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Induction of apoptosis by *Aaptos* sp., fractions in human breast cancer cell line, MCF-7

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ABSTRACT

Breast cancer is a malignant tumor with the highest incidence among women. Reduced chemotherapeutic efficacy of current anticancer drugs result in further screened for novel alternative chemotherapeutic agents of natural origin. Marine secondary metabolites are promising sources of unexploited drugs that have a wide structural diversity and have shown a variety of biological activities. Therefore, the main aim of this study was to determine the cytotoxic effects and mode of cell death exerted by three fractions, F-1, F-2 and F-3 fractions prepared from *Aaptos* sp., marine sponge on human breast cancer cell line, MCF-7. The three fractions produced potent cytotoxicity effects with IC₅₀ values at 72 hr of less than 30 µg/ml in the order of F-1 > F-2 > F-3. MCF-7 cell death exerted by the fraction was found to be mediated by apoptosis based on the exposure of phosphatidylserine and presence of DNA fragmentation in treated cells. Therefore, bioactive compounds present in these fractions are responsible in inducing apoptosis and thus, possessed potential to be candidates for chemotherapeutic drugs.



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INTRODUCTION

Cancer remains number one of the major killer worldwide. Based on report by American Cancer Society (ACS), cancer is the second foremost cause of death after cardiovascular disease, which accounted for 24% of total deaths. A total of 2,543,279 deaths were recorded in the United States in 2012, of which 582,623 (23%) were from cancer. However, breast cancer is still the leading cancer diagnosed in women with 29% of new cases reported in 2016. (Siegel et al., 2015; Siegel et al., 2016).

Cancer is multifactorial disease characterized by lack of highly regulated mechanism of cell growth known as apoptosis or programmed cell death. This fundamental process plays a key role in maintenance of tissue homeostasis and deletion of faulty or damaged cells (Wyllie, 1997). Therefore, one of the key characteristics of the developed anticancer drugs is to kill cancer cells via apoptosis to reduce a prolonged and adverse inflammation, which prevents serious side effects. Consequently, many pharmaceutical companies currently focus on apoptosis-based chemotherapy in cancer drug development. Its morphological characteristics include plasma membrane blabbing, cell shrinkage, nuclear condensation, chromosomal DNA fragmentation, and formation of apoptotic bodies (Elmore, S 2007).

Common risk factors associated with breast cancer are family history, age, reproductive status, genetic mutations, and lifestyle (Sudhakar, 2009; Fenga, 2016). Surgery, radiotherapy, chemotherapy and immunotherapy are used for breast cancer treatment. Chemotherapy has been an important component of the clinical treatment of cancer along with radiotherapy, in which the combined effects

being more effective than single therapy. However, the major drawback with chemotherapy is poor bioavailability and severe systemic toxicity, leads to finding of new therapeutics agent for cancer treatment (Gul-e-Saba, 2013; Hasumi *et al.*, 2011, Wang *et al.*, 2009).

Recent studies showed that various therapeutic agents of natural origin induced apoptosis in cancer cells (Sari *et al.*, 2013). There is a significant contribution of natural products in the field of drug discovery, with an estimated 77% of all anti-cancer drugs approved between 1940 and 2014 being small molecule natural products and natural product-derived compounds. Actinomycin D, mitomycin C, trastuzumab emtansine are produced from natural product and their derivatives are used as anti-cancer drugs (Newman & Cragg, 2016). Generally, marine invertebrates such as sponges utilize their secondary metabolites or natural products as a form of chemical defence against predators and as a competitive advantage in their perpetual battle for limited resources, such as nutrients and space (Bolton *et al.*, 2013). The dominant sources of marine natural products that are utilized commercially for their beneficial pharmacological properties in biomedical research (Fusetani, 2010).

Among these, numerous anticancer agents derived from marine sources especially sponges have entered preclinical and clinical trials (Mayer *et al.*, 2010; Miller *et al.*, 2010) and have shown potential cytotoxic activity against various tumor types (Da Rocha *et al.*, 2001).

Various potent triterpenes, isolated from the Red Sea Sponge identified as siphonolone A, siphonolone A, neviotine A and siphonolone L were found to be cytotoxic against human breast and liver cancer cell lines (Al-Massarani *et al.*, 2015). Similarly, dysiherbols A–C and dysideanone E as tetracyclic meroterpenes isolated from a marine sponge were found to possess NF- κ B inhibitory and cytotoxic activity (Wei-Hua *et al.*, 2016). Hyattella cribiformis (EA) fraction acts as apoptosis inducing agent promoting tubulin polymerization as evidenced mitotic arrest also belongs to marine sponges family (Pazhanimuthu *et al.*, 2015).

