Induction of apoptosis by Stichopus chloronotus and Holothuria nobilis fractions in the human cervical cancer cell line, HeLa

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ABSTRACT

Cancer is an uncontrolled growth of rapidly dividing cells. Decreased efficacy of current anticancer drugs urges to further screening and investigation for a better alternative to current chemotherapeutics. Natural products of marine origin are great sources of potential new drugs of enhanced biological activities. Thus, the work aims to investigate the cytotoxic effects along with the mode of cell death exerted by SC-8, SC-9, HN-3, HN-4 and HN-5 fractions prepared from Stichopus chloronotus and Holothuria nobilis marine-sponge on the human cervical cell line, HeLa. The fractions produced effective cytotoxicity with IC50 values at 72hr of less than 30 μg/ml in the order of HN-3 > HN-4 > SC-9 > SC-8 > HN-5. These fraction induced cytotoxicity via mediating apoptosis in HeLa cells. The early apoptosis was done by fractions via exposure of protein phosphatidylserine (PS) to the outer leaflet of the plasma membrane and late apoptosis confirmed due to the presence of fragmented DNA in treated cells. The presence of potentially bioactive compounds in these fractions might be responsible for inducing apoptosis and, thus, own potential to be a successful candidate for exploring forthcoming chemotherapeutic drugs.

INTRODUCTION

Cancer is the second leading cause of death worldwide. There is an estimated record of 2,626,418 deaths in the United States, 23% of which were due to cancer in the year 2014 (Siegel et al., 2016). The cervical cancer is the second leading cause of cancer death, emphasizing the need to improve screening rates as well as increase acceptance of and access to human papilloma virus vaccination (Lowndes, 2006). Cancer is an uncontrolled growth of cells, which occur due to rapid cell division. In multicellular organisms, the timely accomplishment of programmed cell death is critical for several natural physiological processes, including embryogenesis, post-embryonic development and homeostasis (Fuchs and Steller, 2011). Deregulation of cell death is a common feature of many human diseases, such as hyper proliferative conditions in cancer. Therefore, anticancer drugs have the potential to kill cancer cells via induction of apoptosis, causing less harm to surrounding cells and thus, eventually prevent lethal side effects (Jan, R, and Chaudhry, Gul-e-Saba, 2019).

The cell death occurred by apoptosis showed a remarkable characteristic feature of apoptosis includes; cell shrinkage, the cytoplasm becomes dense followed by chromatin condensation, plasma membrane blebbing and budding formation, which eventually triggered the formation apoptotic bod-
ies (Elmore, 2007). Several common risk factors associated with cervix cancer can cure and prevented by early screening and detection of stages. The presence of the Human Papilloma Virus (HPV) is one of the main epidemiological risk factors regarding cervix cancer (Lowndes, 2006). Poor healthy lifestyle, physical inactivity, alcohol consumption, obesity and tobacco smoking increase the incidence of cervix cancer.

Various clinical treatments for cervix cancer; includes surgery, radiation, and chemotherapy based on the stage of the cancer. A wide range of chemotherapy drugs, specific to cellular targets, such as DNA-targeting drugs, mitotic inhibitors and anti-metabolites, are used to treat cervical cancer. However, along with the poor solubility, cellular toxicity and various adverse side effects of chemotherapy, the drug resistance mechanism is an obstinate issue in cancer treatment. Platinum-based drugs have been used as a cytotoxic agent towards cervical cancer. The poor solubility of cisplatin (Pt-complex) held the efficacy of the potent drug (Gul-e-Saba et al, 2014; Hasumi et al., 2011). The resistance to platinum-based drugs, e.g., cisplatin and carboplatin, could be multi-factorial. The presence of effective repair mechanism results in DNA repair in cancer cells, causes the repression of apoptosis, enhanced drug detoxification, and minimal storage of intracellular carboplatin (Sousa et al., 2014).

The inadequate success of conventional chemotherapy due to serious side effects and reduces mortality; there is a potential need for new and effective therapeutic agents for the treatment of cancer. The new candidate must have the potential to kill the cancer cells by inducing the natural process of cell death apoptosis that will not result in inflammation and no toxic effects to normal cells. Over the past few decades, there has been a search for a new potential candidate in natural products. Therefore, many plant-derived compounds have been developed for treating cancer include; doxorubicin, bleomycin, mitomycin, vincristine, and vinblastine (Adrian and Collin, 2018).

