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Research Article

Methanol extracts of four selected marine sponges induce apoptosis in human breast cancer cell line, MCF-7

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ABSTRACT

Breast cancer is the most frequently diagnosed cancers among women worldwide. However, due to limited chemotherapeutic efficacy of current anticancer drugs, various natural products have been screened for novel alternative chemotherapeutic agents. Marine environment offers vast diversity of living organisms that provide compounds with impressive structural diversity and drug-like properties. Therefore, the main aim of this study was to determine the cytotoxic effects and mechanisms of cell death exerted by marine sponges on human breast cancer cell line, MCF-7. Four species, namely *Aaptos* sp., *Stryphuous ponderosus*, *Theonella* sp. and *Xestospongia* sp, were selected for this study. Methanol extracts from three species produced potent cytotoxicity effects with IC_{50} values at 72hr of less than 30µg/ml in the order of *Aaptos* sp. > *S. ponderosus* > *Theonella* sp. Due to the lack of studies on *S. ponderosus*, extract from this species was then used to determine its mechanisms of cell death. MCF-7 cell death exerted by the extract was found to be mediated by apoptosis based on the presence of DNA fragmentation in treated cells. Treatment of cells with the extract increased the levels of of caspase 3 suggesting that the protein was responsible in triggering the DNA fragmentation. In addition, the extract also induced the levels of caspases 8 and 9 and the levels of caspase 8 was higher than the latter suggesting that extrinsic was the major pathway that involved in inducing apoptosis. The apoptotic-induced cytototoxicity activity of the methanol extract of *S. ponderosus* may be due to the presence 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.

Keywords: Apoptosis; Breast cancer; *Caspases; S. ponderosus*; Marine extract.

INTRODUCTION

Cancer is one of main causes of mortality in the world population. Recently, American Cancer Society reported that cancer is the second leading cause of death after cardiovascular disease (Siegel *et al*., 2015). It was estimated that 14.1 million new cancer cases and 8.2 million cancer deaths occurred in 2012 globally. Breast cancer remains the second most common cancer with 1.7 million cases but ranks fifth in mortality with 522,000 deaths. However, breast cancer is still the leading cancer diagnosed in women both in more and less developed regions with more cases occurring in less developed as compared to more developed regions (Ferlay *et al*., 2015).

It was well established for many years that the induction of apoptosis on cancer cells by any natural compound is the key target for cancer prevention and therapy (Taraphdar *et al*., 2001). Apoptosis is an active and highly regulated form of cell death of which damaged and mutated cells, potentially dangerous to the entire organism, are eliminated naturally without causing adverse side effects (Schumacher *et al*., 2011). The key proteins that are responsible in triggering apoptosis belong to caspase family of cysteinyl-proteases play the key role in the initiation and execution of the pathways (Ghavami *et al*., 2009). Therefore, identification of natural products based on mechanisms of action may hold a great promise in the development of novel alternative chemotherapeutic drugs.

Natural products aided as important chemical prototypes for the discovery of new molecules, and continue to be the most promising source of drug leads, especially in the anticancer field. Studies on marine natural products as the source of drug candidates are still young. However, marine resources have produced a vast quantity of novel and new bioactive compounds (Kamalakkannan, 2015). Sponges represent one of the most promising sources of marine bioactive compounds among them, predominantly for pharmaceutical leads (Jain *et al*., 2008; Blunt *et al*., 2011). Defense mechanisms against predators and ecological factors such as competition for space with other sessile marine organisms and symbiosis are the factors that lead for sponges to produce a plethora of secondary metabo-

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lites with diversified chemical structures (Peters *et al*., 2010; Chaves-Fonnegra *et al*., 2008). Two analogues that were derived from sponge metabolites have been developed and clinically used as antiviral and anti-tumour drugs. Ara-C is used for the treatment of long-term acute non-lymphocytic, chronic myelocytic and meningeal leukemia (Ireland *et al*., 1993; McConnel *et al*., 1994), and, Ara-C is prescribed to patients to treat herpes simplex and herpes zoster viral infection (Mayer *et al*., 2010; Snoeck *et al*., 1999).

Currently, the development of anti-cancer agents using marine resources especially sponges has fast gained popularity since there are several potential therapeutic compounds that have been identified including chemo-therapeutic agents and immunomodulatory (Belarbi & Contreras Gómez, 2003). Therefore, in this study, four species of sponges were selected, to evaluate their cytotoxicity activity and mechanisms of cell death on human breast adenocarcinoma (MCF-7) cell line. In addition, chemical profiling of the extracts was also determined.