Currently, marine invertebrates especially sponges are found to produce significant number of natural products. However, to date, there are still a large number of unexplored secondary metabolites from the marine environment remained unexplored. Therefore, extensive research is required to determine their biological activities. Therefore, in this study, sponge *Aaptos* sp., fractions were selected to evaluate their cytotoxicity activity and mode of cell death on human breast adenocarcinoma (MCF-7) cell line.

MATERIALS AND METHODS

Materials

The materials used for cell culture were purchased from Sigma Aldrich, USA. MCF-7 cell line was purchased from American Type Cell Culture, USA. CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, ApoAlert™ Annexin V, Clontech, USA, DeadEnd™ Fluorometric TUNEL were from Promega USA. All chemicals were of analytical grades.

Preparation of fractions from *Aaptos* sp.,

Three fractions of *Aaptos* sp., were used in this study. F-1 fraction was prepared using Medium pressure liquid chromatography (MPLC), F-2 EA and F-3 (MeOH) prepared by Solid Phase Extraction (SPE) method. Locations of *Aaptos* sp., collection is shown in Table 1. Identification was carried out Dr. Noraznawati Ismail, Institute of Marine Biotechnology, Universiti Malaysia Terengganu. Then, the sample cleaned, chopped and freeze-dried to remove water. After dehydration processing, sample was ground to powder form and 10 g of each sample was subsequently used for extraction. The samples were extracted using cold extraction technique where 10 g of dried sample was macerated in 100 ml of methanol (polar) for 24 hr and repeated for three times. The sample was filtered using vacuum filter (Buchner) set with whatman filter paper. Then, filtrate will be concentrated using rotary evaporator under vacuum pressure below 45 °C to produce methanol crude extract. The filtrate was evaporated to dryness using Rota-vapor (BUCHI, Switzerland) at 40°C.

Table 1: Location and coordinate where the specie of sponge was collected

Species	Location of collection
<i>Aaptos</i> sp.	Teluk Belanga (N 05°36.656' E 103°04.024')

F-1 fraction was prepared by MPLC flash chromatography. 5g of dried methanol extract was reconstituted with 50 ml of MeOH: H₂O + 0.1% TFA. Mixture was then centrifuge at 14000 rpm for 15 mins at 4°C. Supernatant obtained was then flow through HLB cartridge and eluent were collected into tube, dried using dryer (MiVac Quattro). Dried extract was reconstituted with 3 ml of 90:10 H.O: MeOH + 1% TFA. The sample mixture was mixed well and then filtered using 0.2 μ m PTFE filter if un-dissolved sample present. The final mixture was then loaded into MPLC Flash Chromatography (BUCHI, Switzerland).

F-2 fraction was prepared by column chromatography using SPE column cartridge/ column chromatography (pack with silica gel). The methanolic extract was mixed with ethyl acetate and filtered

through column chromatography. Same procedure was repeated using methanol through SPE column cartridge to produce and F-3 (MeOH) fraction. The collected samples were then concentrated by using rotary evaporator (BUCHI, Switzerland).

Determination of cytotoxicity activity of fractions

Cytotoxicity effects of three fractions of *Aaptos* sp., in MCF-7 cell line were determined using MTS assay (CellTiter 96™ Aqueous Non-Radioactive Cell Proliferation Assay) (Tengku Muhammad *et al.*, 2017). Briefly, the cells were cultured onto 96-well plate at a density of 6000 cells/well and incubated at 37°C in a CO₂ incubator overnight. When the cells reached confluency at approximately 80%, the medium was replaced with fresh medium containing various concentrations of the prepared fractions and incubated for 72 hr. DMSO was used to dissolve and dilute the fractions and the final concentration of DMSO in each well was 1% (v/v). Vincristine sulfate was used as positive control and untreated cells (in the presence of only 1% DMSO (v/v)) were designated as negative control. After treatment, 20 ml of MTS was transferred to each well, incubated for 2 hr and absorbance was then read at 490 nm.

Determination of early apoptosis in treated cells

The Annexin V-FITC Apoptosis Detection Kit (APO Alert Annexin V, USA) was used to determine the apoptosis mode of cell death exerted by the fractions on the cells. The MCF-7 cell line was cultured at a cell density of approximately 10,000 cells/chamber in 96-well plate and incubated at 37 °C in a humidified atmosphere in the presence of 5% (v/v) CO₂ overnight. Then, the medium was replaced with fresh medium containing the fraction at concentration of IC₅₀ at 72 hr and incubated at 3 hr, 6 hr and 24 hr.

