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Cytotoxic activity of crude extract and fractions from S*argassum siliquosum* (JG Agardh) and other seaweeds against selected human cancer cell lines

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Abstract

Cancer greatly contributes to human mortality and is considered as a major threat to humankind. Seaweeds and their extracts are now studied as potential sources of bioactive compounds useful in cancer management and control. This study attempts to evaluate the cytotoxic activities of the crude extracts from *Eucheuma denticulatum* (N.L.Burman) F.S.Collins & Hervey, *Kappaphycus alvarezii* (Doty ex P.C. Silva) and *Sargassum siliquosum* against human colon cancer line (HCT-116), breast cancer cell line (MCF-7) and lung adenocarcinoma cell line (A549) using MTT Assay. Hexane partition showed IC_{50} of 17.38 ± 0.60 µg/mL against HCT-116, 18.75 ± 5.77 µg/mL against MCF-7 and 23.11 ± 1.95 µg/mL against A549. Ethyl acetate partition showed cytotoxicity against HCT-116 with IC_{50} of 5.95 ± 0.72 µg/mL, 7.16 ± 0.72 µg/mL against MCF-7 and 5.60 ± 0.47 µg/mL against A549. *S. siliquosum* ethyl acetate gave the highest selectivity index for A549, HCT 116 and MCF-7 cell lines than the non-cancer AA8. This means that tested extracts were less cytotoxic to the non-cancer cell line. Doxorubicin showed lowest selectivity index thus higher cytotoxicity towards non-cancer cell line, AA8. It is suggested that active extracts should be subjected to further purification.

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Introduction

Seaweeds and their extracts are important sources of biologically active compounds. They are used as components in pharmaceuticals, fertilizers, feeds and other biotechnological products. Seaweed extracts provide much benefit for humankind and they are considered as catalysts in the development of new biotechnological products (Chojnacka *et al*., 2012).

Sargassum siliquosum J.G. Agardh (Family Sargassaceae) is an endemic species of Sargassum in the Philippines. It is widely distributed in Batangas, Cagayan, Cavite, Ilocos, La Union, Masbate, Pangasinan, Quezon, Antique, Bohol, Cebu, Misamis Occidental and Zamboanga del Sur (Montaño *et al*., 2006). *S. siliquosum* grows all year round but active growth is observed during the colder months (Largo & Ohno, 1992).

Sulfated polysaccharides extracted from *S. siliquosum* exhibited concentration-dependent free radical scavenging activity. This was found to be dependent on the degree of sulfation of the polysaccharides used in DPPH Assay. Results from the Nitrite Assay proved the ability of the polysaccharides to inhibit LPS-stimulated NO generation in a dose-dependent manner. NO inhibition of up to 80% was observed at 125µg/ml of the extract (Vasquez *et al*., 2012).

S. siliquosum exhibited hydroxyl radical scavenging activity. Hydroxyl radical incorporates itself in the nucleotide sequence of DNA and breaks DNA strands that contributes to carcinogenesis, mutagenesis and cytotoxicity (Moskovitz *et al*., 2002).

Dichloromethane fraction of *S. siliquosum* exhibited 65% inhibition against hydroxyl radical at 1000 µg/ mL concentration. It also exhibited 83% hydrogen peroxide radical scavenging activity at 10mg/mL (Corpuz *et al*., 2013).

Kappaphycus alvarezii is one of the most common and fast growing seaweeds. It thrives in variable habitats. It contributes 80% to the total seaweed export of the Philippines. The Philippines pioneered the commercial cultivation of *K. alvarezii* during the latter half of the 1960s in southern Mindanao in the Philippines using local varieties selected from the wild (Parker, 1974). It is commercially farmed in Tawi-Tawi, Northern Bohol and Pangasinan. It is the main source of kappa carrageenan. It also serves as source of food, minerals like Ca, K, Mg, Na, Cu, Fe, Mn and it is used in bioremediation of Pb- and Cd-polluted areas (Trono, 1992).

Crude ethanolic extract of *K. alvarezii* showed significant cytotoxic activity against Ehrlich ascites carcinoma cell lines (Sundaram *et al*., 2010). The methanol extract of *K. alvarezii* also showed potent α-amylase inhibition activity and cytotoxic activity against *Escherichia coli* (Nagarani and Kumaraguru, 2013).

