

Full Length Research Paper

Apoptosis induced in human breast cancer cell line by *Acanthaster planci* starfish extract compared to tamoxifen

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Breast cancer remains the most common cancer among women all over the world. Current chemotherapeutic drugs for breast cancer such as tamoxifen show severe side effects. Therefore, there is a focus toward new chemotherapeutic agents to be obtained from natural sources. This study was carried out to evaluate cytotoxic and apoptotic effects of the *Acanthaster planci* starfish extract compared to tamoxifen in human breast cancer MCF-7 cell line. The extract was obtained using phosphate buffered saline. MTS assay and Annexin V assay were performed to evaluate the cytotoxic effect and apoptotic effect, respectively. The extract was found to inhibit the growth of MCF-7 cells ($IC_{50}=15.6 \mu\text{g/ml}$) and induced apoptosis (52.9% after 1 h treatment and 63.9% after 2 h treatment). This apoptotic effect was more potent and earlier than the apoptotic effect induced by tamoxifen. These data suggest that *A. planci* starfish extract may be utilized as potential chemotherapeutic agents to be used in the treatment of human breast cancer.

Key words: *Acanthaster planci*, cytotoxicity, apoptosis, tamoxifen, MCF-7 cell line.

INTRODUCTION

Chronic diseases are linked with high mortality and morbidity (Sahib et al., 2009). Cancer, as a chronic disease, represents the largest cause of mortality in the world and over 6 million lives are claimed each year (Chermahini et al., 2010). Breast cancer is the most common cancer and the leading cause of cancer death in women between 35 to 54 years (Yu et al., 2005). Cancer is the result of either improper cell proliferation or

improper cell death, and apoptosis and necrosis are the two major mechanisms of cell death. In cancer chemotherapy, apoptosis does not lead to cell lysis and inflammatory responses in contrast to necrosis. Therefore, it is ideal to increase apoptosis without increasing necrosis during chemotherapeutic treatment of cancer (Lin et al., 2008).

In the area of anti-cancer drugs development, some of the effective drugs have been originated from natural products. These include agents derived from plants and they have been reviewed in the recent volume "Anticancer Agents from Natural Products" (Cragg et al., 2005), such as vinblastine and vincristine (vinca alkaloids)

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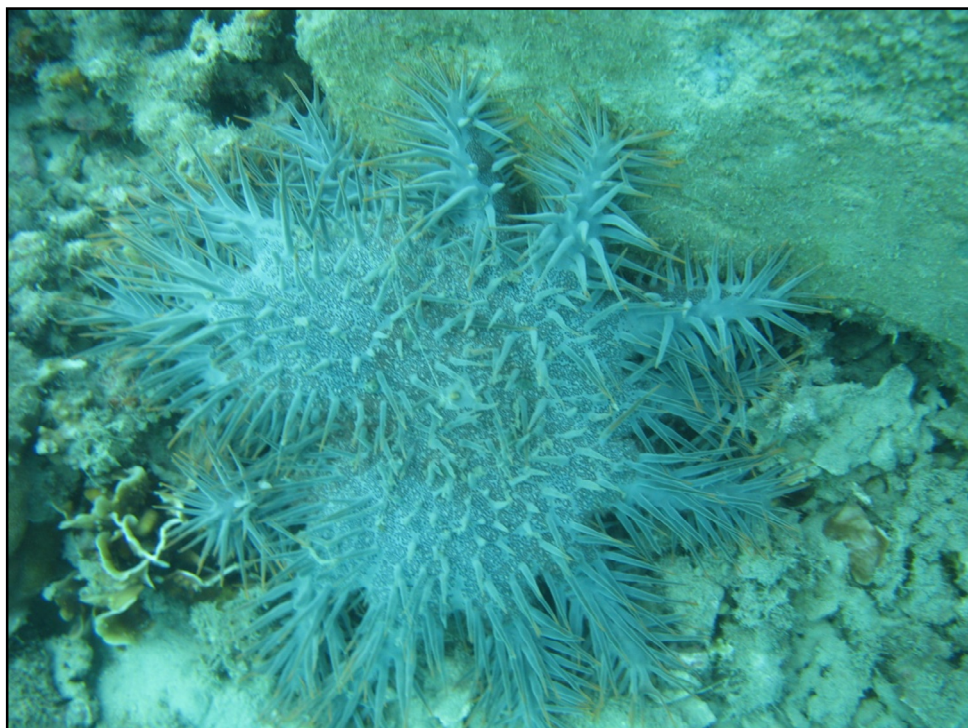


Figure 1. The crown-of-thorns starfish, *A. planci* (Linnaeus, 1759). Voucher specimen, PPSK/USM/CTI-0-07-2009-APLC.