After treatment, cells were washed with 1× binding buffer. Subsequently, the cells were incubated in 200 µL of binding buffer with 5 µL of Annexin V-FITC and 10 µL of PI at 37°C for 5-15 mins. Vincristine sulfate was used as positive control and untreated cells (in the presence of only 1% DMSO (v/v)) were designated as negative control. The slides were then observed under ImageXpress Micro XLS Widefield High-Content Analysis System (HCS) (Sunnyvale, USA), for images.

Determination of late apoptosis in treated cells

The DeadEnd™ Fluorometric Apoptosis Detection System (Promega, USA) was used to determine DNA fragmentation induced apoptotic cell death in treated cells. The assay was done according to the manufacturer's instructions. The MCF-7 cell line

was cultured in Labtek Chamber Slides (Nunc, Denmark) at a cell density of approximately 10,000 cells/chamber and incubated at 37 °C in a humidified atmosphere in the presence of 5% (v/v) CO₂ overnight until the cells reached 80 to 90% confluency. Briefly, treated cells were fixed by 4% paraformaldehyde solution in PBS (pH 7.4) for 25 mins at 4°C followed by washing with phosphate buffer saline (PBS) for 5 mins. Then the fixed cells were permeabilize for 5 min by immersing the slide in 0.2% triton X-100 and rinsed with PBS. Reaction mixture and enzyme solution and were added as provided by manufacturer. Green fluorescence of FITC-labeled apoptotic cells detected by Image Xpress Micro XLS Widefield High-Content Analysis System (HCS) (Sunnyvale, USA).

Statistical Analysis

Cytotoxicity experiments were carried out in triplicates and results were expressed as percentage growth inhibition of control. IC₅₀ values for growth inhibition was derived from a nonlinear regression model (curvefit) based on sigmoidal dose response curve (variable) and computed using GraphPadPrism (Graphpad). Data are given as mean ± S.E.M. ANOVA *P<0.05, **P<0.01 (Dunnett post-test).

RESULTS AND DISCUSSION

The cytotoxic effects of three fractions, F-1, F-2 and F-3 fractions prepared from *Aaptos* sp., on human breast carcinoma MCF-7 cell line were investigated. As shown in Figure 1-3, all fractions produced a dose-dependent inhibition of MCF-7 cell growth over the treatment period of 72 hr.

The F-1 fraction significantly inhibited growth of MCF-7 cell line at concentrations 0.391 µg/ml and above when treated for 24, 48 and 72 hr (Figure 1). The fraction produced a potent cytotoxicity effect on MCF-7 cell line at concentrations 12.5 µg/ml and above where more than 50% of cell populations were killed. Interestingly, at 72 hr treatment, concentration at 12.5 µg/ml and higher was also able to inhibit the cells of more than 75% as compared to control. The IC₅₀ values were decreased as the incubation periods were increased from 24 hr (11.48 µg/ml) to 48 hr (6.55 µg/ml) and to 72 hr (5.064 µg/ml).

Aaptos sp. F-2 fraction also produced a potent inhibitory activity on MCF-7 cell line. At 24 hr incubation, the cell growth was significantly inhibited at concentrations of 6.25 µg/ml and above. Interestingly, at 24, 48 hr and 72 hr, the inhibition was more significantly increased (P<0.01) at 12.50 µg/ml and above as compared to control (Figure 2). The F-2 fraction exhibit increased cytotoxicity activity against MCF-7 cell line with IC₅₀ values of

Table 2: IC50 values of three fractions of Aaptos sp., and vincristine sulfate (µg/ml)

Aaptos sp., Fraction	IC ₅₀		
	24 hr	48 hr	72 hr
F-1	11.48	6.55	5.06
F-2	86.80	44.64	15.06
F-3	>100	39.81	25.00
Vincristine Sulphate	3.16	2.81	0.20