Eucheuma is considered as one of the top three sources of carrageenan in the Philippines and in the tropical Asia and Western Pacific. This species can be found living in coarse sandy-corally to rocky substrates and intertidal zones. It is commercially cultured in Northern Bohol and Pangasinan for seaweed export. It is the main source of iota carrageenan. *E. denticulatum* is used as food source and controls heavy metal pollution such as Pb and Cd (Trono, 1992). According to Food and Agriculture Organizations of the United Nations, the Philippines has the highest harvest of carrageenophyte resources in 2001 amounting to 115,000 tonnes dry weight (McHugh, 2003).

There are still few literatures and researches regarding the cytotoxic activities of Philippine seaweeds against human cancer cell lines. This study aimed to evaluate the cytotoxic activities of crude extracts and fractions from Philippine seaweeds, *S. siliquosum, K. alvarezii* and *E. denticulatum* against selected human cancer cell lines. In order to identify the seaweeds with potential bioactive molecules against cancer cells, MTT assay was performed using the seaweed crude extracts and fractions.

Materials and methods

Extraction of crude extract

Specimens of *S. siliquosum* were collected from the University of the Philippines Marine Science Institute (MSI) Bolinao Marine Laboratory in Pangasinan in May 2013. The collected specimen was identified by Dr. Gavino C. Trono. *E. denticulatum* and *K. alvarezii* were collected by UP MSI Bolinao Marine Laboratory staff in July 2013 and were identified by Dr. Ronald Villanueva.The seaweeds were washed using distilled water and air dried at room temperature. The dried samples were crushed using a blender. Dried and crushed samples were soaked in 100% ethanol for three days. The filtrate (crude ethanolic fraction) was concentrated using rotary evaporation and reserved for solvent partitioning. Thereafter, the air dried samples were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 4 mg/mL for use in the subsequent assays (Canoy *et al.*, 2011).

Cell viability assay (MTT assay)

The assay to determine cell survival/toxicity was conducted after the method of Mosman (1983) with some modifications. In detail, AA8, A549, HCT116 and MCF7 cells were seeded separately at 4 x 10^4 cells/mL/well in sterile 96-well microtiter plates. Cells were incubated overnight at 37°C and 5% CO_2.

The 4 mg/mL extracts were serially diluted to concentrations 1000 µg/mL, 500 µg/mL, 250 µg/mL and 125 µg/mL in a master dilution plate (MDP). From the MDP, 10 µL were obtained and dispensed on to the plated cells to obtain the final concentrations 50 µg/mL, 25 µg/mL, 12.5 µg/mL and 6.25 µg/mL. Doxorubicin, a cancer chemotherapeutic drug, served as positive control while DMSO, the solvent for the extracts, served as negative control. Three replicate wells were used per concentration. The treated cells were then incubated for 72 hours at 37°C and 5% CO_2.

After incubation the media was withdrawn and 20 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye at 5 mg/mL PBS was added to every well. The cells were again incubated at 37°C and 5% CO_2 for four hours, after which 150 µL DMSO was added to each well. Absorbance at 570 nm was read using ELISA plate reader. The concentration required to kill 50% of the cell population or Inhibition Concentration 50 (IC_{50}) was computed using linear regression of the graph of absorbance against concentration. The selectivity index (SI) was calculated by dividing the IC_{50} value for the non-cancer cell line AA8 by the value of the IC_{50} for cancer (A549, HCT-116 and MCF-7) cell lines (Al-Quabaisi *et al.*, 2011). This value indicated the specificity of the extracts to cancer cells. A value of 2 or more indicated high specificity.

Solvent partitioning

Extracts with high cytotoxic activity were subjected to liquid-liquid partitioning using hexane and ethyl acetate. This procedure separated the compounds in the extracts based on their polarity thus the fractions with higher cytotoxicity can be determined.

Approximately 300mL of the crude extract concentrated using rotary evaporation was placed in a 1000mL separatory funnel. Equal amount of 95% n-hexane was added. After mixing, the solution separated into layers, the upper organic layer contains the compounds soluble in hexane. This procedure was done repeatedly until the organic layer turned colorless indicating that the entire hexane fraction was already acquired. Rotary evaporation was performed in order to recover the hexane and to obtain hexane fraction.

