2]. Ekor naga (*Rhaphidophora pinnata* (Lf) Schott), Araceae family, has been used as , antibacteria, anticancer, rheumatism and cough.

Apoptosis is a process of programmed cell death (PCD) that may occur in DNA. Gene that plays a role in a apoptosis process is p53 gene that acts as a cancer suppressor. A good anticancer compound is a compound that can induce apoptosis[3].

Pre elemintory research on ekor naga leaf showed the chemical compounds from class of alkaloids, terpenoid/steroid, saponins and polyphenol compounds. Fisrt screening test of toxicity ethanol extract (*crude*), extracts n-hexane and ethyl acetate of ekor naga leaf by using BST (*Brine Shrimp Lethality Test*) method to *Artemia salina* Leach gave score LC_{50} of 19.686 µg/mL, 505.82 µg/mL, 28.84 µg/mL[4,5]. Cytotoxic activity of ethanol extract (*crude*), chloroform fraction and ethylacetate fraction to MCF-7 cancer cells has IC_{50} of 112.24 µg/mL; 59.082 µg/mL; 812.663 µg/mL, respectively[6]. Based on the tests which have done, it is necessary to do the next investigation from chloroform fractions of ekor naga leaf in the proliferacy cell inhibition and apoptosis induction of MCF-7 cancer cells by *in vitro* as well as functional groups in the fractions by infra red spectrophotometer.

Method

Chloroform fraction isolation by Column Chromatography

Chloroform fractions were separated by column chromatography using silica gel 60 as stationary phase and mobile phase obtained from the thin layer chromatography (TLC), chloroform: acetone (3:7). Silica gel was inserted into the column and added written mobile phase to solidify, then mobile phase was added until 2 cm from the surface of the column and left for 24 hours. A total of 5.0089 g of chloroform fraction was immersed slowly to the column. Faucet was opened slowly and mobile phase (a mixture of $CHCl_3$-aceton; 100:0 to 0:100 / gradient elution) was added portion by portion. Elution was collected every 20 ml. The separation results were grouped in the same spot pattern by thin-layer chromatography (TLC) of the eluate until nine isolates (A – I isolates) were obtained.

In vitro Cytotoxic activity test on MCF-7 cancer cells

Cytotoxic activity test done for chloroform fraction (A-I isolates). MCF-7 cells were implanted into 96 well *microplate* and incubated at 5% CO_2 incubator for 24 hours, to obtain confluent growth, in order to obtain the density of 10^4 cells/well. Then the media was replaced with the new ones and added with 100 µL of test solution with different concentrations (500, 250, 125, 62.5, 31.25 µg/mL). incubated again for 24 hours. At the end of incubation, removed the media was and then the cells were washed with a solution of Phosphate Buffer Saline (PBS). Each well was added with 100 µL of cultur media and 10 µL of MTT reagent (3-(4,5-dimetiltiazol-2-yl) -2.5-diphenyl tetrazolium bromide) 0.5%. Cells were incubated again for 4-6 hours in a 5% CO_2 incubator at 37° C to form formazan. The reaction was stopped by 100 µL of MTT reagent stopper (sodium dodecyl sulfate). The cells were incubated for one night at room temperature, and then analysed by ELISA reader at λ 595 nm[7].

Proliferation kinetic assay (*doubling time of cells*)

Proliferation kinetic assay was performed on D isolates with three concentrations. MCF-7 cells were implanted into 96 well microplate and incubated at 5% CO_2 incubator for 24 hours to obtain confluent growth, in order to obtain the density of $5x10^3$ cells/well. Then the media was replaced with the new ones and added with 100 µL of test solution with different concentrations and incubated for 24, 48, and 72 hours. At the end of incubation, the media removed and then the cells were washed with solution of phosphate buffered saline (PBS). Each well was added with 100 µL of cultur media and 10 µL of reagent MTT (3 - (4,5-dimetiltiazol-2-yl) -2.5 -diphenyl tetrazolium bromide) 0.5%. Cells were incubated again for 4-6 hours in a 5% CO_2 incubator at 37°C to form formazan. The reaction was stopped by 100 µL of MTT reagent stopper (sodium dodecyl sulfate). The cells were incubated for one night at room temperature, and then analysed by ELISA reader at λ595 nm[7].