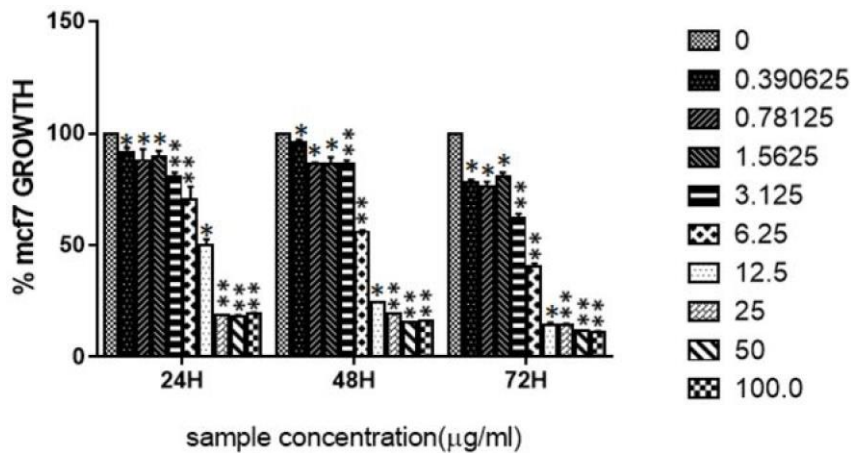


Figure 1: Percentage of growth inhibition ± standard deviation for F-1 fraction of Aaptos sp., against MCF-7 at 24 hr, 48 hr, and 72 hr

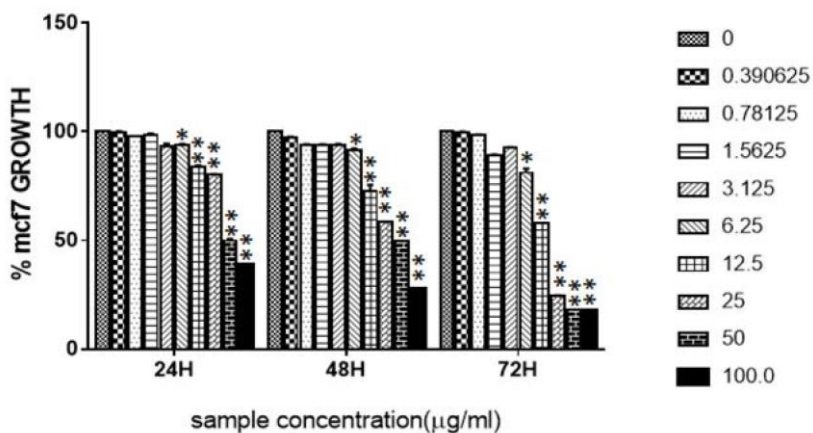


Figure 2: Percentage of growth inhibition ± standard deviation for F-2 fraction of Aaptos sp., against MCF-7 at 24 hr, 48 hr, and 72 hr

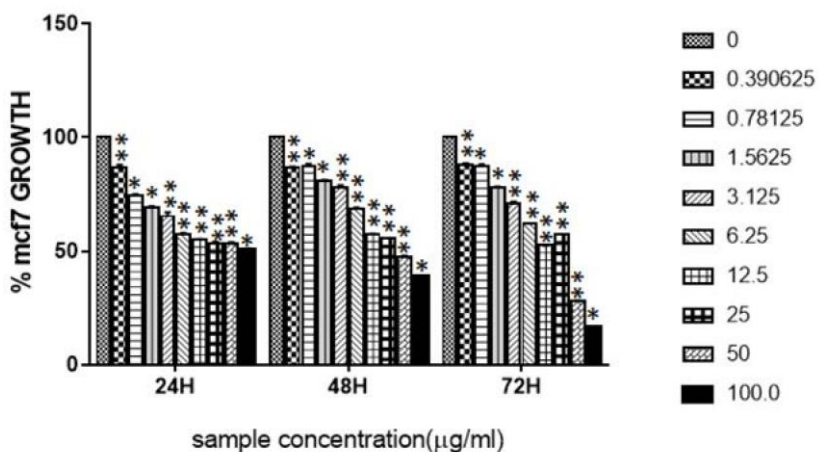


Figure 3: Percentage of growth inhibition ± standard deviation for F-3 fraction of Aaptos sp., against MCF-7 at 24 hr, 48 hr, and 72 hr