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 ATCC. CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Caspase-Glo™ 3 Assay, Caspase-Glo™ 8 Assay, Caspase-Glo™ 9 Assay, DeadEnd™ Colorimetric Apoptosis Detection System were purchased from Amresco, USA Fischer Scientific, UK Promega, USA, respectively. All chemicals are of analytical grades.

Preparation of methanol extract from sponges

Four different species of sponges were used in this study, *Stryphuous ponderosus, Aaptos* sp*., Theonella* sp*.,* and *Xestospongia* sp*.*, and the locations that the sponges were collected are shown in Table 1. Species identification was carried out Dr. Noraznawati Ismail, Institute of Marine Biotechnology, Universiti Malaysia Terengganu. Sponges were then cleaned, chopped and freeze-dried to remove water. After dehydration processing, sponges were ground to powder form and 10 g of each sample was subsequently used for extraction.

Samples were extracted using cold extraction technique where 10 g of dried sample was macerated in 100 ml of methanol for 24hr and repeated for three times. Extracts were filtered and filtrates were evaporated to dryness using rotavapor (BUCHI, Switzerland) at 40 °C. The extracts were de-salted to get crude methanol extract (Gami & Parabia, 2011).

Determination of cytotoxicity activity of the extracts

Cytotoxicity effects of the extracts in MCF-7 cell line were determined using MTS assay (CellTiter 96™ Aqueous Non-Radioactive Cell Proliferation Assay) as described by Sandhya *et al*., (2014).

Briefly, the cells were cultured onto 96-well plate at a density of 6000 cells/well and incubated at 37°C in an incubator overnight. When the cells reached confluency at approximately 80%, the medium was replaced with fresh medium containing various concentrations of the prepared extracts and incubated at 24, 48 and 72hr. DMSO was used to dissolve and dilute the extracts 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)) was designated as negative control. After treatment, 20 ml of MTS was transfered to each well, incubated for 2 hr and absorbance was then read at 490 nm.

Determination of apoptotic cell death in treated cells

The DeadEnd™ Colometric Apoptosis Detection System (Promega, USA) was used to determine apoptotic cell death in treated cells. MCF-7 cell line was cultured in Labtek Chamber Slides (Nalge 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) CO_2 overnight until the cells reached 80 to 90% confluency. Subsequently, the medium was replaced with fresh medium containing the extract at concentration of IC_{50} at 72hr and incubated at 12, 24 and 36hr. Untreated negative control cells were incubated in the presence of 1% DMSO (v/v) and positive control cells were treated with 1U/ml DNase I. After treatment, the cells were processed according to the manufacturer's protocol. The slides were then observed under light microscope.

Determination of the levels of Caspases in treated cells

The cells were cultured at a cell density of approximately 6,000 cells/well in 96-well plate and incubated overnight until the cells reached confluency between 80 - 90%. The medium was then removed, replaced with fresh medium in the presence of extract and incubated over the period of 36hr. The levels of caspases 3/7, 8 and 9 were determined by using Caspase-Glo™ Assay (Promega, USA) as described by the manufacturer's protocol.

Determination of the extract chemical profiles using thin layer chromatography

The extract was diluted in an appropriate amount of solvent, spotted on the TLC plate and placed in a developer chamber with a mixture of solvent. After the spotted extracts were allowed to migrate to a required length, the plate was taken out, left to dry and visualized under UV light. Subsequently, the plate was then heated on the hot plate and visualized using iodine vapour, dragendorff's reagent and anisaldehyde reagent as described by Wagner & Bladt (1996).

Table 1: Locations and coordinates where the species of sponges were collected in this study

Species	Location of collection
Stryphuous ponderosus	Pulau Karah (N 05°35.857' E 103°03.725')
Aaptos sp.	Teluk Belanga (N 05°36.656' E 103°04.024')
Theonella sp.	Teluk Belanga (N 05°36.656' E 103°04.024')
Xestospongia sp.	Teluk Belanga (N 05°36.672' E 103°04.021')