Phylum: Echinodermata
 Class: Asteroidea
 Order: Valvatida
 Family : Acanthasteridae
 Genus : Acanthaster (Gervais, 1841)
 Species: *A. planci* (Linnaeus, 1758)

alkaloids) that were extracted from *Catharanthus roseus* (Gueritte and Fahy, 2005). There are many varieties of fruits and vegetables also have been evaluated for their protective activity against cancer (Ampasavate et al., 2010).

At present, there are no anti-cancer drugs derived from the marine area that have yet to be approved to be used commercially. However, there is a significant number of compounds presently being further evaluated in the clinical trials for various application as anti-cancer drugs such as, aplidine which was derived from *Aplidium albicans* (Henriquez et al., 2005), discodermolide derived from the Caribbean deep water sponge *Discodermia dissolute* (Gunasekera and Wright, 2005), bryostatin derived from the bryozoan *Bugula neritina* which is found in the Gulf of California (Newman, 2005), ecteinascidin 43 isolated from the tunicate *Ecteinascidia turbinata*, collected initially in the Caribbean (Henriquez et al., 2005) and finally, dolastatin 10, from the Indian Ocean nudibranch *Dolabella auricularia* (Flahive and Srirangam, 2005).

The crown of thorns starfish, *Acanthaster planci* (Figure 1) is widely distributed in the Indo-Pacific region, where outbreaks of this starfish cause severe damage to the coral reef (Endean, 1982).

It has been reported that the crude toxin, extracted from the *A. planci* starfish spines, exhibits mouse lethality, hemolytic activity, myonecrotic activity, hemorrhagic activity, increase capillary permeability activity, edema-forming activity, phospholipase A2 (PLA2) activity, anticoagulant activity and cardiovascular actions (Yara et al., 1992; Shiroma et al., 1994; Karasudani et al., 1996; Shiomi et al., 1998). It has also been reported that a cytostatic compound (Asterosaponin 1) from the starfish *Culcita novaeguineae* functions by inducing apoptosis in human glioblastoma U87MG cells (Cheng et al., 2006).

In the view of these scientific reports and the traditional usages of natural products, the present study was aimed to investigate the cytotoxic and apoptotic effects of the *A. planci* starfish extract on human breast cancer MCF-7 cell line, and to compare with the effects induced by

tamoxifen.

MATERIALS AND METHODS

Dulbecco's modified eagle medium (DMEM), trypsin ethylene diamine tetraacetic acid (EDTA) 0.25%, fetal calf serum and penicillin/streptomycin were purchased from GIBCO (Grand Island, NY, USA). CellTiter 96[®] AQUEOUS non-radioactive cell proliferation assay kit was purchased from Promega (Madison, WI, USA). Tamoxifen and phosphate buffered saline tablets were obtained from Sigma (St. Louis, MO, USA). Cell culture flasks and 96-well μ l plates were purchased from Corning Incorporated (Corning, NY, USA). Chemicals used in this study were analytical or cell culture grade.

Methods

Starfish

The *A. planci* starfish specimens were harvested at the coasts of Pulau Redang (5°47.404"N; 102°59.596"E) and Pulau Lang Tengah (5°47.593"N; 102°53.483"E) Eastern Peninsular Malaysia, in July, 2009 and were taxonomically identified by Associate Professor Dr. Farid Che Ghazali. Post harvesting, they were immediately kept in ice, then transported to our laboratory and frozen at -20°C. A voucher specimen registered number (PPSK/USM/CTI-0-07-2009-APLC) was deposited at the School of Health Sciences, Universiti Sains Malaysia.

Preparation of extract

A frozen sample of *A. planci* (300 g), after homogenization by a homogenizer, was extracted twice with two volumes of phosphate buffered saline (0.01 M, pH 7.0) under room temperature. The supernatant was filtered and then evaporated by rotary evaporator and the extract was then freeze dried and weighed. The dried yield of the extract was 5.515 g. The extract was then dissolved in deionized distilled water.

Cell culture

The MCF-7 cell line was a kind gift from Dr. Amin Malik Shah and was originally obtained from American Type Culture Collection (Rockville, Md, USA). Cells were cultured in DMEM containing 10% fetal calf serum and 1% penicillin/streptomycin. Cells were maintained in 25 cm² cell culture flasks in an incubator at 37°C containing 5% CO₂.