Marine organisms signify a vast unexploited potential source of potential anti-cancer compounds (Adrian, 2007; Correia-Da-Silva et al., 2017). Sea cucumbers potent Holothuroidea from phylum Echinodermata, famous for containing valuable nutrients such as multi-vitamins; vit-A, B-complex (B1, B2, and B3) along with iron, magnesium, calcium and zinc (Aminin et al., 2015). The potential secondary compound, Triterpenoid glycosides isolated from sea cucumber species possess anticancer activity (Li et al., 2013). Similarly, another compound Frondoside A has shown potent anticancer effects in various solid malignancies. Frondoside A, limit cancer cell growth via the arrest cell cycle and induces apoptosis. Like previously mentioned compounds, saponin is also bioactive metabolites. The sea cucumber Holothuria Nobilis Selenka’s potential source of Echinoside A shows potent anticancer activities. Aqueous extract of Holothuria arenicola reduces the size of the tumor via inducing intrinsic apoptosis (Baharara et al., 2016). Sphingoid bases from Stichopus variegate induce the cell death via the mechanism of apoptosis. The mechanism involves the caspase-3 and Bax activation, which are pro-apoptotic enzymes and downregulation of activation of anti-apoptotic AKT (Sugawara et al., 2006; Hossain et al, 2013). Echinoside A isolates from Holothuria Nobilis and Peasonothuria Graeifie Induce apoptosis via downregulation of anti-apoptotic proteins of the Bcl-2 family, and up-regulate the activation of caspase-3 (Zhao, 2012; Li et al, 2010).

The screening for anticancer agents across various marine organisms has exposed several active compounds (Bordbar et al., 2011). Here, our interest is echinoderms, which only located in the marine, for example, sea cucumbers, sea urchins, sea stars, sand dollars and sea lilies. Echinoderms are found to have a number of natural products; extensive research is required to determine their cytotoxicity and mode of cell death. Therefore, in this study, sea cucumbers species Stichopus chloronotus and Holothuria nobilis fractions were selected to evaluate their cytotoxic abilities in the cancer cell with investigating the and mode of cell death in human cervical cancer (HeLa) cell line.

MATERIALS AND METHODS

The chemicals used were purchased from Sigma Aldrich, USA. The cancer cell line (HeLa cell line) was purchased from American Type Cell Culture, USA. For cytotoxic studies, the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay kit used purchased from Promega, USA. The ApoAlertTM Annexin V, Clontech, USA and DeadEnd™ Fluorometric TUNEL were from Promega USA. All chemicals used were of analytical grades.

Preparation of Fractions from Stichopus chloronotus and Holothuria nobilis

The fractions of Stichopus chloronotus and Holothuria nobilis were prepared and further evaluated in this study. The fraction of Stichopus chloronotus and Holothuria nobilis was prepared by using Medium pressure liquid chromatography
(MPLC) using a previously reported method (Gul-E-Saba et al., 2018). Locations of sample collection are shown in Table 1. Briefly, the study sample was chopped after cleaning and then undergoes freeze-drying for removal of water content. Subsequently, the powder form of the sample used, 10 gm of each sample, was later used for the extraction process (Hudayah et al., 2017).

Cytotoxicity activity of Fractions

The cytotoxic effects of Stichopus chloronotus and Holothuria nobilis fractions on the HeLa cell line were determined by using MTS assay (CellTiter 96TM Aqueous Non-Radioactive Cell Proliferation Assay) (G-S Chaudhry, R Jan 2019a). The cell line was culture according to scientific procedures done in Animal cell culture Lab, ISO certified (MS/ISO/IEC 17025:2015 SAMM NO:796) Institute of Marine Biotechnology, IMB Briefly, the cells were plated on 96 well plates and incubated at 37°C in a 5% CO2 incubator. Next, the old medium was replaced with a new medium containing serial dilution concentrations of the prepared fractions and incubated for 24hr, 48hr and 72hr. Subsequently, after treatment, 20μl volume of MTS was added to each well and incubated for 3 hr. Finally, the absorbance was observed at 490 nm (Multiskan, Thermo Fisher US) ELISA reader.

Phosphatidylserine (PS) Externalization (Early apoptosis) in treated cells

The Annexin V-FITC Apoptosis Detection Kit was used to observe the apoptosis mode of cell death exerted by the fractions on the HeLa cells. The process of staining cells was the same as to mention in our previously reported method. Briefly, the cancer cells were plated with a density of 10,000 cells/well in 96-well plates. The Annexin V-FITC and PI (5μL + 10μL) of at 37°C for 5-15 mins. The slides were then observed under ImageXpress Micro XLS Widefield High-Content Analysis System (HCS) (Sunnyvale, USA) for images.