After the last round of hexane partitioning, the bottom layer was collected and subjected to ethyl acetate partitioning. Collected extract at 300mL was placed in a 1000mL separatory funnel and mixed with 300mL of ethyl acetate and 300mL of distilled water. The solution was allowed to separate and the upper layer containing the ethyl acetate fraction was collected and air dried to recover the ethyl acetate extract. Hexane and ethyl acetate partitions of cytotoxic crude extract were subjected to MTT assay.

Statistical analysis

The results were expressed as mean ± SD of three independent experiments. IC_{50} values from MTT Assay were subjected to statistical analyses.

Kolmogorov-Smirnov and Shapiro-Wilk Test for Normality was performed to determine if the IC_{50} values are in normal distribution. One Way Analysis of Variance was performed to determine whether there are any significant differences between the IC_{50} means of the extracts and the positive control, Doxorubicin©. Differences with $P<0.05$ values were considered as significantly different. Tukey HSD multiple comparison was also performed to determine which particular pair of extracts differ significantly from one another.

Results

Researches on natural products have provided hits for potential development of chemotherapeutic medicines. This research is a study of comparative cytotoxic activities of crude extract, hexane and ethyl acetate fractions of seaweeds *S. siliquosum*, *K. alvarezii, E. denticulatum* as well as the standard drug Doxorubicin against human colon cancer cell line (HCT-116), breast cancer cell line (MCF-7) and lung adenocarcinoma cell line (A549).

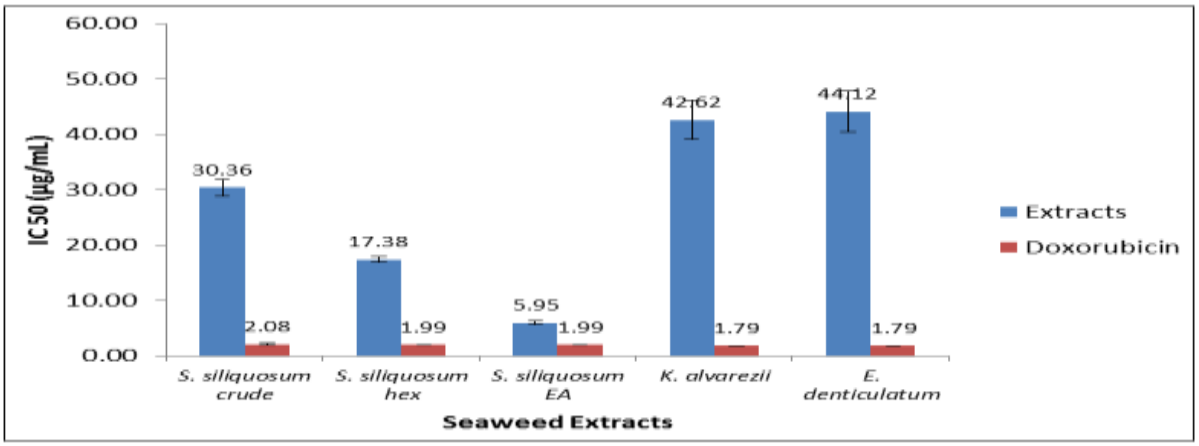

Fig. 1. Mean (± SD) IC50 values of seaweed extracts against colon carcinoma HCT116. Doxorubicin was used as positive control while DMSO was used as negative control. Three trials were done with three replicates for each concentration.

IC_{50} values of crude extracts and fractions against HCT 116 are in Figure 1 with *S. siliquosum* crude extract (30.36 ± µg/mL), *S. siliquosum* hexane (17.38 ± 0.60 µg/mL), *S. siliquosum* ethyl acetate (5.95± 0.72 µg/mL), *K. alvarezii* crude extract (42.62 ± 5.98µg/mL) and *E. denticulatum* crude extract (44.12 ± 6.43µg/mL).