MTS cytotoxicity assay

CellTiter 96w AQUEOUS non-radioactive assay kit was used to assess cell viability according to the manufacturer's instructions (Promega, USA). Cells were cultured in 96-well plates (8.0 × 10³ cells/well) overnight prior to the treatment with extract or tamoxifen (a chemotherapeutic drug used in the treatment of breast cancer). The cells were then treated with extract (6.25 to 100 μ g/ml) and tamoxifen (5 to 20 μ g/ml). After 48 h, 20 μ l of the kit reagent solution was added directly to each well and incubated for 5 h. The

absorbance was then measured at 490 nm with a reference wavelength at 630 nm using the enzyme-linked immunosorbent assay (ELISA) microplate reader Power Wave_x 340 (Bio-Tek Instruments, Inc. USA). The cell viability inhibition was calculated according to the following formula:

$$\text{Cell viability inhibition (\%)} = \frac{\text{Absorbance in control wells} - \text{Absorbance in test wells}}{\text{Absorbance in control wells}} \times 100\%$$

The average cell viability inhibition obtained from triplicate determinations at each concentration was plotted as a dose response curve. The IC₅₀ (50% inhibition concentration) of extract and tamoxifen was determined as the concentration which reduced cell growth by 50% in treated cells compared to untreated cells.

Detection of apoptosis

Annexin V assay was used to detect apoptosis which was performed using an apoptosis detection kit (Annexin V-FITC Apoptosis Detection Kit; BioVisionResearch Products, CA, USA) and FACSCanto II flow cytometer (BD Biosciences, CA, USA). Cells were cultured (1.5 × 10⁵ cells) overnight in 25 cm² cell culture flasks prior to the treatment with extract or tamoxifen. Cells were then treated with IC₈₅ of the extract (30 μ g/ml) and with IC₈₅ of tamoxifen (15 μ g/ml) for 1, 2 and 4 h. Cells were harvested by treatment with trypsin EDTA 0.25%, washed with serum-containing media, re-suspended in binding buffer and incubated with fluorescein-conjugated Annexin V and propidium iodide at room temperature for 5 min in the dark.

Statistical analysis

The cytotoxic results were expressed as mean \pm standard deviation using one way analysis of variance (ANOVA). Values at p<0.01 were considered as statistically significant. The flow cytometric results were analyzed using BD FACSDiva software.

RESULTS

Starfish extract induced cytotoxicity in MCF-7 cells

Treatment with various concentrations of starfish extract caused a dose-dependent cytotoxicity in MCF-7 cells (Figure 2). The extract was found to be significantly potent cytotoxic on MCF-7 cells and showed a low IC₅₀ value (15.6 μ g/mL, p < 0.01). IC₅₀ value for tamoxifen was found to be 11.84 μ g/ml, p < 0.01 (Figure 3).

Starfish extract induced apoptosis in MCF-7 cells

To provide more accurate data representation, two types of plots (dot plot and contour plot) were used to express the flow cytometric results analysis. The left lower quadrant represented living cells, the right lower quadrant represented early apoptotic cells and the right upper quadrant represented late apoptotic or necrotic cells. Control experiments for the extract and tamoxifen showed

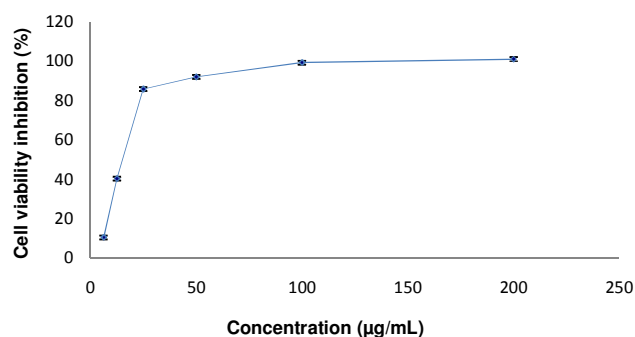


Figure 2. The cytotoxicity effect of *A. planci* extract on MCF-7 cells

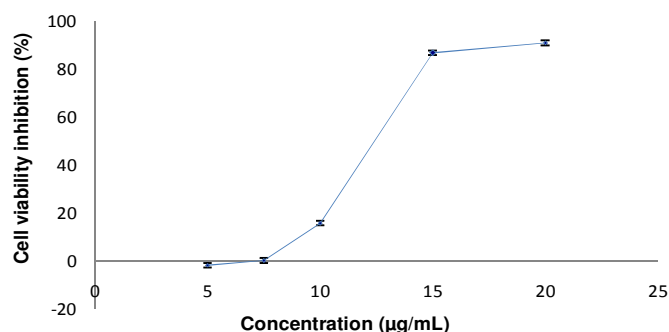


Figure 3. The cytotoxicity effect of tamoxifen on MCF-7 cells.