Fluorometric TUNEL System (Late apoptosis) in treated cells

The DeadEndTM Fluorometric Apoptosis Detection System (Promega, USA) was used to determine the fragmentation in DNA, which shows that the induction of apoptosis in cancer cells via DNA fragmentation. The HeLa cell line was cultured approximately 10,000 cells/chamber and incubated at 37°C in the presence of 5% (v/v) CO2 for 24hr. The method used was the same as our previously reported method (Chaudhry, GS, Jan, R, Zafar, MN, Habsah, M, Muhammad, TST, 2019b). The treated cells were fixed by using 4% paraformaldehyde solution in PBS (pH 7.4) followed by washing and then permeabilize for 5 min by using 0.2% triton X-100. The green fluorescence of fragmented DNA represents apoptotic cells, which was noticed by Image Xpress Micro XLS Widefield High-Content Analysis System (HCS) (Sunnyvale, USA).

Statistical Analysis

The Cytotoxicity or cell viability assay was carried out in triplicates in two independent experiments. The IC50 values were calculated from a nonlinear regression model (curve-fit) using GraphPad Prism 7.0 (Graphpad). The values are given as mean ± S.E.M where, ANOVA *P<0.05 and **P<0.01 (Dunnett post-test).

Table 1: Location and coordinate where the samples were collected

| Species               | Location of collection          |
|-----------------------|---------------------------------|
| Stichopus chloronotus | Bidong Island, Terengganu (5°37’12"N 103°3’48"E) |
| Holothuria nobilis    | Bidong Island, Terengganu (5°37’12"N 103°3’48"E) |

RESULTS AND DISCUSSION

The cytotoxicity effects of five fractions, SC-8, SC-9, HN-3, HN-4 and HN-5fractions prepared from Stichopus chloronotus and Holothuria nobilis sp., on human cervical cancer HeLa cell line were investigated Table 2. All fractions produced a dose-dependent based growth inhibition of cell at each incubation period of the 24hr, 48hr and 72hr.

Figure 1: Percentage of growth inhibition ± standard deviation for SC-8 fraction of Stichopus chloronotus against HeLa cells at 24hr, 48hr, and 72hr.

The SC-8 fraction of Stichopus chloronotus sp., significantly inhibited HeLa cell growth at 7.50 μg/ml and above at 24hr, and at 0.937 μg/ml and above when treated for 48hr and 72hr (Figure 1). The fraction showed a cytotoxicity effect on the HeLa cell line at 7.50 μg/ml, where 20%, 25% and 50% of cell populations were killed after 24hr, 48hr and...
Table 2: IC50 values of three fractions of Stichopus chloronotus and Holothuria nobilis fractions and vincristine sulfate (µg/ml)

| Fractions          | 24hr | 48hr | 72hr |
|-------------------|------|------|------|
| 1 Holothuria nobilis HN-3 | 9.04 | 4.794| 2.545|
| 2 Holothuria nobilis HN-4 | 15.51| 8.586| 3.918|
| 3 Holothuria nobilis HN-5 | 36.56| 24.42| 14.76|
| 4 Vincristine Sulfate   | -    | -    | 0.005|

72 hr, respectively. Interestingly, at 72 hr treatment, 15 µg/ml inhibited 80% of cell growth as compared to control. The IC50 values were reduced from 19.91 µg/ml (24 hr) to 15.74 µg/ml (48 hr) and to 8.543 µg/ml (72 hr).

Similarly, the SC-9 fraction of Stichopus chloronotus sp. also showed a potential inhibitory activity on the HeLa cell line. Initially, at 24 hr incubation, the percentage of cell growth was significantly inhibited at 15 µg/ml and above. Moreover, at a lesser concentration of 7.50, the inhibition was significantly increased by 50% and 75% after 48 hr and 72 hr, respectively. Interestingly, 3.75 µg/ml reduced 40% of cell growth after 72 hr incubation (Figure 2). The fraction exhibited potent cytotoxicity activity against HeLa cell line with IC50 values of 49.18 µg/ml (24 hr), 23.64 µg/ml (48 hr), and 6.71 µg/ml (72 hr).

