Evaluation of anti-cancer activity of *Acanthester planci* extracts obtained by different methods of extraction

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Abstract: *Acanthaster planci*, the crown-of-thorns starfish, naturally endowed with the numerous toxic spines around the dorsal area of its body. Scientific investigations demonstrated several toxico-pharmacological efficacies of *A. planci* such as, myonecrotic activity, hemorrhagic activity, hemolytic activity, mouse lethality, phospholipase A2 (PLA2) activity, capillary permeability-increasing activity, edema-forming activity, anticoagulant activity and histamine-releasing activity from mast cells. The present study was performed to evaluate the cytotoxic activity of *A. planci* extracts obtained by different methods of extraction on MCF-7 and HCT-116, human breast and colon cancer cell lines, respectively. Results of the cell proliferation assay showed that PBS extract exhibited very potent cytotoxic activity against both MCF-7 and HCT-116 cell lines with IC_{50} of 13.48 µg/mL and 28.78 µg/mL, respectively, while the extracts prepared by Bligh and Dyer method showed moderate cytotoxicity effect against MCF-7 and HCT-116 cell lines, for chloroform extract, IC_{50} = 121.37 µg/mL (MCF-7) and 77.65 µg/mL (HCT-116), and for methanol extract, IC_{50} = 46.11 µg/ml (MCF-7) and 59.29 µg/ml (HCT-116). However, the extracts prepared by sequential extraction procedure from dried starfish found to be ineffective. This study paves the way for further investigation on the peptide composition in the PBS extract of the starfish to discover potential chemotherapeutic agents.

Keywords: *Acanthaster planci*, cytotoxicity, MTS assay, human cancer cell lines.

INTRODUCTION

Cell proliferation plays an important role in cancer development, and it has been demonstrated that some natural agents have properties to control hyper-proliferation of cells in the target organs during the initiation as well as post-initiation phases of cancer (Mori *et al.,* 1999; Mori *et al.,* 1997). In the area of anticancer 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 the vinca alkaloids vinblastine and vincristine that were isolated from the Madagascar periwinkle, *Catharanthus roseus* (Gueritte and Fahy, 2005). Etoposide and teniposide were derived semi-synthetically from epipodophyllotoxin, an epimer of podophyllotoxin, isolated from roots of *Podophyllum* species (Lee and Xiao, 2005). Paclitaxel (Taxol) was derived from the bark of the Pacific yew tree from the Pacific Northwest, *Taxus brevifolia*, and the analogue, docetaxel (Kingston, 2005). Camptothecin was isolated from the bark of *Camptotheca acuminata*, a precursor to the semi-synthethetic drugs topotecan (Hycamptin) and irinotecan (Camptosar) (Rahier *et al.,* 2005).

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 (a) aplidine which was isolated from the Mediterranean tunicate *Aplidium albicans* (Henriquez *et al.,* 2005), (b) discodermolide, from the Caribbean deep water sponge *Discodermia dissolute* (Gunasekera and Wright, 2005), (c) bryostatin, derived from the bryozoan *Bugula neritina* which is found in the Gulf of California (Newman, 2005), (d) 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).

Acanthaster planci belongs to the family Acanthasteridae (also known as crown-of-thorns starfish), and is a red colored starfish that occurs in coral reefs in the Red Sea. It has been found that this starfish is damaging coral reefs by inhabiting tropical and subtropical waters and preys on coral polyps. This starfish has a number of venomous spines on the dorsal surface of its body (Ota *et al.,* 2006). Invasive contact with these spines inflicts noxious symptoms such as severe pain, redness, swelling and protracted vomiting (Shiomi *et al.,* 1998). Some studies

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showed that the crude toxin extracted from the spines exhibits some biological activities such as myonecrotic activity, hemorrhagic activity, hemolytic activity, mouse lethality, phospholipase A2 (PLA2) activity, capillary permeability-increasing activity, edema-forming activity (Shiomi *et al.*, 1985), anticoagulant activity (Karasudani *et al.*, 1996), cardio-vascular actions (Yara *et al.*, 1992; Shiroma *et al.*, 1994) and histamine-releasing activity from mast cells (Shiomi *et al.*, 1989). Accordingly, a hypothesis was formulated that *A. planci* could exhibit some cytotoxic potency.