showed that about 89.0% of the cells were live, about 4.5% were early apoptotic cells and about 6.5% were late apoptotic/necrotic cells (Figures 4A and D and 5a and c). Phosphatidylserine externalization occurred after 1 and 2 h treatment with 30 µg/ml of the extract, live, early apoptotic and late apoptotic (or necrotic) cells were determined after 1 h treatment to occupy 29.9, 52.9 and 17.2%, respectively (Figures 4B and E), and after 2 h treatment, determined to occupy 15.8, 63.9 and 20.3%, respectively (Figures 4C and F). Most of the cells were late apoptotic or necrotic after 4 h treatment with the extract. Phosphatidylserine externalization occurred after 4 h treatment with 15 µg/ml tamoxifen, cells were determined to occupy 52.2, 42.3 and 5.5%, respectively (Figures 5b and 5d). Apoptosis was not detected after 1 and 2 h treatment with tamoxifen.

DISCUSSION

A large number of compounds synthesized by marine organisms, plants and microorganisms have been studied to evaluate their potential biological therapeutic

activities (Ruggieri, 1976; Cundliffe, 1981). Owing to the cytotoxic efficacies, some natural products exhibited cytotoxic properties and were found to be potent inhibitors of cellular metabolic pathways. Natural products were found to be excellent sources of novel and tangible drugs for chemotherapy such as vinblastine, vincristine and Taxol (Gueritte and Fahy, 2005; Kingston, 2005).

In this study, the cytotoxic and apoptotic effects of the *A. planci* starfish extract were evaluated in human breast cancer MCF-7 cell line. The starfish crude extract significantly exhibited potent cytotoxic activity on MCF-7 cells with a low IC_{50} value (15.6 µg/ml, $p < 0.01$). The National Cancer Institute (NCI) has reported that crude extract which exhibits cytotoxicity with IC_{50} less than 20 µg/mL is considered as an active compound against cancer cells (Geran et al., 1972; Chen et al., 1988). Apoptosis, a process of programmed cell death, leads to the elimination of individual cells from the midst of viable tissue (Wyllie et al., 1980; Raff, 1992). It is ideal to search for chemotherapeutic compounds that induce potent apoptosis during treatment of cancer. Tamoxifen, a nonsteroidal selective estrogen receptor modulator, inhibits proliferation and induces apoptosis in MCF-7 breast cancer cells by estrogen receptor-dependent modulation of gene expression and is widely used as a chemotherapeutic drug in the treatment of breast cancer (Fisher et al., 1998; Radmacher and Simon, 2000; Zheng et al., 2007). The present study clearly demonstrated that the *A. planci* extract induces apoptosis in human breast cancer MCF-7 cells. Interestingly, it was found that the observed apoptotic effect induced by the extract after 1 and 2 h treatment (52.9 and 63.9%, respectively) is more potent and earlier than the apoptotic effect induced by tamoxifen after 4 h treatment (42.3%). Thus, it has been suggested that the extract of *A. planci* starfish, as a potential cytotoxic agent that could be employed as a chemotherapeutic agent, might be more effective than tamoxifen in the treatment of breast cancer.

Conclusion

The outcomes of the aforementioned study demonstrated that the crude extract of *A. planci* starfish has potent cytotoxic as well as apoptotic effects on human breast cancer MCF-7 cell lines. Therefore, *A. planci* starfish crude extract could be a promising chemotherapeutic agent to be used more effectively than tamoxifen in the treatment of breast cancer but further investigation is required.