The HN-3 fraction of Holothuria nobilis sp. reduced the cell growth in a dose-dependent manner over the period of 24 hr, 48 hr and 72 hr. The fraction significantly reduced the growth of HeLa cells at concentrations 0.93 µg/ml and above, after 48 hr and 72 hr incubation (Figure 3). Interestingly, 50% of growth inhibition was noticed at 7.5 µg/ml after 24 hr incubation, which was further reduced in 48 hr and 72 hr. Interestingly, 45% of inhibition was recorded at 0.93 µg/ml after 72 hr incubation. IC50 value of the was 9.04 µg/ml, 4.794 µg/ml and 2.545 µg/ml at 24 hr, 48 hr, and 72 hr treatment, respectively, which specify the potential cytotoxicity effect of fractions on HeLa cell line at 72 hr, categorized as cytotoxic (IC50 < 30 µg/ml) by the National Cancer Institute, USA against cancer cell line at 72 hr (Geran et al., 1972).

The similar pattern of inhibition was observed in HN-4 fraction of Holothuria nobilis sp., HN-4 fraction significantly inhibited the cell growth of MCF-7 cells as compared to control when treated at concentrations of 1.87 µg/ml and above (24 hr) and 0.93 µg/ml and above (48 hr and 72 hr). At a concentration of 3.75 µg/ml, more than 50% of growth inhibition was observed at 72 hr incubation. The fraction exhibited potent cytotoxicity activity towards the HeLa cell line with IC50 values of 15.51 µg/ml (24 hr), 8.58 µg/ml (48 hr) and 3.918 µg/ml at 72 hr treatment (Figure 4).
60% and 80% of growth inhibition were observed at 48hr and 72hr incubation, respectively. Interestingly, 0.937 µg/ml exhibited 55% inhibition of growth at 72hr. The fraction showed cytotoxicity activity against HeLa cell line with IC50 values of 36.56 µg/ml (24hr), 24.42 µg/ml (48hr) and 14.76 µg/ml at 72hr (Figure 5). Vincristine sulfate was used as a positive control in the cytotoxicity study showed the IC50 value of 0.005 µg/ml at 72hr.

Figure 4: Percentage of growth inhibition ± standard deviation for HN-4 fraction of Holothuria nobilis against HeLa cells at 24hr, 48hr, and 72hr.

Overall, fractions of Stichopus chloronotus and Holothuria nobilis sp. produced a potent cytotoxicity effect on the human cervical cancer cell line, HeLa, at all treatment periods. (Table 1). The relative potential of cytotoxicity of the fractions at 72hr as follows, Holothuria nobilis HN-3 > Holothuria nobilis HN-4 > Stichopus chloronotus SC-9 > Stichopus chloronotus SC-8 > Holothuria nobilis HN-5.

To determine the mode of cell death responsible for producing the cytotoxicity effects of fractions of Stichopus chloronotus and Holothuria nobilis sp., apoptosis study was done includes early and late apoptosis (DNA fragmentation).

Figure 5: Percentage of growth inhibition ± standard deviation for HN-5 fraction of Holothuria nobilis against HeLa cells at 24hr, 48hr, and 72hr.

HeLa cells were treated with fractions of Stichopus chloronotus and Holothuria nobilis sp., and vincristine sulfate at the concentrations of IC50 72hr. Cells treated with SC-8, SC-9, HN-3 and HN-4 fractions were positive to green stain, indicating the occurrence of early apoptotic cells after 3hr incubation. The increase in annexin V-FITC binding (time-dependent) was observed in the outer membrane of cells after treated with fractions from 3hr to 24hr incubation. Interestingly, at 24hr treatment, most of the cells were positive to annexin V (V+), which indicates the induction of early apoptosis. However, the presence of late apoptotic cells in SC-8 and SC-9 was higher than NH-3 and HN-4 treated samples, showed by the presence of red stain of propidium iodide (PI) binds to DNA of treated cells after 24hr (Figure 6). Untreated control HeLa cells were viable and negative-stain to annexin V and PI. Thus, the results intensely show that all the five fractions of Stichopus chloronotus and Holothuria nobilis sp. produced the cytotoxicity effects on the HeLa cell line via induction of apoptosis.

In order to further confirm, the apoptosis was induced by fractions in the HeLa cell line, TUNEL (nick end labeling DNA fragmentation) detection. (Figure 7A-D) shows that the cells treated with SC-8, SC-9, HN-3 and HN-4 and vincristine sulfate (positive control) at the concentrations of IC50 72hr. The observation of green stain of DNA fragmentation at 36hr indicating the presence of DNA fragmentation, through catalyzed polymerization via Terminal Deoxynucleotidyl Transferase (TdT) of fluorescence-labeled nucleotides to the site of cleavage DNA. Similarly, the green nucleus was also detected in the positive control (Vincristine) treated cells. However, untreated (as control) cells were not green stained under High Content Screening. Thus, the results strongly indicate that the fraction of Stichopus chloronotus and Holothuria nobilis sp., induced late apoptosis in HeLa cells via DNA fragmentation.