Against this backdrop, the present study was conducted to determine the cytotoxic activity of extracts obtained by three different methods of extraction from the starfish *A. planci* against two human cancer cell lines, MCF-7 breast cancer and HCT-116 colon cancer.

MATERIALS AND METHODS

Materials

Diethyl ether, chloroform, methanol and ethanol were purchased from Fisher Scientific (Loughborough, UK). RPMI 1640 medium, Dulbecco's modified Eagle medium (DMEM), fetal calf serum, penicillin/streptomycin and trypsin EDTA 0.25% were purchased from GIBCO (Grand Island, NY, USA). MTS assay kit (CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay) was purchased from Promega (Madison, WI, USA). Phosphate Buffered Saline (PBS) was purchased from Sigma (St. Louis, MO, USA). Cell culture flasks ($25cm^2$) and 96-well microtiter plates were purchased from Corning Incorporated (Corning, NY, USA). 5-fluorouracil (5-FU) was a kind gift from Penang General Hospital. Chemicals used in this study were analytical or cell culture grade.

Starfish

The specimens of *A. planci* were harvested along 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 was taxonomically identified by Associate Professor Dr. Farid Che Ghazali. Post harvesting, they were ice frozen and transported to the laboratory and stored at -20˚C until use. A voucher specimen registered as number PPSK/USM/CTI-0-07-2009-APLC was deposited at the Centre for Sea Cucumber Research, School of Health Sciences, Universiti Sains Malaysia (fig. 1).

Extraction

In the present study, the extracts were prepared by three different extraction methods as follows:

Verrall method

Five extracts were prepared sequentially using different organic solvents following the procedure described by Verrall with slight modifications (Verrall, 1985). One dried starfish was crushed to powder and 90 g was extracted with 300 ml diethylether at room temperature for 24 h and was then filtered. The solvent was evaporated by a rotary evaporator, the extract was then lyophilized and weighed and the dried pellet was

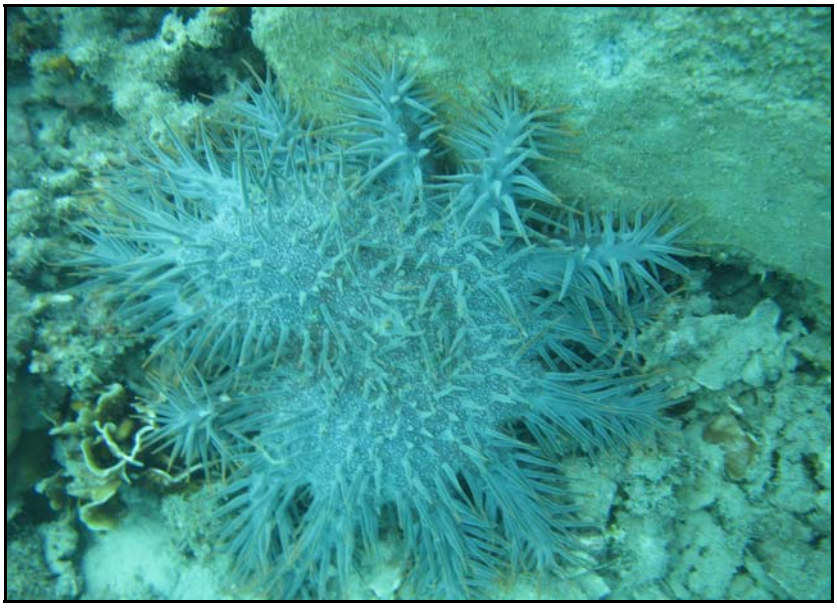

Fig. 1: The Crown-of-Thorns starfish, *Acanthaster planci* (Linnaeus, 1759)