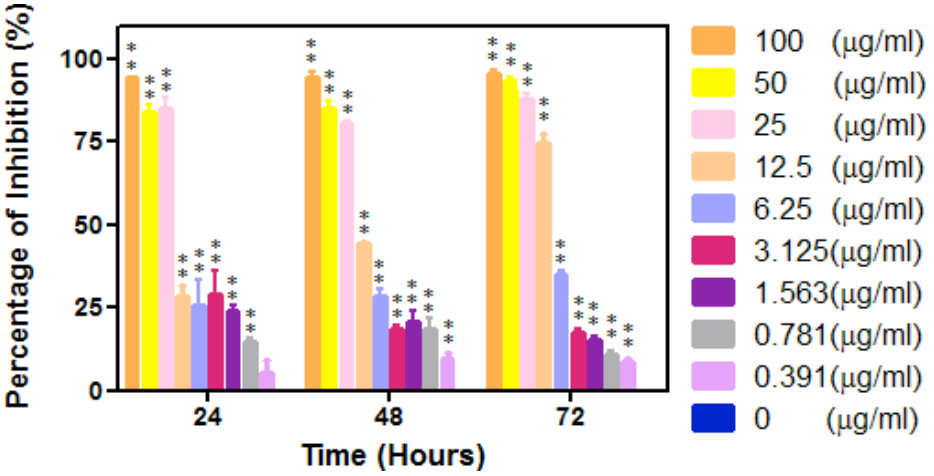

Figure 1A: Percentage of growth inhibition ± standard deviation for S. ponderosus methanol extracts against MCF-7 at 24hr, 48hr, and 72hr

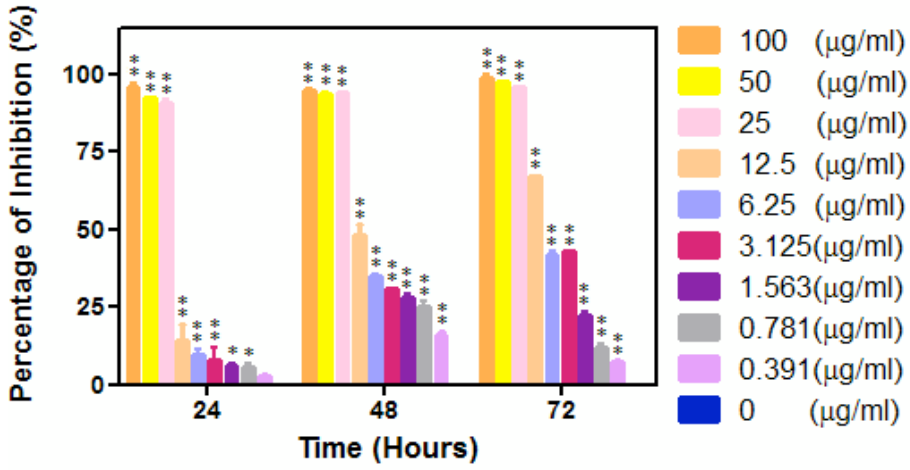

Figure 1B: Percentage of growth inhibition ± standard deviation for Aaptos sp. crude methanol extracts against MCF-7 at 24hr, 48hr and 72hr

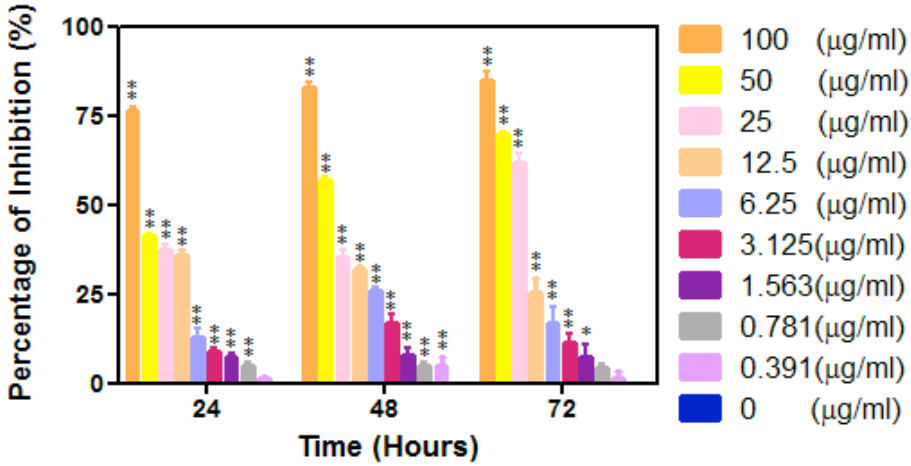

Figure 1C: Percentage of growth inhibition ± standard deviation for Theonella sp. crude methanol extract against MCF-7 at 24hr, 48hr, and 72hr