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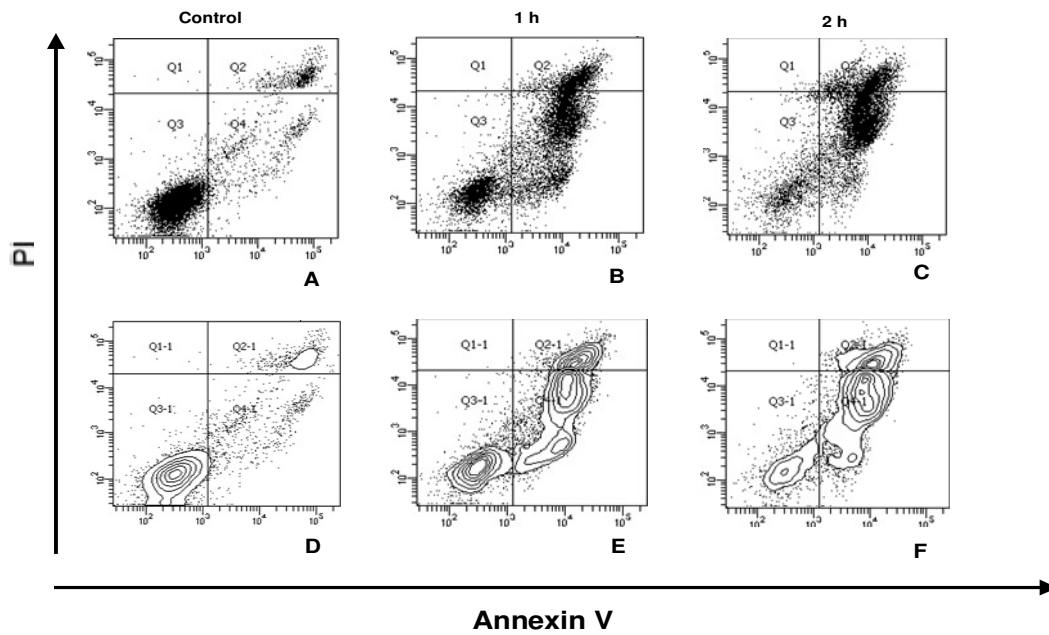


Figure 4. Representative dot plots (A, B, C) and contour plots (D, E, F) showing flow cytometric results analysis of MCF-7 cells treated with extract then stained with FITC-conjugated Annexin V and propidium iodide.

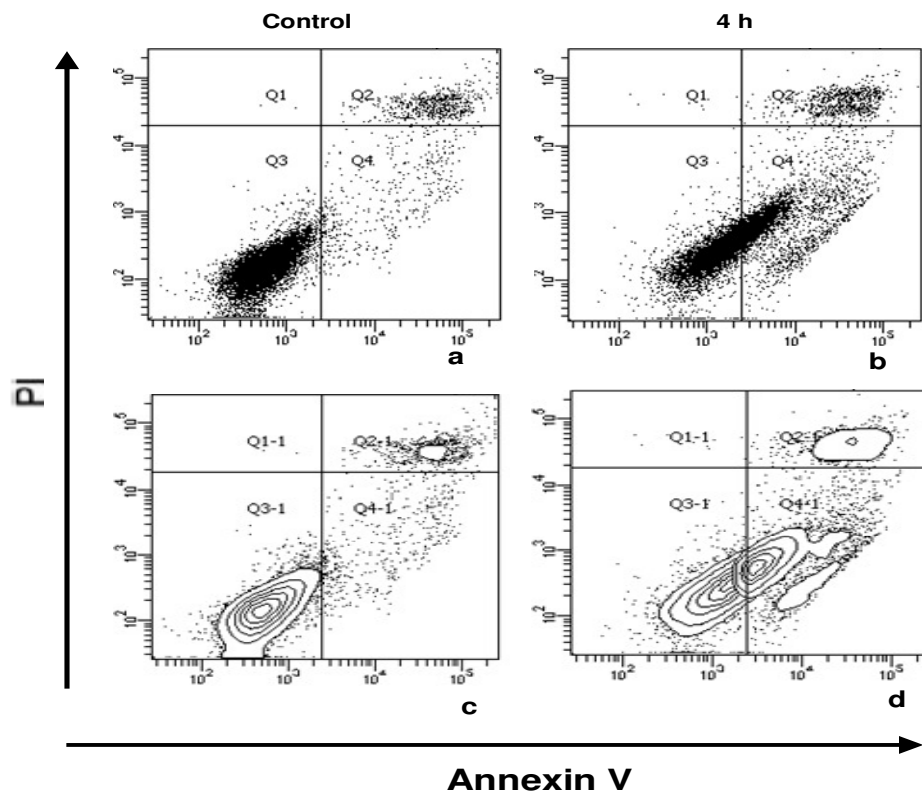


Figure 5. Representative dot plots (a, b) and contour plots (c, d) showing flow cytometric results analysis of MCF-7 cells treated with tamoxifen then stained with FITC-conjugated Annexin V and propidium iodide.

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