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

Voucher specimen: PPSK/USM/CTI-0-07-2009-APLC

extracted with 300 ml chloroform at room temperature for 24 h. After filtration, the solvent was evaporated and the extract was lyophilized and weighed and the dried pellet was extracted with 300 ml of 80% methanol using water bath at 80°C for 5 min and was stirred at room temperature for 24 h. Following filtration, evaporation of the solvent was done, the extract was lyophilized and weighed and the dried pellet was extracted with 300 ml 80% ethanol using water bath at 80°C for 3 h and was stirred at room temperature for 24 h. After filtration, the solvent was evaporated, the extract was lyophilized and weighed and the dried pellet was extracted with 150 ml distilled water at room temperature for 24 h. The water was evaporated following filtration, and the final extract was lyophilized and weighed. The yield of the extracts were 0.4682 g, 0.4215 g, 1.436 g, 0.683 g and 0.77 g for diethyl ether, chloroform, methanol, ethanol and water, respectively.

Bligh and Dyer method

Two different extracts were prepared from a frozen sample according to the method of Bligh and Dyer with slight modifications (Bligh and Dyer, 1959). The starfish (400 g) was homogenized and extracted twice with a combination of chloroform and methanol (2:1). After filtration, the two solvents were separated by a separatory funnel. Then, the solvents were evaporated by using a rotary evaporator and the two extracts were lyophilized and weighed. The yield of the chloroform extract was 7.08 g and of the methanol extract was 3.524 g.

Preparation of PBS extract

A frozen sample of *A. planci* (300 g), after homogenization, was extracted twice with two volumes of 0.01 M phosphate buffer (pH 7.0) (Shiomi *et al.*, 2004). The buffer was filtered and then evaporated by using a rotary evaporator and the extract was then lyophilized and weighed. The yield of the PBS extract was 5.515 g.

Cell Culture

HCT-116 and MCF-7 cell lines were a kind gift from Dr. Amin Malik Shah and was originally obtained from American Type Culture Collection (Rockville, Md, USA), Cells were cultured in RPMI 1640 medium and DMEM, respectively. Both media were supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Cells were grown in 25 cm^2 cell culture flasks at 37˚C in an incubator containing 5% CO_2.

MTS cell proliferation assay

CellTiter 96w AQ_{ueous} non-radioactive assay was used to assess cell proliferation using tetrazolium compounds according to the manufacturer's instructions (Promega, USA). Cells were grown overnight in 96-well microtiter plates. After 48 h treatment with different concentrations of the extracts (6.25-200 µg/mL), positive control (5-FU) and negative control (1% DMSO), 20 µl of the MTS/PMS solution was added directly to each well and incubated for 5 h. The absorbance was measured at 490 nm with a reference wavelength at 630 nm using the ELISA microplate reader Power Wave$_x$ 340 (Bio-Tek Instruments, Inc. USA). The blank corresponded to 90 µl of culture medium and 10 µl 1% DMSO.

STATISTICAL ANALYSIS

The data are expressed as mean ± standard error means (SEM) using one way ANOVA. Values at $p < 0.01$ were considered as statistically significant (*).

RESULTS

Cytotoxicity effect of the extracts prepared by Verrall method

All the extracts exhibited very insignificant cytotoxicity effect against both cancer cell lines. The IC_{50} values for all the extracts calculated were more than 200 µg/mL (table 1), whereas the 5-FU demonstrated very strong cytotoxic activity against MCF-7 and HCT-116 cell lines with IC_{50} values 1.51 and 2.59 µg/mL, respectively (tables 1, 2 and 3).

Table 1: IC_{50} values of the extracts prepared by Verrall method against MCF-7 and HCT-116 cell lines.

Samples	IC_{50} (µg/mL)	
	MCF-7	HCT-116
Diethyl Ether extract	>200	>200
Chloroform extract	>200	>200
Methanol extract	>200	>200
Ethanol extract	>200	>200
Water extract	>200	>200
5-FU	1.51*	2.59*

Table 2: IC_{50} values of the extracts prepared by Bligh and Dyer method against MCF-7 and HCT-116 cell lines.