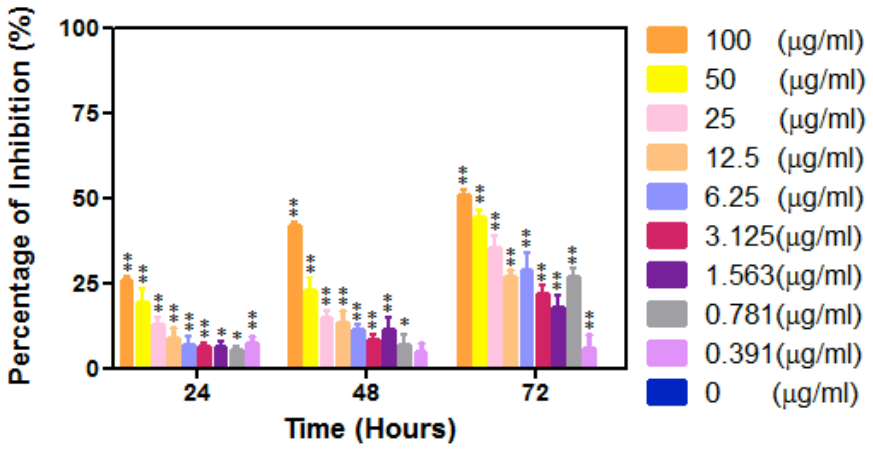

Figure 1D: Percentage of growth inhibition ± standard deviation for Xestospongia sp. crude methanol extracts against MCF-7 at 24hr, 48hr, and 72hr

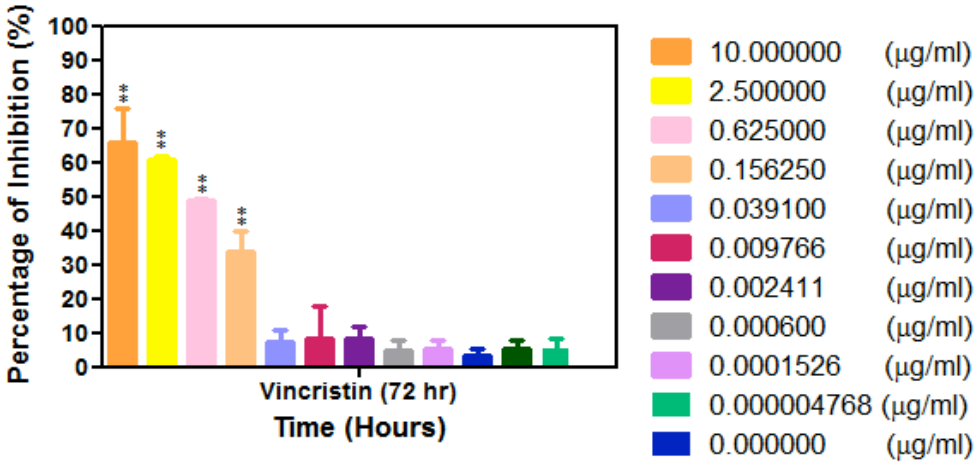

Figure 1E: Percentage of growth inhibition ± standard deviation for Vincristine sulphate against MCF-7 at 72 hr