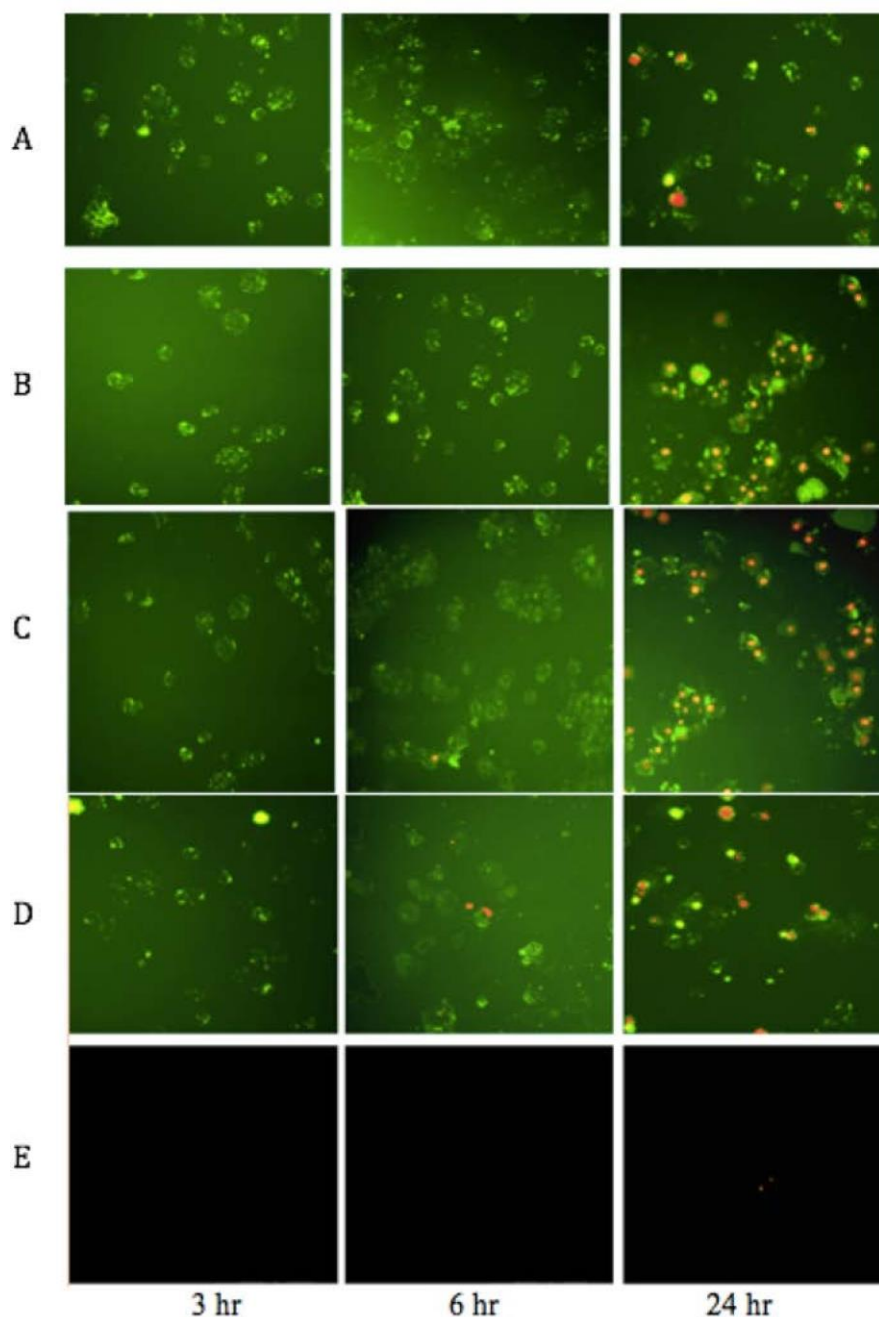


Figure 4: (A-E): The green stain (annexin-FITC) early apoptosis; Red stain, PI (propidium Iodide) late apoptosis indicates induction of apoptosis in MCF-7 treated row A-E. (A) F-1 fraction; (B) F-2 fraction; (C) F-3 fraction; (D) Vincristine sulfate and (E) Nega

86.80 (24 hr), 44.64 (48 hr) and 15.06 $\mu\text{g/ml}$ (72 hr).

Similarly, the inhibitory effects were also observed when the cells were treated with *Aptos* sp. F-3 fraction over the period of 72 hr. The fraction significantly inhibited the growth of MCF-7 at concentrations 0.390 $\mu\text{g/ml}$ and above, after 24, 48, and 72 hr incubation (Figure 3). Interestingly, IC_{50} value of the fraction was >100 $\mu\text{g/ml}$; 39.881 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$ at 24 hr, 48 hr, and 72 hr treatment, respectively, which clearly indicate that the fraction produced potent cytotoxicity effect on MCF-7 cell line at 72 hr. as judged by the criterion

set by the National Cancer Institute, USA of which the extract is categorized as cytotoxic against cancer cell line when the IC_{50} value at 72 hours is lower than 30 $\mu\text{g/ml}$ (Geran *et al.*, 1972).