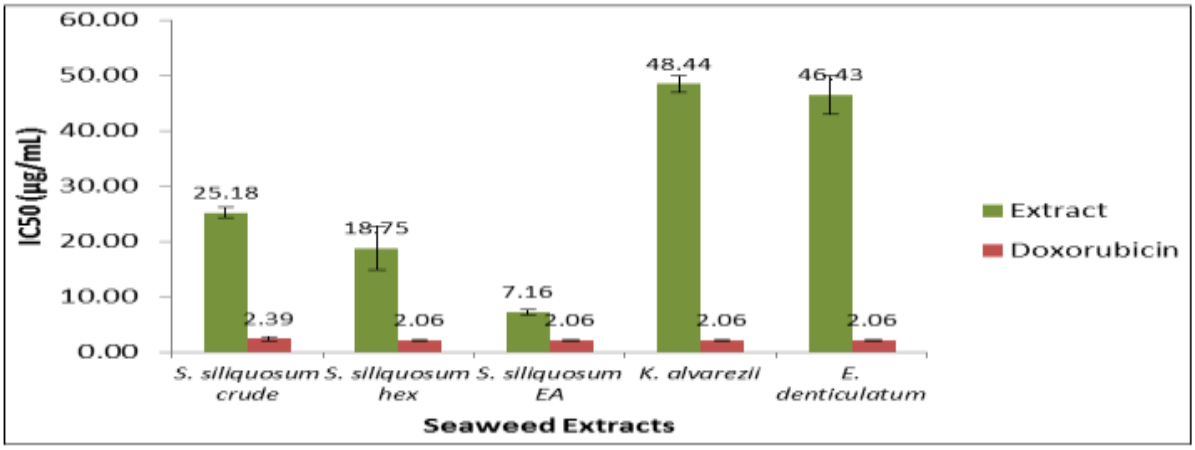

Fig. 2. Mean (± SD) IC50 values of seaweed extracts against breast cell line MCF 7. Doxorubicin was used as positive control while DMSO was used as negative control. Three trials were done with three replicates for each concentration.

IC_{50} values against breast cell line MCF-7 are presented in Figure 2 with *S. siliquosum* crude extract (25.18 ± 1.61µg/mL), (*S. siliquosum* hexane (18.75 ± 5.77 µg/mL), *S. siliquosum* ethyl acetate (7.16± 0.72µg/mL), *K. alvarezii* crude extract (48.44 ± 2.70µg/mL) and *E. denticulatum* crude extract (46.43 ± 6.18 µg/mL).

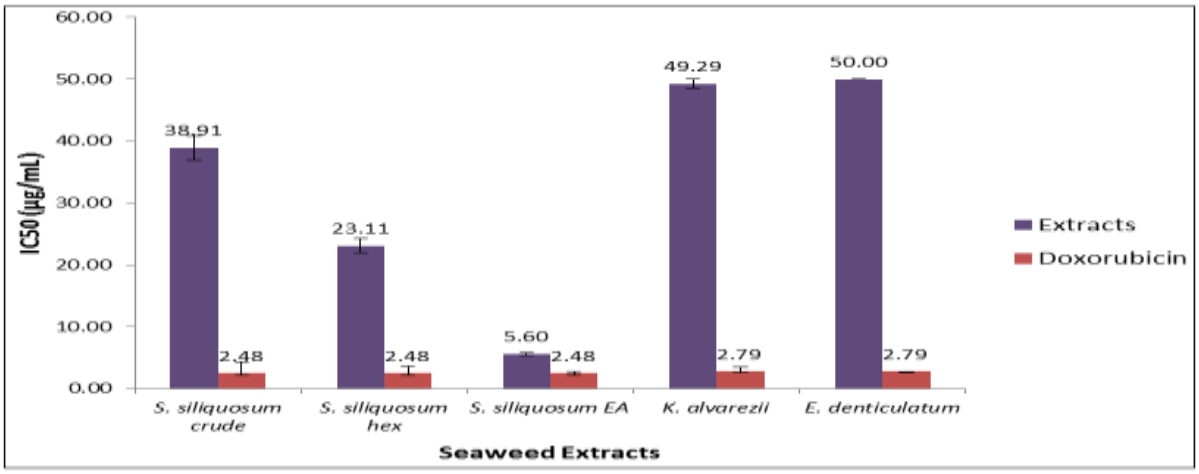

Fig. 3. Mean (± SD) IC50 values of seaweed extracts against lung adenocarcinoma, A549. Doxorubicin was used as positive control while DMSO was used as negative control. Three trials were done with three replicates for each concentration.