Apoptosis assay

Apoptosis assay was performed on D isolates with two concentrations (IC_{50} and ½ IC_{50}). MCF-7 cells were implanted in a 24 wells microplate using coverslips and incubated in 5% CO_2 incubator for 24 hours to obtain confluent growth in order to obtain the density of 4 x 10^4 cells / well, then incubatet with the test solution for 24 hours. The media was removed and the cells were washed with PBS solution. *Cover slip* was placed over the object glass and added with 10 µL of *ethidium bromide-acridine orange* solution and allowed to stand for 5 minutes, then observed under a fluorescence microscope. Living cells gave green fluorescence and dead cells gave orange fluorescence.

Characterization using Infrared Spectrophotometer

Characterization using infrared spectrophotometer was performed to find out the functional groups in a compound. Characterization was done by mixing 1 mg of sample with 150 mg of potassium bromide using a mixture vibrate, (then the mixture was pressed into pellets at a pressure of 11.5 ton/cm^2 to form KBr plate). KBr plate was inserted into the sample compartment of infrared spectrophotometer and the absorbance was measured at wave numbers 4000 - 400 cm^{-1}.

Analysis Data

Absorbance data obtained from the kinetics of proliferation and cytotoxic assay were calculated by using the following formula:

$$\% \text{ Living Cells} = \frac{Absorbance\ Treatment - \ Absorbance\ Media\ Control}{Absorbance\ Control\ cel - Absorbance\ Media\ Control} \text{ X } 100\ \%$$

IC_{50} concentrations were then calculated by probit analysis using SPSS 17, from the linear relationship between log concentration and percentage of live cells. IC_{50} is the concentration that causes death of 50% cell population.

Result and Discussion

Separation by column chromatography resulted nine isolates (A-I isolates), obtained from the grouping of the came spot pattern by thin layer chromatography. These fractions were tested for anticancer activity on MCF-7 cancer cells.

Cytotoxic Activity of resulted fractions.

Cytotoxic activity test was performed to determine the potential toxicity of A - I isolates. The cytotoxic activity result of the nine isolates (A-I) on MCF-7 cancer cells. Can be seen in Table 1.

Table 1. Cytotoxic activity of $CHCl_3$ fractionation on MCF-7 cells

$CHCl_3$ Fractions	IC_{50} (µg/mL)
Isolate A	190.550
Isolate B	134.014
Isolate C	204.474
Isolate D	87.831
Isolate E	96.016
Isolate F	34.161
Isolate G	9.621
Isolate H	758.572
Isolate I	245.446

Referring to Table 1, it shows cytotoxic activity in D - G isolates gave IC_{50} values <100 µg/mL, which can be said to have anticancer activity, and the results of chemical screening (chemical compound screening showed it contained terpenoida group). Toxic effect will increase with the increasing of concentration. Toxic effects increased was indicated by the small percentage of living cells. High concentration of the isolate showed small absorbance which meant smaller number of the living cells.

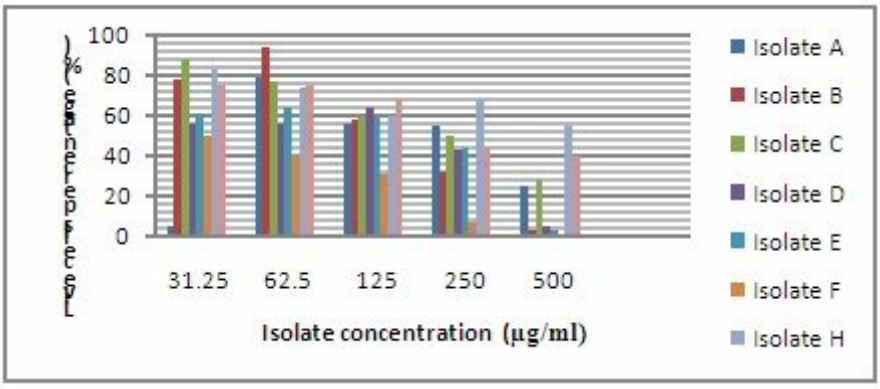

Figure 1: Concentration of isolates vs mean of living cells percentage

The cell morphology after addition of A - I isolates on MCF-7 cells showed many dead cells. It can be seen in

Figure 2.