Vincristine sulphate control was used as a positive control in the cytotoxicity study showed the IC_{50} value of 0.20 $\mu\text{g/ml}$ at 72 hr. Overall, three fractions, F-1, F-2 and F-3 fractions prepared from *Aptos* sp., produced a potent cytotoxicity effects on human breast carcinoma, MCF-7 cell line based on IC_{50} value of less than 30 $\mu\text{g/ml}$ at 72 hr (Table

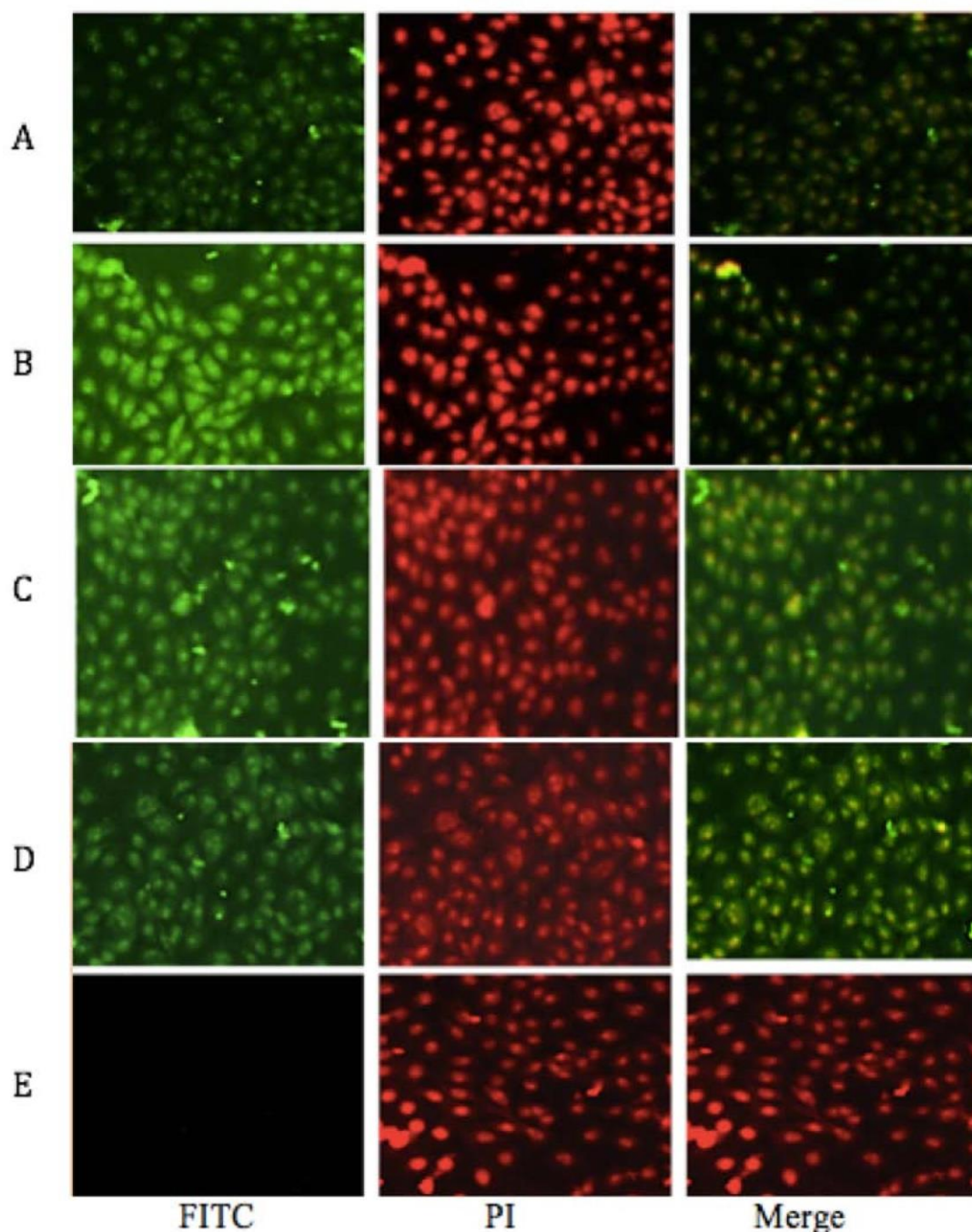


Figure 5: (A-E): The presence of green stain (FITC), which indicates DNA fragmentation in MCF-7 cells that was treated with row A-E. (A) F-1 fraction; (B) F-2 fraction; (C) F-3 fraction; (D) Vincristine sulfate and (E) Negative control, after 24 hr

2). The relative potential of cytotoxicity of the fractions is as follows: *Aptos* sp. fraction, F-1 > F-2 > F-3 fraction.