IC_{50} values against A549 are presented in Figure 3 with *S. siliquosum* crude extract (38.91 ± 3.33 µg/mL), *S. siliquosum* hexane fraction (23.11 ± 1.95 µg/mL), *S. siliquosum* ethyl acetate fraction (5.60 ± 0.47µg/mL), *K. alvarezii* crude extract (49.29 ± 1.22 µg/mL) and *E. denticulatum* crude extract (50.00 µg/mL).

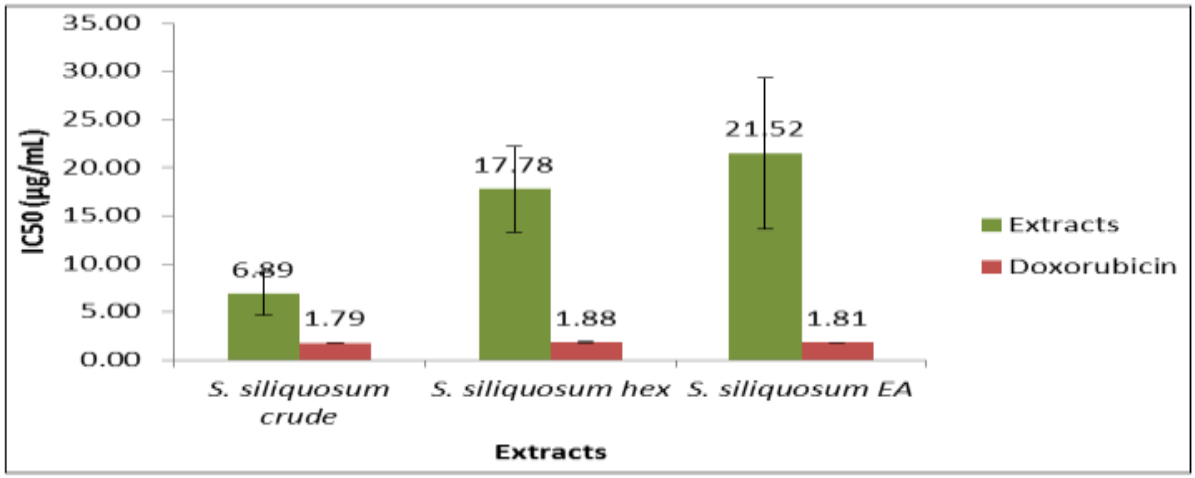

Fig. 4. Mean (± SD) IC_{50} values of seaweed extracts against non-cancer Chinese hamster ovarian fibroblast AA8. Doxorubicin was used as positive control while DMSO was used as negative control. Three trials were done with three replicates for each concentration.

S. siliquosum extracts that exhibited high cytotoxic activities should be selective against cancer cell lines. It is important that they are non-toxic against normal or non-cancer cell lines. To determine the selectivity of the seaweed extracts, they were tested against the non-cancer cell line Chinese hamster ovary (AA8). Comparatively, the cytotoxic activities of all the seaweed extracts were lower than Doxorubicin thus

showing more selective cytotoxicity. IC_{50} of *S. siliquosum* crude extract was 6.89 ± 3.86 µg/mL, *S. siliquosum* hexane fraction, 17.78 ± 7.72 µg/mL and *S. siliquosum* ethyl acetate 21.52 ± 13.60 µg/mL.

Figure 5 shows the selectivity index of all the cytotoxic seaweed extracts used in this study. *S. siliquosum* ethyl acetate fraction showed high selectivity for HCT 116, MCF-7 and A549 cell lines.

Photomicrographs of HCT 116, MCF-7 and A549 cells treated with different extracts are shown in Figure 6, 7, and 8 respectively while AA8 cells treated with cytotoxic extracts are shown in Figure 9.