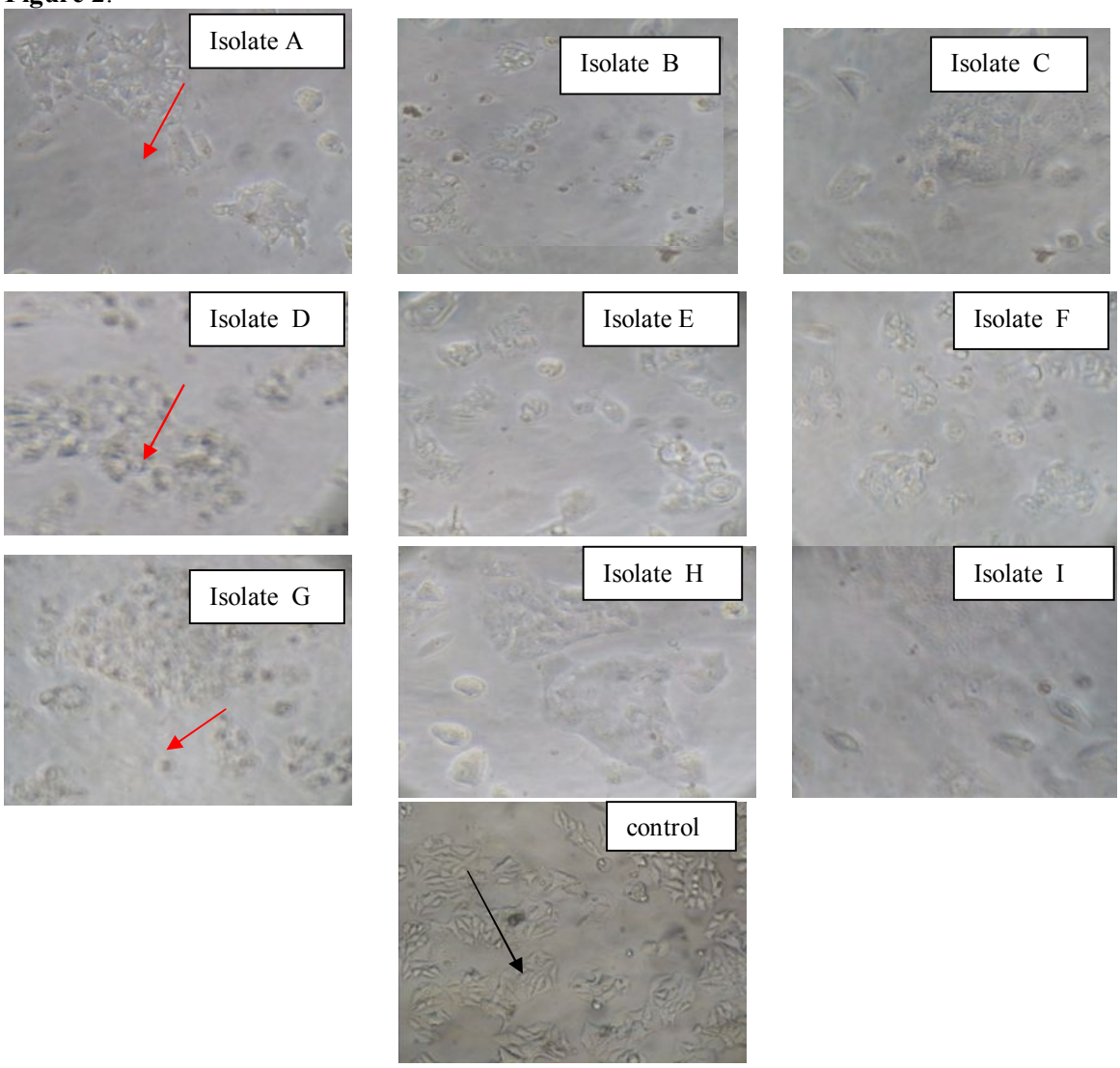

Figure 2: Morphology of MCF-7 cells after incubated with A – I isolates for 24 h (magnification 10 x 10). Living cells were indicated by black arrows (⟶) while dead cells was shown by red arrows (⟶)

Cytotoxic activity is used as a reference for tracking mechanism of D isolate in inhibiting the growth of cancer cells. Therefore, it is necessary to test the kinetic of cell proliferation and to do apoptosis observation to determine the profile of inhibition mechanism of cell growth by D isolate.