In order to determine the mode of cell death that was responsible in mediating the cytotoxicity effects of three fractions of *Aptos* sp., Annexin V and TUNEL assays were performed according to the manufacturer's instruction.

The Annexin V-FITC staining method was used to confirm the presence of early apoptosis in cells. Figure 4 A-D shows that the cells treated with fractions; F-1, F-2, F-3 and vincristine sulfate at the

concentrations of IC_{50} 72 hr showed the presence of green fluorescence at the cell membrane of MCF-7 cells. A time-dependent increase in annexin V FITC binding to the cell membrane was observed when cells were treated with the fraction in increasing treatment period. Untreated control MCF-7 cells were viable and negative to annexin V and PI. Cells treated with F-1, F-2 and F-3 fractions at 3 hr and above were stained with annexin V-FITC only, indicating the presence of early apoptotic cells. Interestingly, at 24 hr treatment cells were most of the positive to annexin V and PI, indicating the presence of late apoptotic cells. However, the

presence of late apoptotic cells in F-3 treated sample was less than to that of F-1 and F-2. Thus, the results strongly indicate that the all the three fractions of *Aaptos* sp., exerted the cytotoxicity effects on MCF-7 cell line via apoptosis.

To further validate the fractions induced apoptosis in MCF-7 cell line was performed by TUNEL detection assay. Figure 5 A-D shows that the cells treated with F-1, F-2, F-3 of *Aaptos* sp., and vincristine sulfate at the concentrations of IC₅₀ 72 hr (5.06 µg/ml) for F-1, 15.06 µg/ml and 25 µg/ml for F-2 and F-3 respectively. The green stain of DNA fragmentation typically localized in morphologically identifiable nuclei of MCF-7 cells at 24 hr indicating the presence of DNA fragmentation, through catalysing polymerization of labelled nucleotides to cleavage DNA free 3'-OH DNA ends, which is a hallmark of apoptosis (Bowen *et al.*, 1998). Similar green stained nuclei was also observed when the cells were stained with vincristine sulfate as positive control (Talib *et al.*, 2012). However, nuclei of untreated (control) MCF-7 cells were not positive for green stain when observed under High Content Screening. Thus, the results strongly indicate that the fraction of *Aaptos* sp., induced apoptosis of MCF-7 cells, line via DNA fragmentation.

DISCUSSION

Cancer is multifactorial disease with uncontrolled division of cells occurred. The number of new cancer cases is expected to rise by 70%, from 14 million to 22 million, in couple of decades (World Health Organization 2014). Natural products and their various analogs covered approximately 50% of the drugs, presently used for clinical purposes within which 63% are of anticancer drugs (Cragg & Newman, 2009; Newman & Cragg, 2016). There are increasing evidences that suggest that the marine environment contain different classes of biologically active compounds with strong anticancer properties (Mayer & Gustafson, 2006). This characteristic of marine invertebrate leads these organisms to develop a vast number of biologically active secondary metabolites that suggests a dramatic potential for drug discovery. It is also interesting to note that the immense pool of marine organisms is still a largely untapped source of novel compounds with potent antitumor activity (Andavan & Lemmens-Gruber, 2010). One of the marine organisms that produce various natural products is sponge. Besides producing alkaloidal elements, sponge also biosynthesizes other interesting classes of natural products such as terpenoids, glycosides, phenols, phenazines, polyketides, fatty acids, peptides, amino acid analogues, nucleosides, porphyrins, aliphatic cyclic peroxides and sterols (Gordaliza, 2010; Montaser & Luesch, 2011; Mehbub *et al.*, 2014).

Due to highly complex frameworks and are precisely relevant chemical structures, these substances can display strong and often highly specific biological activities such as antibacterial, antiviral, antifungal, anti-prion, antimalarial, anti-inflammatory and immune or neuro-suppressive qualities (Blunt *et al.*, 2015). They exhibit distinct cytotoxic activity towards certain types of malignant cell lines, which make them potential source of drug development for the treatment of cancer.

Exposure of phosphatidylserine from inner to outer membrane and DNA fragmentation resulting apoptosis are leading mode of cell death of several anticancer agents. Thus, In order to be a drug, candidate used to possess unique characteristics in inducing apoptosis that leads to cell death (Su *et al.*, 2012).