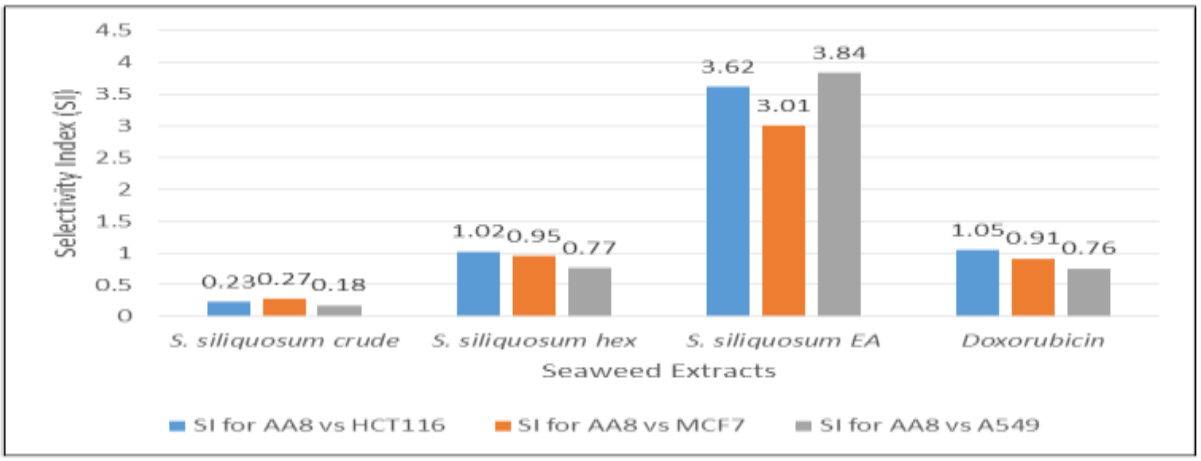

Fig. 5. Selectivity Index of seaweed extracts against AA8, HCT-116, MCF-7 and A549.

Discussion

Results of the MTT assay showed differences in the sensitivity of the cancer cell lines to the seaweed extracts. MCF-7 was the most susceptible to all the extracts utilized in this research. A549 cell line exhibited the highest resistance against the active extracts from *S. siliquosum*. Despite the generally higher IC_{50} value of the *S. siliquosum* extract against A549 relative to the other two cancer cell lines, it is interesting to note that the extract still showed potency against this lung cell line which has developed various mechanisms of resistance against anticancer drugs (Scagliotti *et al.*, 1999).

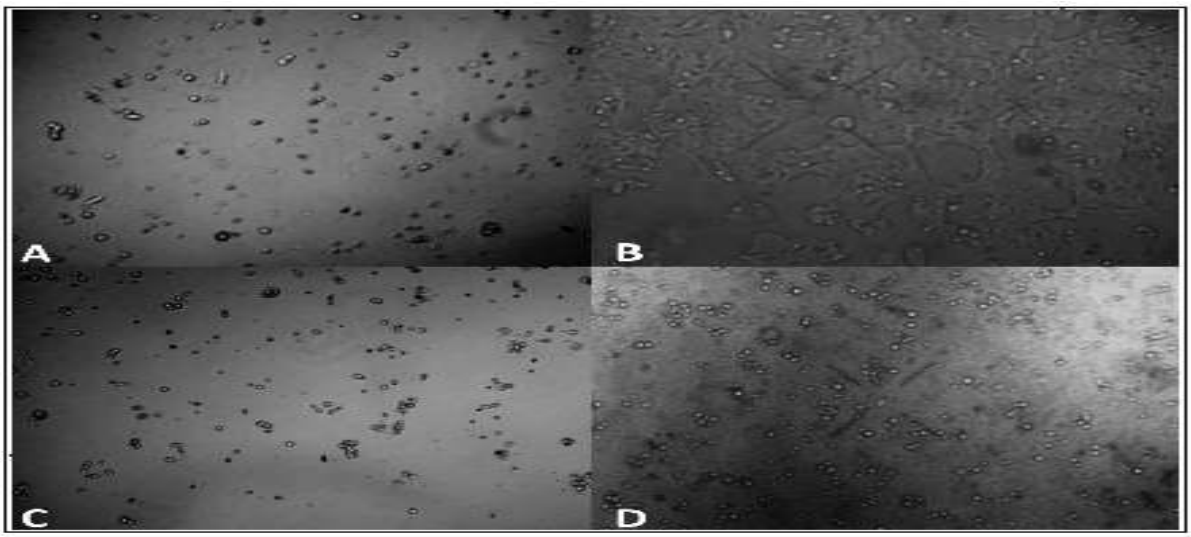

Fig. 6. Photomicrographs of HCT 116 cells treated with (A) Doxorubicin (B) DMSO (C) 50µg/ml *S. siliquosum* hexane fraction (D) 50µg/ml *S. siliquosum* ethyl acetate fraction and subjected to MTT assay after 24 hours of incubation (100x). Doxorubicin was used as positive control while DMSO was used as negative control.