Samples	IC_{50} (µg/mL)	
	MCF-7	HCT-116
Chloroform extract	121.37	77.65
Methanol extract	46.11*	59.29*
5-FU	1.51*	2.59*

Table 3: IC_{50} values of PBS extract against MCF-7 and HCT-116 cell lines.

Samples	IC_{50} (µg/mL)	
	MCF-7	HCT-116
PBS extract	13.48*	28.78*
5-FU	1.51*	2.59*

Cytotoxicity effect of the extracts prepared by Bligh and Dyer method

Out of the two extracts, the methanol extract exerted significant cytotoxicity effect against both cell lines in

dose dependent manner (fig. 2), its IC_{50} value was 46.11 µg/ml (MCF-7) and 59.29 µg/ml (HCT-116). On the other hand, the chloroform extract showed toxicity only at higher concentrations (fig. 3). The IC_{50} value of chloroform extract for both cell lines was more than that of methanol extract. Table 2 shows the IC_{50} values of tested samples.

Cytotoxicity effect of PBS extract

The PBS extract found to be the most potent cytotoxic

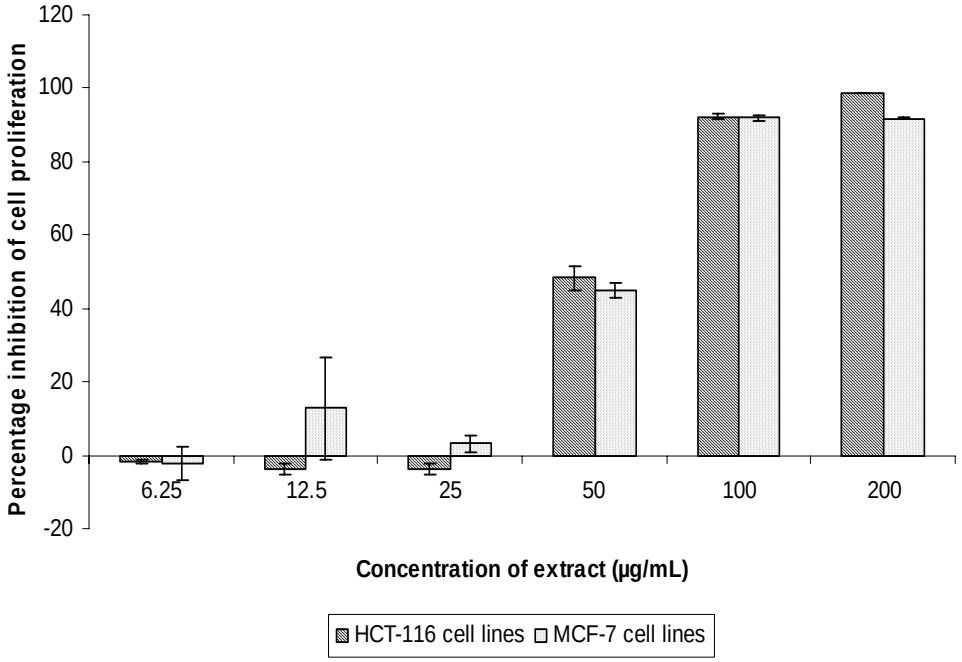

Fig. 2: The effect of methanol extract on HCT-116 and MCF-7 cell lines.