Antiproliperative effect of D fraction

Doubling time test was conducted to view the activity of MCF-7 cell proliferation treated by D isolate. Compounds that can extend the doubling time demonstrated the ability of these compounds to inhibit proliferation of cancer cells through a mechanism of cell cycle arrest. Doubling time test was performed by counting the number of live cells treated with D isolate in each unit of time. Doubling time is the time required by cells to double itself into two. The results showed that the level of 45 µg/mL D isolate was able to inhibit cell proliferation significantly. Mean while, the level of 90 and 180 µg/mL, the isolate could suppress the cell growth up to 100% (Figure 3).

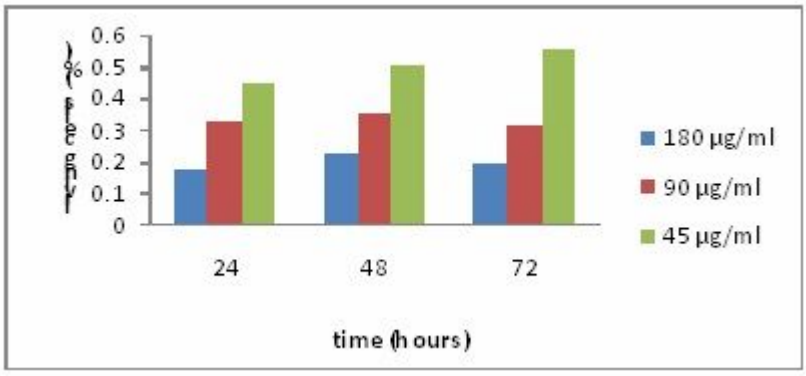

Figure 3: Profile of MCF-7 cell growth due to the treatment of D isolate observed at 24, 48 and 72 hour.

Apoptosis Induction Activity of D Isolate

Observation of apoptosis was conducted using double staining gave *ethidium bromide- akridine orange* (EB - OR) method. The results showed that the control cells green fluorescence which absorbed the acrydine orange reagent. For the treated cells, it showed yellow-orange red fluorescence. It indicated the loss of membrane permeability in some cells by giving D isolate, which caused ethidium bromide can enter the cell and caused orange red fluorescence as an indicator of cell death, also a fragmentation of nucleus was seen.

(A) MCF-7 control (B) 45 µg/ml of D isolate

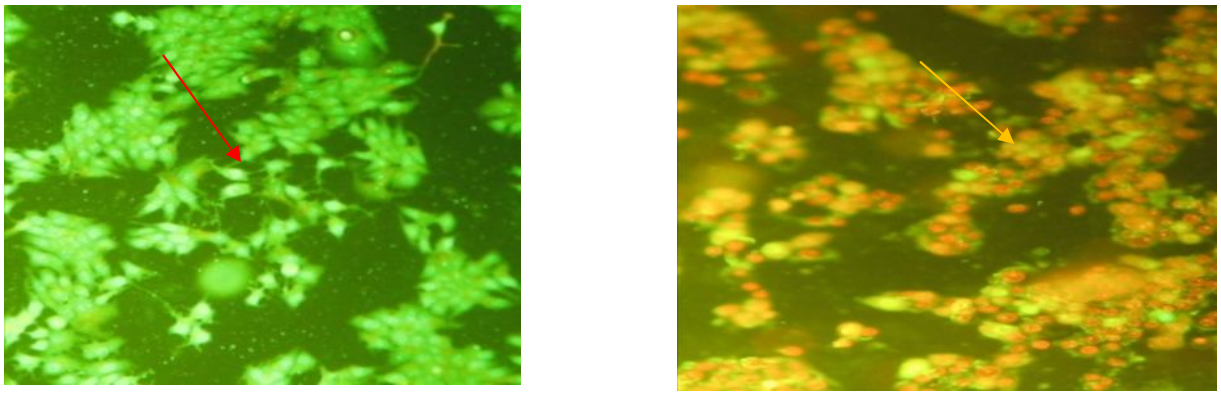

Figure 4: D isolate induced apoptosis in MCF-7 cells after 72 hours incubation viewed with a fluorescence microscope (100x magnification) → control MCF-7 cells, → the cells undergo apoptosis