According to the Suffness and Pezzuto (2009), a crude extract can be considered as cytotoxic against carcinoma cells *in vitro* and can be used for anticancer drug development if the standard IC_{50} value is less than 30 µg/mL. Based on this criterion, *S. siliquosum* hexane and *S. siliquosum* ethyl acetate are considered as highly cytotoxic against A549, HCT-116 and MCF-7 cell lines.

Crude extracts from *K. alvarezii* and *E. denticulatum* did not show cytotoxic activity against all human cancer cell lines used in this study. This lack of cytotoxic activity might be due to masking of biological activity by the presence of some inhibitory compounds in the crude extract (Nasrin *et al.*, 2015). Based on the standard values of toxicity by Suffness and Pezzuto (2009), *K. alvarezii* and *E. denticulatum* are not possible sources of cytotoxic compounds.

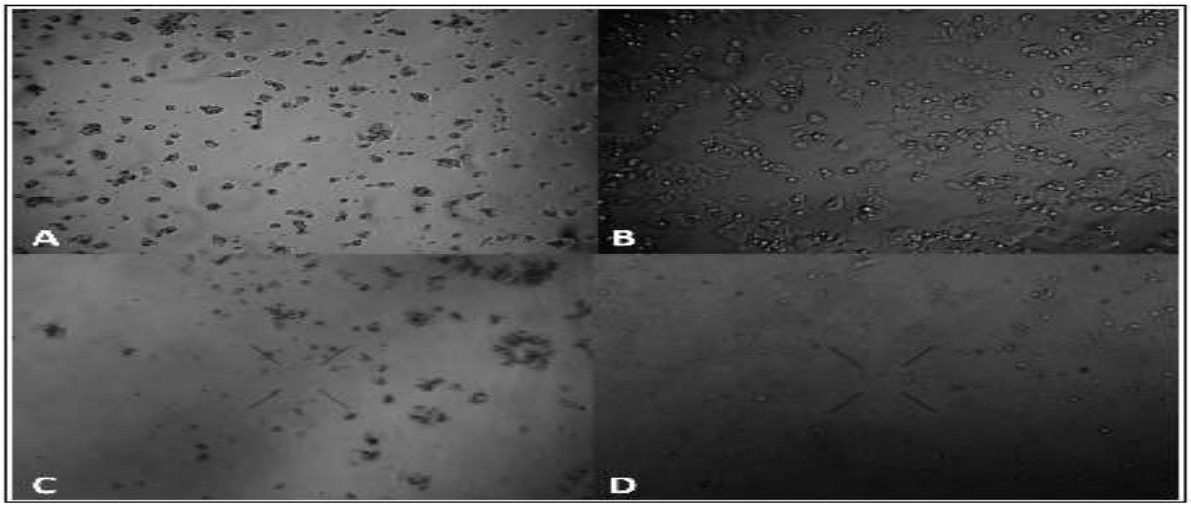

Fig. 7. Photomicrographs of MCF-7 cells treated with (A) Doxorubicin (B) DMSO (C) 50µg/ml *S. siliquosum* hexane fraction (D) 50µg/ml *S. Siliquosum* ethyl acetate fraction and subjected to MTT assay after 24 hours of incubation (100x). Doxorubicin was used as positive control while DMSO was used as negative control.

Statistical analysis revealed that there is no significant difference between the IC_{50} values of *S. siliquosum* ethyl acetate extract and doxorubicin against all cancer cell lines used in this study.

This means that the cytotoxicity of *S. siliquosum* ethyl acetate extract is comparable to that of doxorubicin. These results support the impression of potential anticancer compounds present in *S. siliquosum* ethyl acetate extract.

A previous study revealed 88% antitumor activity at 190µg/ml fucoidan isolated from *S. siliquosum* using MTT Assay. It also exhibited cytotoxic activity against HepG2 and renal carcinoma cell line. Phytochemical analysis of *S. siliquosum* is recommended to determine the presence of several known bioactive compounds in the crude extract.