Our previous studies reported that of *Aaptos* sp., extract produced cytotoxicity effect on MCF-7 cell line with IC₅₀ value of 7.61 µg/ml. (Tengku Muhammad *et al.*, 2017). In this study, 3 fractions of *Aaptos* sp., F-1, F-2 and F-3 produced a potent cytotoxicity activity of IC₅₀ 5.06 µg/ml, 15.06 µg/ml and 25 µg/ml, respectively, on human breast carcinoma cell line, MCF-7 when treated for 72 hr.

It was widely reported that various active extracts and compounds such as alkaloids, polyketides and terpenes isolated from marine sponge induce cytotoxicity in various cancer cells. In human breast cancer cells, ethyl acetate extract of *Hyattella cribiformis*, a marine sponge found in Thondi coast of India produced a potent cytotoxicity effect on MCF-7 and MAD-MB-231 cell lines with IC₅₀ values of 4.1 and 17.4 µg/ml, respectively (Goh *et al.*, 2014). In addition, a combination of butanol and chloroform partitions of methanol extract of *Geodia cydonium* collected from Fulf of Naples, Italy also exerted cytotoxicity activity on similar human breast cancer cell lines (Calcabrini *et al.*, 2017). Moreover, various bioactive compounds were also identified to inhibit the proliferation of human breast cancer cell lines. For examples, agelasine B (Pimentel *et al.*, 2012) and ingenamine (De Oliveira *et al.*, 2004) isolated from *Agelas clathrodes* and *Pachycalina* sp. killed MCF-7 cell line in dose-dependent manner with IC₅₀ value of 2.99 µM and 11.3 µg/ml, respectively.

Interestingly, two known compounds are used clinically to treat cancer. Eribulin mesylate, a synthetic analogue of helicondrin B isolated from Porifera halichondria *okadai* is used for the treatment of breast cancer by inhibiting the microtubules (Mayer *et al.*, 2010). Another anticancer agent, Ara-C, a derivative of the nucleosides spongostatin and spongouridine of *Tectitethya crypta* (Proksch and Edrada, 2002) is used for the treatment of leukemia (Schwartzmaan, 2000).

There are various mechanisms that are responsible in mediating the cytotoxicity effects of natural products isolated from marine sponges such as DNA protection, cell cycle, apoptosis and inflammation (Calcabrini *et al.*, 2017). Interestingly, the fractions exerted cytotoxicity effects on MCF-7 via apoptosis. Apoptosis is defined as programmed cell death, whereby series of intracellular enzymatic reactions are initiated provoking the disorganization of protein and subsequent damage to DNA. In the early stage of apoptosis, the integrity of the membrane is maintained, preventing intracellular components from being released, thus avoiding direct tissue damage (Navarrete & Ibáñez, 2008). In addition, one of the important features during this stage is translocation of phosphatidylserine (PS) from the inner to the outer leaflet within the membrane (Vermes *et al.*, 1995).

Our study showed that F-1, F-2, F-3 of *Aaptos* sp., at the concentrations of 5.06 µg/ml, 15.06 µg/ml and 25 µg/ml respectively triggered the translocation of PS which indicates the presence of early apoptosis at 3 hr, 6 hr in MCF-7 cells. In a parallel study, late apoptosis was also induced by all three fractions based on the presence of DNA fragmentations after 24 hr treatment (Pec *et al.*, 2003). In agreement with this study, various compounds isolated from sponges also exhibited apoptotic-mediated cell death on human breast cancer cells.

Guanidine Alkaloid, monanchocidin B isolated from marine sponge induced DNA fragmentation in malignant cells that leads to cell death (Dyshlovoy *et al.*, 2016). In addition, both spongistatin 1 (Schneiders *et al.*, 2009) and agelasine B (Pimentel *et al.*, 2012) killed MCF-7 cells by triggering the apoptosis via the activation of caspases. Therefore, it is tempting to speculate that the compounds presence in the three fractions used in this study may induce the apoptosis by similar mechanisms of action.

CONCLUSION

This study demonstrated that three fractions prepared from *Aaptos* sp., produced cytotoxicity effects (IC₅₀ less than 30 µg/ml at 72 hr) on human breast cancer MCF-7 cell line. MCF-7 cell death exerted by the fraction was found to be mediated by apoptosis based on the exposure of phosphatidylserine on the outer surface of cell membrane as early apoptosis and presence of DNA fragmentation as late apoptosis in treated cells. The apoptotic-induced cytotoxicity activity of the three fractions of *Aaptos* sp., may be due to the presence bioactive compounds such as alkaloid and terpenoid compounds, and thus, may have the potential to be developed further as candidates for chemotherapeutic drugs for the treatment of breast cancer.

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