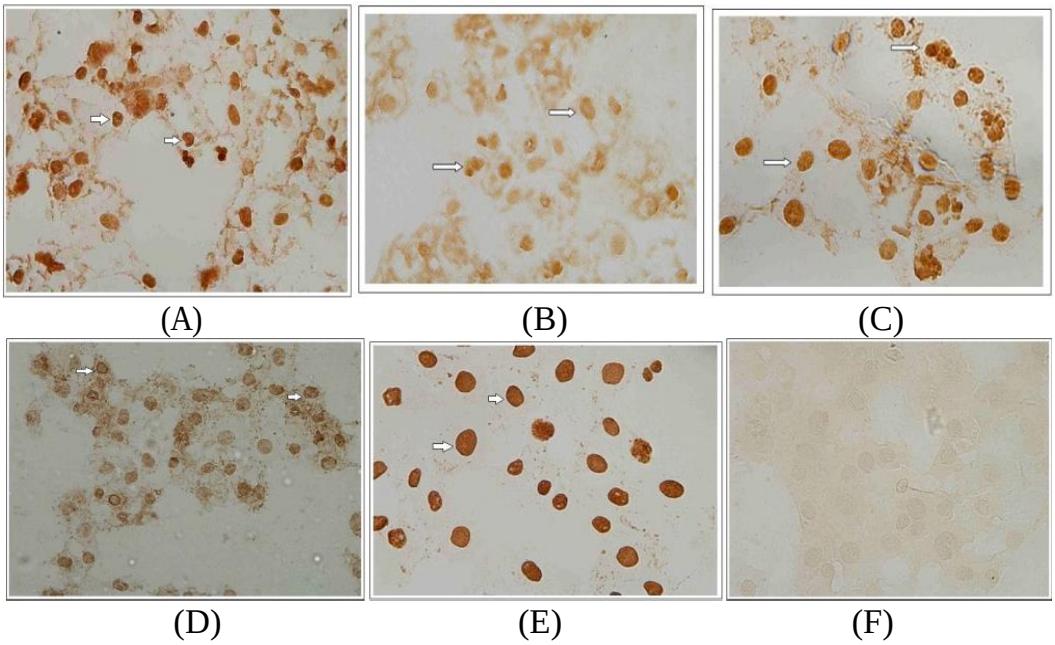

Figure 2: The presence of DNA fragmentation in nuclei which indicates apoptosis that was treated in MCF-7 cell line treated with crude methanol extract of S. ponderosus for 12 hr (A), 24 hr (B) and 36 hr (C); with vincristine sulphate for 24 hr (D); with DNase I (E); with DMSO for 24 hr (F). The arrow indicates DNA fragmentation.

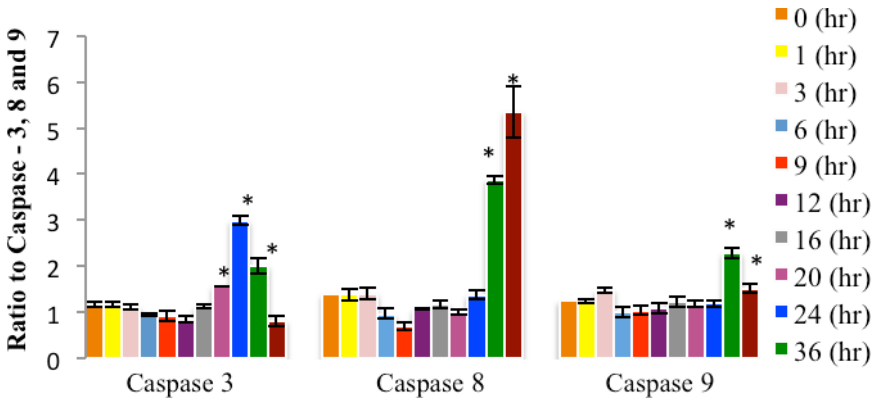

Figure 3: The expression of caspase- 3, 8 and 9 in MCF-7 cells treated with S. ponderosus SP1 extract

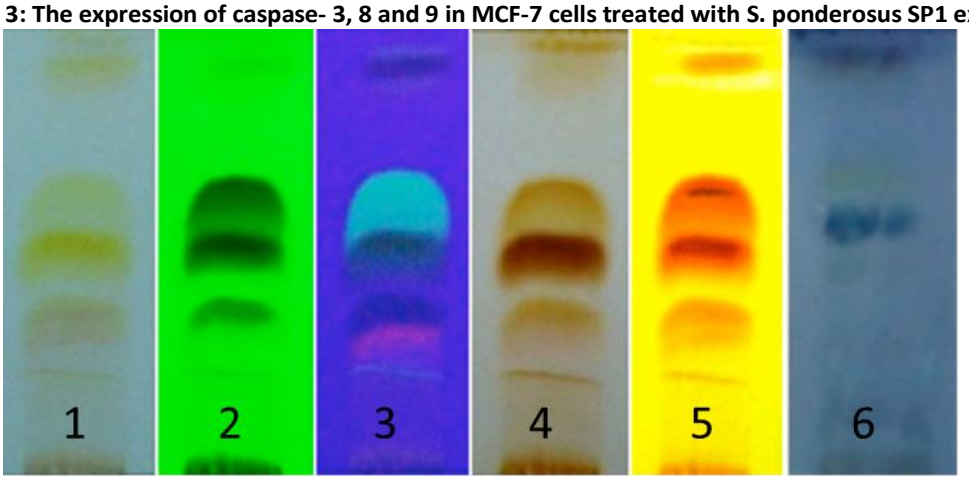

Figure 4: TLC profiling of S.ponderosus methanol extract under; (1) before sprayed, (2) viewed under UV254, (3) viewed UV356, (4) Iodine vapour, (5) Dragendorff's reagent, (6) Anisaldehyde reagent

Table 2: IC_{50} values of methanol extracts prepared from four sponge species and vincristine sulfate

Sl.NO	Extract Sample	IC_{50}		
		24hr	48hr	72hr
1	*Stryphuous ponderosus*	22.36	14.61	8.49
2	*Aaptos* sp.	16.72	14.22	7.61
3	*Theonella* sp.	47.86	37.15	21.88
4	*Xestospongia* sp.	ND	ND	97.72
5	Vincristine Sulphate	ND	ND	0.64

Statistical Analysis

Cytotoxicity experiments were carried out in triplicates and results were expressed as percentage growth inhibition of control. IC_{50} values for growth inhibition was derived from a nonlinear regression model (curvefit) based on sigmoidal dose response curve (variable) and computed using GraphPad Prism (Graphpad). Data are given as mean ± S.E.M. A NOVA *P<0.05, **P<0.01 (Dunnett post-test).