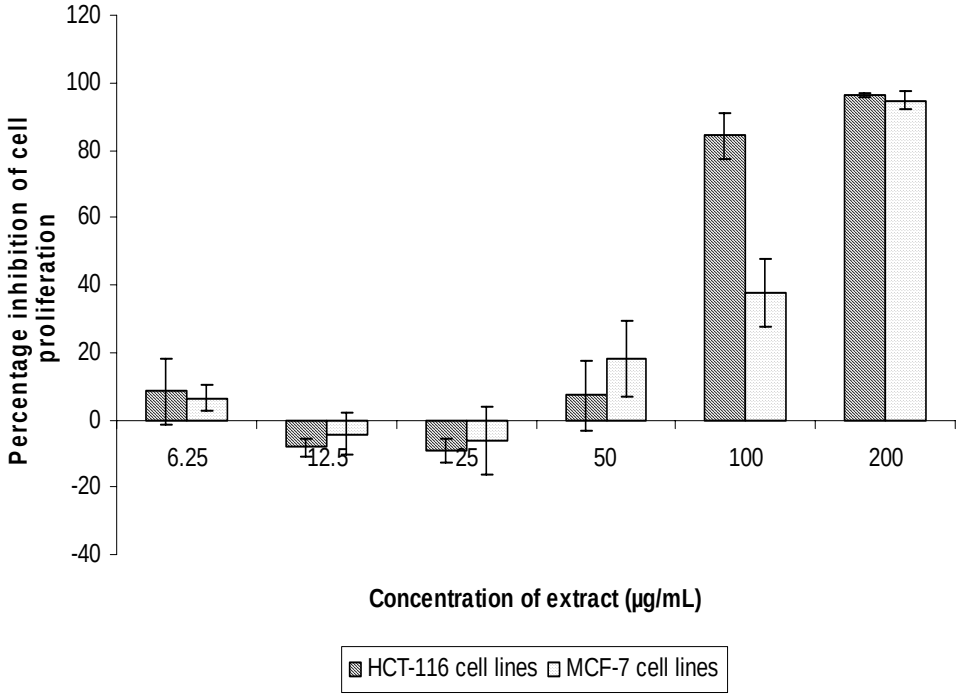

Fig. 3: The effect of chloroform extract on HCT-116 and MCF-7 cell lines

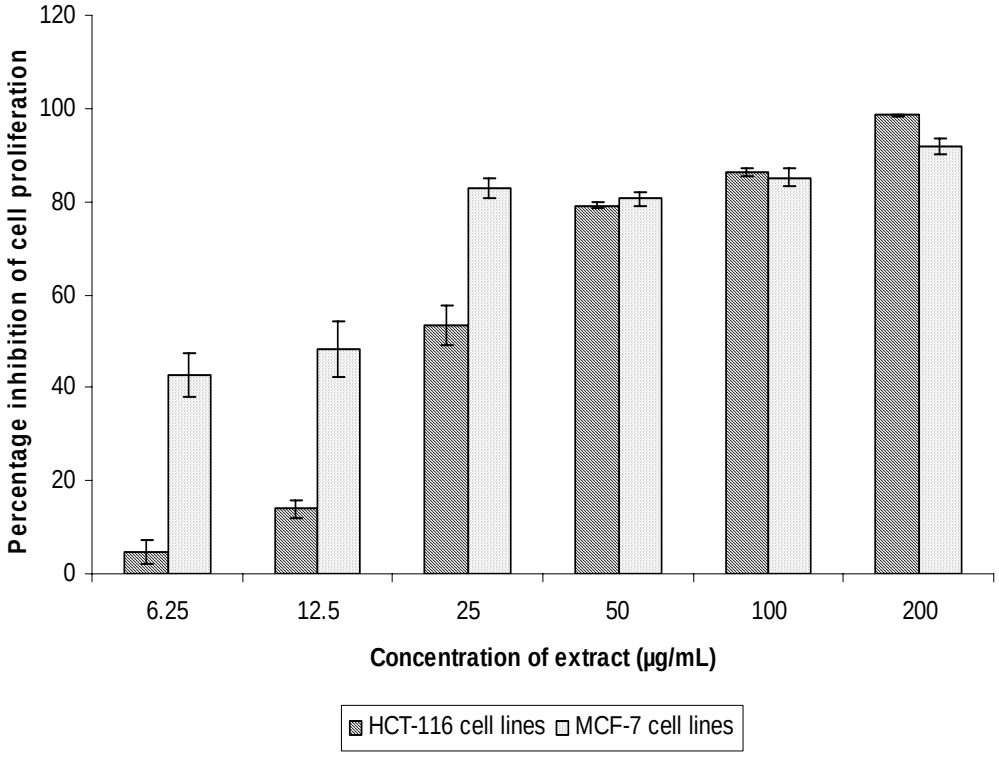

Fig. 4: The effect of PBS extract on HCT-116 and MCF-7 cell lines.