The results of characterization by Infrared Spectrophotometer

Infrared spectrophotometer was used to determine the functional groups of chloroform fractions (D, E, F, and G isolates). If a molecule is irradiated with infrared ray on the different frequency or wave number, the functional groups at the appropriate energy level will vibrate. Functional groups will absorb appropriate infrared

ray and will be recorded in the form of spectra. Infrared spectra of the $CHCl_3$ fraction, D, E, F, and G isolates are shown in the Figure 5.

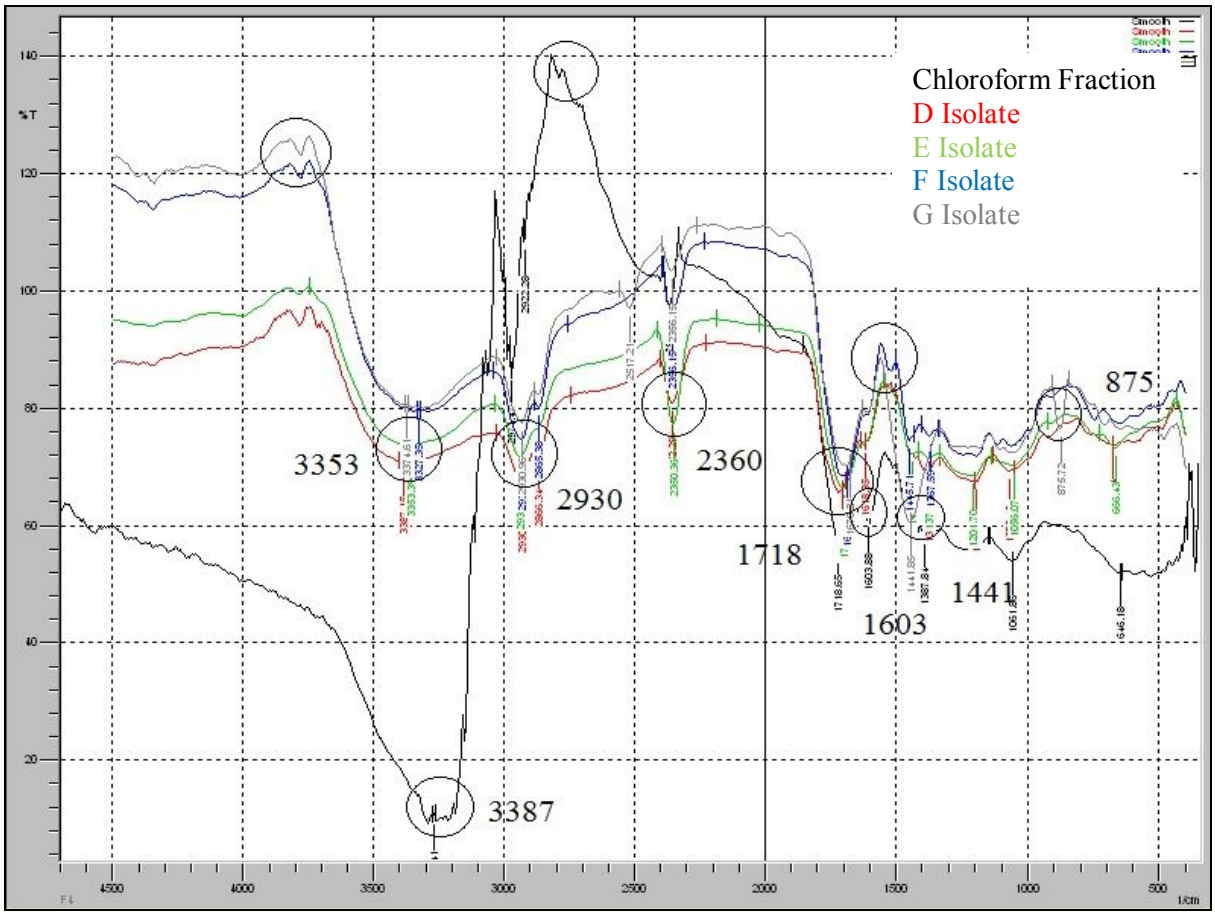

Figure 5: Infrared spectrum of chloroform fraction,(D, E, F, and G isolates)