RESULTS

Cytotoxicity effects of methanol extracts of sponges on MCF-7 cell line

The cytotoxic effects of methanol extracts prepared from four marine sponges *Stryphuous ponderosus*, *Aaptos* sp., *Theonella* sp., and *Xestospongia* sp. on human breast carcinoma MCF-7 cell line were investigated. As shown in Figure 1A-1D, all extracts produced a dose-dependant inhibition of MCF-7 cell growth at each of the three incubation periods.

The extract of *Stryphuous ponderosus* significantly inhibited MCF-7 cell line at concentrations 0.781µg/ml and above at 24hr, and at concentrations 0.391µg/ml and above when treated for 48 and 72hr (Figure 1A). The extract produced a potent cytotoxicity effect on MCF-7 cell line at concentrations above 12.5 ¥ g/ml where more than 50% of cell populations were killed. Interestingly, at 72hr treatment, concentration at 12.5µg/ml was also able to inhibit the cells more than 50% as compared to control. The IC_{50} values were decreased as the incubation periods were increased from 24hr (22.36µg/ml) to 48hr (14.61 ¥ g/ml) and to 72hr (8.49µg/ml).

Aaptos sp. also produced a potent inhibitory activity on MCF-7 cell line. At 24hr incubation, the percentage of cell growth was significantly inhibited at concentrations of 0.781µg/ml and above. Interestingly, at 48hr and 72hr, the inhibition was significantly different at all concentrations as compared to control (Figure 1B). The extract exhibited potent cytotoxicity activity against MCF-7 cell line with IC_{50} values of 16.72µg/ml (24hr), 14.22µg/ml (48hr), and 7.61µg/ml (72hr).

Similar pettern of inhibitory effects of *Theonella sp*. were also observed when the cells were treated with methanol extract over the period of 72hr. The extract significantly inhibited the growth of MCF-7 at concentrations 1.563µg/ml and above, after 24, 48, and 72hr incubation (Figure 1C). Interestingly, IC_{50} value of the extract was 47.86µg/ml; 37.15µg/ml and 21.88µg/ml at 24hr, 48hr, and 72hr treatment, respectively, which clearly indicate that the extract only exerted a potent cytotoxicity effect on MCF-7 cell line at 72hr, 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 30µg/ml (Geran *et al.,* 1972).

Although the crude methanol extract of *Xestospongia* sp. significantly inhibited the growth of MCF-7 cells as compared to control when treated at concentrations of 0.391µg/ml and above (24hr) and 0.781µg/ml and above (48hr and 72hr), the extract did not show any cytotoxicity effect at all concentrations used. The extract only inhibited more than 50% of cell population at 100µg/ml with the IC_{50} value of 97.72µg/ml at 72hr treatment (Figure 1D).

Vincristine sulphate was used as a positive control in the cytotoxicity study showed the IC_{50} value of 0.64µg/ml at 72hr (Figure 1E). Overall, methanol extracts of *Stryphuous ponderosus*, *Aaptos* sp., and *Theonella* sp., produced a potent cytotoxicity effects on human breast carcinoma, MCF-7 cell line at all treatment period except at 24hr for *Theonella* sp. (Table 2). The relative potential of cytotoxicity of the extracts is as follows: *Aaptos* sp. > *Stryphuous ponderosus* > *Theonella* sp. By contrast, methanol extract *Xestospongia* sp. did not exhibit any cytotoxicity activity against MCF-7 cells (Table 2).

The study on the determination of the biological activities of *Stryphuous ponderosus* is still very limited, therefore, methanol extract of this species was selected for to investigate the mechanisms of cell death on MCF-7 cells.

Induction of apoptosis exhibited by methanol extracts of Stryphuous ponderosus

In order to determine the mode of cell death that was responsible in mediating the cytotoxicity effects of methanol extract of *S. Ponderosus*, a modified TUNEL assay using The DeadEnd™ Colometric Apoptosis Detection System was utilized according to the manufacturer's instruction.