Over the past few decades, much effort has been dedicated in regards to discovering various novel active bio-compounds from natural sources. Amongst, marine ecosystems might be an essential resource of potentially active compounds (Lordan et al., 2011). However, their active mechanisms of bioactive compounds need to be investigated. Several naturally occurring compounds have great potential as lead compounds for drug development in pharmaceutics (Venugopal, 2009; Althunibat et al., 2009).

Various species of sea cucumber exerted cytotoxicity on various human cancer cell lines. The aqueous extract of Stichopus chloronotus inhibited the growth of human non-small lung carcinoma (A549) and cervical cancer cells (C33A). However,
Figure 6: (A-E): The green stain (annexin-FITC) early apoptosis; Red stain, PI (propidium iodide) late apoptosis indicates induction of apoptosis in HeLa cells; (A,B,C) SC-8 (D,E,F) SC-9 (G,H,I) HN-3 (J,K,L)HN-4 (M,N,O) HN-5 (P,Q,R) positive control (S,T,U) Negative control (untreated) for 3hr, 6hr and 24hr respectively.
Figure 7: (A-E): The presence of green stain (FITC), which indicates DNA fragmentation in HeLa cells treated with row A-E. (A) SC-8; (B) SC-9; (C) HN-3; (D) HN-4; (E) HN-5; (F) Vincristine sulfate and (G) Negative control, after 36 hr.
Our study showed that SC-8 and SC-9 fraction of less direct tissue damage (the leakage of intracellular components results in membrane integrity remains constant, preventing outer leaϑlet within the membrane, whereas the translocation of phosphatidylserine (PS) to the outer leaϑlet proteins and DNA fragmentation (1992) of cell death involves exposure of plasmamembrane (Allen et al., 1993a, b)). During the early stage of apoptosis induction, translocation of phosphatidylserine (PS) to the outer leaϑlet within the membrane, whereas the membrane integrity remains constant, preventing the leakage of intracellular components results in less direct tissue damage (Allen et al., 1993). Our study showed that SC-8 and SC-9 fraction of Stichopus chloronotus and HN-3, HN-4 and HN-5 fractions of Holothuria nobilis sp., at the concentrations of 8.543, 6.71, 2.545, 3.918 and 14.76 μg/ml respectively triggered the exposure/translocation of PS which indicates the presence of early apoptosis at 3 hr, 6 hr and 24 hr in HeLa cells is in agreement with the previous study, the induction of apoptosis by total and individual saponins was increased by time (Yu et al., 2015). Similarly, late apoptosis was also induced by fractions based on the presence of DNA fragmentations. The fragmentation of DNA is the hallmark of apoptosis (Wadskog et al., 2004). The TUNEL positive (green) was observed with a fluorescence microscope, which indicated DNA fragmentation (Hwang et al., 2012). The extracts in this study were mainly hydrophobic compounds (organic extract); they could be sphingoid bases, which have been found to have anticancer properties (Sugawara et al., 2006). Sphingoid bases isolated from sea cucumber induced DNA fragmentation results in apoptotic cell death. Stichopouloside C isolated from sea cucumbers (holothurians) induced apoptosis via activation of Fas receptor protein, up-regulation of caspase-3 and caspase-8, activation of Bid through cleavage, and mitochondrial damage in cancer cells (Yun et al., 2012). Therefore, our studied fractions might be possible to go through apoptosis via extrinsic or intrinsic pathways of program cell death by similar mechanisms of action.

CONCLUSIONS

This study demonstrated that SC-8 and SC-9 fraction of Stichopus chloronotus and HN-3, HN-4 and HN-5 fractions of Holothuria nobilis sp., produced potential cytotoxicity effects on human cervical cancer HeLa cell line. The cell death was mainly due to mediated by apoptosis via early (exposure of phosphatidylserine) and late apoptosis (presence of fluorescence Nucleus DNA fragmentation) fractions treated cells. However, further investigation needed to be done to study the effect of fractions in vivo, including toxicity study (serum enzymes) as well as normal cell line study. The apoptotic-induced cytotoxicity activity might be due to the presence of secondary metabolites as bioactive compounds such as alkaloid and terpenoid compounds. Thus, a mechanistic study needs to be done to fully understand the potential of these active compounds as future chemotherapeutic drugs for the treatment of cervical cancer.
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