among all the extracts obtained from the other extraction methods. It demonstrated very significant anti-proliferative activity against both cell lines in dose dependent manner (fig. 4). It showed very low IC_{50} value for both cancer cell lines. Table 3 shows the IC_{50} value of the PBS extract on MCF-7 cells (13.48 µg/ml) and on HCT-116 cells (28.78 µg/ml).

DISCUSSION

A great number of bioactive secondary metabolites synthesized by marine organisms, plants and microorganisms have been studied for the existence of potential biological therapeutic activities (Cundliffe, 1981; Ruggieri, 1976). Most of these natural products are potent inhibitors of cellular metabolic pathways, therefore they exert cytotoxic property. Due to the cytotoxic efficacies, most of the biologically active natural products have become excellent sources [0]of novel and tangible drugs for chemotherapy, such as Taxol, vinblastine and vincristine (Gueritte and Fahy, 2005; Kingston, 2005).

In this study, we reported the cytotoxicity effect of various extracts, obtained by three different extraction procedures, against the human breast and colon cancer cell lines. Among all the extracts, the PBS extract exhibited excellent cytotoxic activity against both cancer cell lines, while the extracts prepared by Bligh and Dyer method showed moderate cytotoxic activity. However, the extracts prepared from the dried starfish material using organic solvents sequentially demonstrated very insignificant cytotoxic activity. Extraction of frozen starfish using PBS gives the extract which is rich in proteins, including venomous enzymes with strong catalytic activity, such as lethal hepatotoxic glycoprotein, neurotoxic phospholipase A_2 (I and II) and anti-coagulant peptide (Shiomi *et al*., 1990; Shiomi *et al*., 1998 and Karasudani *et al*., 1996). Thus, the main reason for the cytotoxic potency of PBS extract may probably due to presence of these catalytic toxins. The extract prepared using Bligh and Dyer method is reported to be rich in lipids (Bligh and Dyer method, 1959). Extraction and identification of several bioactive lipids were reported from the starfish *Asterias amurensis* using this method (Hossain *et al*., 2006). In this starfish, several unique glycosphingolipids and neutral glycosphingolipids have been reported. There are several reports that the steroidal glycosides and related compounds are principal metabolites in starfish and have a broad variety of biological activities (Andersson *et al*., 1989; Prokof'eva *et al*., 2003).

Sphingolipid derivatives like sphingosine that inhibit protein kinase C is an essential enzyme in cell regulation and signal transduction. Sphingolipids affect significant cellular responses and exhibit antitumor activities in

various mammalian cells. These molecules may function as endogenous modulators of cell function and possibly as second messengers (Hannun and Bell, 1989). It has been reported that ceramides such as sphingosine and sphinganine induce apoptosis in HT-29 and HCT-116 human colon cancer cell (Ahn *et al*., 2002). In this context, the extract prepared by Bligh and Dyer method may probably be abundant in the bioactive lipids, and by the virtue of these lipids the extract exhibited significant cytotoxic activity against human breast and colon cancer cells.

CONCLUSION

Results of the present study demonstrated that PBS extract as well as the extract prepared by Bligh and Dyer method showed significant cytotoxicity effect against human breast (MCF-7) and colon (HCT-116) cancer cell lines. Since PBS extract exerted very potent cytotoxicity effect with lowest IC_{50} value, thus, the peptides from the starfish *Acanthaster planci* could be used as a potential source of chemotherapeutic agents, but further work should be needed to confirm the cytotoxicity effect, and then the anti-cancer activity of this extract.

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