The cells treated with crude methanol extract of *S. Ponderosus* at the concentrations of IC_{50} 72hr (8.49µg/ml) for 12hr, 24hr and 36hr produced dark brown-stained nuclei. Interestingly, the nuclei of MCF-7 cells were also darkly stained when treated with DNase I indicating the presence of DNA fragmentation which is a hallmark of apoptosis (Bowen *et al*., 1998). Similar dark brown-stained nuclei was also observed when the cells were stained with vincristine sulfate as positive control (Talib *et al*., 2012). However, nuclei of DMSO-treated MCF-7 cells were not stained when observed under light microscope. Thus, the results strongly indicate that the extract of *S. Ponderosus* exerted the cytotoxicity effects on MCF-7 cell line via apoptosis.

Regulation of the levels of caspase-3, 8 and 9 proteins in MCF-7 cells treated with Stryphuous ponderosus extract

In order to determine the mechanisms of cell death induced by methanol extract of *S. ponderosus,* the levels of caspase proteins that are responsible in triggering apoptosis either via extrinsic or/and intrinsic pathways were determined. Figure 3 shows the levels of caspase-3, 8 and 9 proteins when MCF-7 cells were treated with crude methanol extract at concentration of IC_{50} 72hr (8.49µg/ml) over a period of 48 hours. Overall, it was found that the protein level of caspase-3 was significantly increased in cells that were treated crude methanol extracts at 20, 24, 36hr. The level of caspase-3 reached the highest level at 24 hours (3-fold increase as compared to untreated control) and decreased again at 36 hours and 48 hours. The level of caspase-8 was also significantly increased 3.9-fold and 5.3-fold as compared to untreated control, at 36hr and 48hr, respectively. Similarly, the protein level of caspase-9 was significantly increased at 36hr (2.3-fold increase) and reduced again at 48hr treatment although the protein level was still above the untreated cells.

Identification of chemical profiles of the extract using thin-layer chromatography

In order to determine the types of compounds present in the extract of *S. ponderosus*, TLC profiling was carried out. The results clearly indicate that the extract contained compounds with C=C double bonds when viewed under UV light (Figure 4). Tan-brown stained spots when viewed iodine vapour indicate that the extract contained organic compounds (Figure 4). Orange and green spots were observed when separated extracts were reacted in dragendorff's and anisaldehyde reagents indicate that alkaloids and terpenes were present in the extract, respectively.

DISCUSSION

In developed and developing countries, breast cancer is ranked the second most common types of cancer

and fifth as cause of death (Ferlay *et al*., 2015). To date, there is still an urgent need to search for new alternative anti-cancer drugs due to lack of effectiveness of the current medications to cure and improve the health of the patients. Natural products have been the main source for the discovery of new drug candidates (Cragg & Newman, 2013). Since mid-1970s, systematic studies on marine organisms as sources of novel biologically active compounds revealed that these habitats offer a rich source of bioactive compounds with diverse and novel structural and chemical classes (Newman & Cragg, 2004; Blunt *et al*., 2012). The lack of natural defenses such as innate immune system, 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 (Schumacher *et al*., 2011). It was estimated that 20,000 new compounds have been purified and about 500 new compounds are discovered each year from marine organisms (Hu *et al*., 2011; Leal *et al.*, 2012). Almost half of these bioactive compounds have been isolated from sponges and therefore, considered as the most bio prospective marine taxon (Hu *et al*., 2011; Leal *et al.*, 2012).

Studies reported many compounds isolated from various species of sponges exerted potent cytotoxicity activities (Sepcic *et al*., 2010). In this study, 3 species of sponges namely *Aaptos* sp., *Theonella* sp. and *Stryphuous ponderosus* produced a potent cytotoxicity activity on human breast carcinoma cell line, MCF-7. The role of *Aaptos* sp. in providing natural products that serve as cytotoxicity agents against cancer cells has been described elsewhere. Our result is in agreement with the previous study which demonstrated that methanol extract from *Aaptos aaptos* produced cytotoxicity effect on MCF-7 cell line with IC_{50} value of 8 µg/ml. In addition, naphthyridine, known as aaptamine, isolated from *Aaptos aaptos* (Jang *et al*., 2007) was found to possess antineoplastic activity and cancer cell growth inhibitory activity (Ohizumi *et al*., 1984; Jang *et al*., 2007). However, cytotoxicity studies on *Theonella* sp. are more limited. To date, only one study was carried out to determine the cytotoxicity activity of natural products isolated from *Theonella* sp. on human breast cancer. It was demonstrated acetyltheonellasterol purified from produced cytotoxicity activity on MCF-7 cell line with IC_{50} value of 11 µg/ml (Guo *et al*., 2012).