Discussion

Ekor naga leaf D isolate activity can inhibit the growth of cancer cells,tudy MCF-7. It provides excellent results with IC_{50} values less than 100 ug / ml which potential to be developed as anticancer. Based on the observation of the kinetics of cell proliferation, D isolate showed an increase in number of dead cells with the increasing of incubation time (Figure 3). This suggests that D isolate can inhibit genes work or work of proteins that regulate cell cycle.

From figure 3, it showed that there was no decrease in the percentage of live cells within observation time (24, 48, and 72 hours). This suggests that D isolate have interacted with MCF-7 cells to inhibit the growth of these cells. According to Ueda[8], if the extracts had IC_{50} values $\leq$ 100 mg/mL, it can be said to have the potential of antiproliferation. Based on the above reference, it can be said that the D isolate with IC_{50} value of 58.567 µg/mL significantly inhibited cell proliferation in MCF-7 that needs to be followed up with a bioactive material isolation.

Death of MCF-7 cells cancer due to the treatment of ekor naga leaf D isolate could be investigated through apoptosis assay. Concentration used for the apoptosis assay was 90 and 45 µg/mL, both of the concentration were on the IC_{50} and half IC_{50} value of MTT cytotoxicity assay method, therefore death cells and live cells could be differentiated at observation time.

In DNA *double staining* method using *acridine orange-ethidium bromide*, both of the reagent were used simultaneously wihch can produce contrast colors, therefore making it easier to observe under a fluorescent microscope. Observation of cell morphology should be done immediately because if the *double staining* solution interact too long with cells, the life cells will die. *Ethidium bromide-acridine orange* can penetrate cell membranes and interact with the cell's DNA. The observation under a fluorescent microscope

showed that control cells were green, and cells with treatment appeared orange red (figure 4). The nuclei were contracted, blebing was occurred and there were cells or incomplete cells which showed cells death were occurred by a mechanism of apotopsis. This indicated that the test substance was able to stimulate apoptosis of the MCF-7 cells. These results were in accordance with the results of the doubling time test which showed the level of 45 µg/ml of the test substance provided growth inhibition. It proved that the D isolate was capable to stimulate apoptosis and inhibit the growth of MCF-7cancer cells.

Based on the data from the infrared spectrum, chloroform fraction, (D, E, F, and G isolates) showed a sharp absorption at wavenumber region 3387-3230 cm^{-1} showing the O-H C-H aliphabetic group (CH_3 and CH_2), strengthen with the absorption at 875 which is the -CH_2 absorption. Wave number of 1709 cm^{-1} with strong intensity showed a carbonyl group (C=O), followed by wave numbers 1201 cm^{-1}, indicating a C-O group, while the *double bond* duplicate at wavenumber 1676 cm^{-1} indicate the presence of group C= C aromatic followed by wave numbers 1445 cm^{-1} indicate the presence of aromatic C-C group[9,10].

In MCF-7 control, apoptosis was not occured because the apotopsis regulator of MCF-7 cells, the p53 protein was bounded and degraded by E6 protein. Isolate with concentration of 45 µg/ml indicated the mechanism of cell cycle arrest and cell death was suspected. The DNA staining data could support the possibility of cell death through apoptosis mechanism

Conclusion

The result obtained by column chromatography was nine isolates that had cytotoxic activity. The ekor naga leaf (*Rhaphidophora pinnata* (Lf) Schott) D isolate had antiproliferative activity and promote apoptosis of the MCF-7 cells. The results of infrared spectrum were the functional groups of O-H; C-H, C = O, and C = C aromatic of A-I isolates that had anticancer properties.

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