By contrast, no study has been carried out to investigate the potential role of natural products prepared from *Stryphuous ponderosus* in exerting cytotoxicity activity on human breast cancer cell line. In our study, it was clearly shown that methanol extract from this sponge species exhibited a potent cytotoxicity activity against human breast carcinoma cell line, MCF-7 with IC_{50} values of 22.36, 14.61 and 8.49µg/ml at 24, 48 and 72hr, respectively, which were less than 30 µg/ml, a criterion set by National Cancer Institute (Geran *et al*., 1972). Interestingly, the extract killed MCF-7 cells via apoptosis which is one of the important cellular target for the development of anti-cancer drugs (Hassan *et al*., 2014). Natural products isolated from sponges have been shown to induce apoptosis in breast cancer cell lines. Agelasine B, a toxin isolated from marine sponge *Agelas clathrodes*, kills MCF-7 cell line via apoptosis (Pimentel *et al*., 2012).

One of the earliest and most consistent observed features of apoptosis is the induction of a series of cytosolic proteases, caspases (Shi, 2002). Among all caspases, caspase-3 is the most important caspases and is a common effector of most of the apoptotic pathways (Nanda kumar *et al*., 2011). It relies on the actions of the initiator caspases including caspase-8 and caspase-9 to mediate its actions (Heo *et al*., 2011; Cohen, 1997). The function of caspase-8 is well established. It is essential for the extrinsic cell death pathway initiated by the TNF family members (Ghavami *et al*., 2009). On the other hand, caspase-9, the apical/initiator caspase within the apoptosome dependent cascade, is reliable for intrinsic cell death pathway. When the intrinsic pathway is activated, cytochrome c is released from the mitochondria, and is recruited to the cytoplasmic receptor, Apaf1 (Zou *et al*., 1997; Rodriguez & Lazebnik, 1999).

Methanol extract of *S. ponderosus* was shown to induce the level of caspase-8 by 5.3-fold as compared to only 2.3-fold in caspase-9 suggesting that both pathways are involved, however, extrinsic was the major pathway used by methanol extract in inducing apoptosis in MCF-7 cells. Pimentel *et al*., (2012) also reported the activation of caspase-8 and extrinsic pathway as the mechanism of action of marine sponge *Agelas clathrodes* compound, agelasine B, in inducing apoptosis in cancer cells. In addition, extracts from *Haliclona* sp. also induced apoptosis via caspase-8 in cancer cells (Bae *et al*., 2015)

In TLC profiling analysis, methanol extract of *S. ponderosus* contained alkaloids and terpenes. This finding is in parallel with many previous studies. For example, some of sponges produced alkaloid compounds as antitumour such as *Neo*-kauluamine (Sayed *et al*., 2002). Triterpenoids are among the most abundant secondary metabolites present in marine environment, such as marine sponges. A large number of biologically active triterpenoids is found to have cytotoxicity against a variety of tumor cells. Several triterpenoids, including ursolic and oleanolic acid, betulinic acid, celastrol, pristimerin, lupeol, and avicins possess antitumor and anti-inflammatory properties (Petronelli, 2009). Therefore, it is tempting to speculate that the same types of compounds present in methanol extract of *S. ponderosus* may be responsible in exerting the cytotoxicity effects on human breast carcinoma cell line, MCF-7, by inducing apoptosis mainly via extrinsic pathway. Thus, from this study, it is interesting to note that there is enormous potential for the extracts prepared from *S.*

ponderosus to be developed as chemotherapeutic agents in the treatment of cancer.

CONCLUSION

This study demonstrated that three extracts prepared from *Aaptos* sp., *Theonella* sp. and *Stryphuous ponderosus* produced cytotoxicity effects (IC_{50} less than 30 mg/ml at 72hr) on human breast cancer MCF-7 cell line. By contrast, extract from *Xestospongia* sp. did not produce any cytotoxicity activity. Modified TUNEL assay showed that *Stryphuous ponderosus* extract killed MCF-7 cell line via apoptosis by the activation of caspases. However, the levels of caspase-8 was higher than caspase-9 in treated cells indicating that extrinsic pathway was the major route used by the extract in inducing apoptosis. TLC profiling of methanol extract of *S. ponderosus* showed that the presence of alkaloid and terpenoid compounds which may be responsible in mediating the apoptotic cell death in MCF-7 cell line.

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