A selectivity or specificity index according to Al-Qubaisi *et al.* determines the selective cytotoxicity of an extract. Higher SI means that an extract possesses selective cytotoxicity (Al-Quabaisi *et al.*, 2011). However, SI values lower than 2.0 indicates that a certain extract is a general toxin which means that though the extract has a high cytotoxicity to cancer cell lines, it also kills the normal cell lines (Koch *et al.*, 2005). *S. siliquosum* ethyl acetate fraction is considered to have a low cytotoxicity to normal cells. It is not classified as general toxin because it exhibited selectivity index value greater than 2.0.

Morphological characterization of cancer cell lines treated with different extracts is shown in Figures 6, 7 and 8. Through morphological comparison, *S. siliquosum* hexane and ethyl acetate fractions were confirmed to exhibit high cytotoxic activities against A549, HCT 116 and MCF-7. Cancer cells treated with

S. siliquosum hexane and ethyl acetate extracts have significantly low percentage of cancer cell population. Dead cells can be distinguished from live cell because of their apparent shrinkage and relatively smaller size compared to live cells.

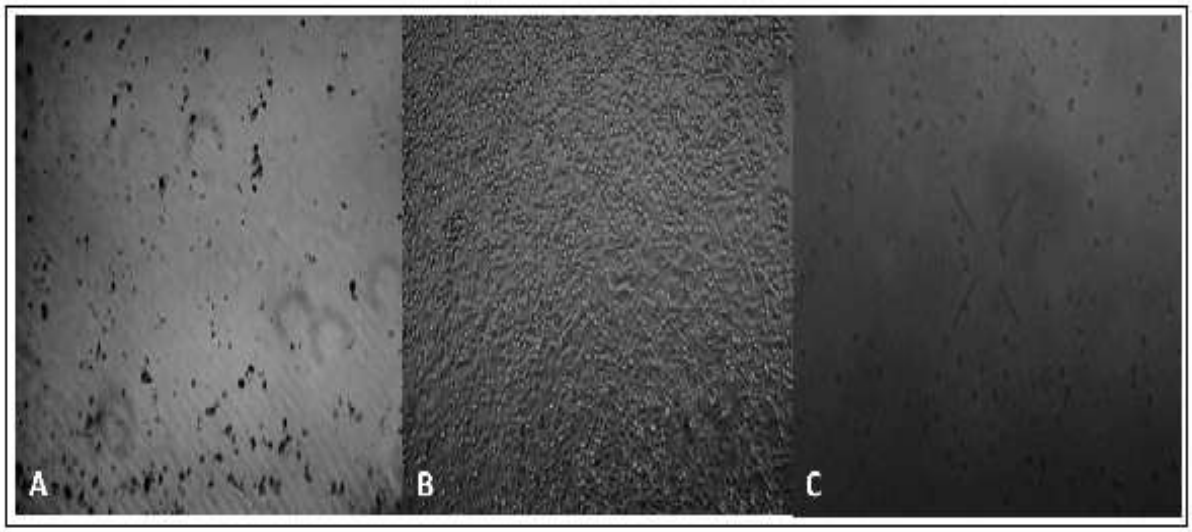

Fig. 8. Photomicrographs of A549 cells treated with (A) Doxorubicin (B) DMSO (C) 50µg/ml *S. siliquosum* ethyl acetate fraction and subjected to MTT assay after 24 hours of incubation (100x). Doxorubicin was used as positive control while DMSO was used as negative control.

Conclusion

Based on the results obtained, *S. siliquosum* and its fractions were considered highly cytotoxic against selected human cancer cell lines. *S. siliquosum* ethyl acetate fraction exhibited the highest cytotoxicity against HCT 116, MCF 7 and A549 cancer cell lines. It also exhibited high selectivity against these cancer cell lines and were classified as non-toxin for normal cells.

Further studies can be conducted utilizing the active extracts from this study. It is suggested that the cytotoxic extracts be further subjected to isocratic silica gel column chromatography and gradient silica gel column chromatography to determine the fraction with highest cytotoxic activity and eventually isolate the active compound. Further studies are needed to evaluate the chemopreventive potentials of the beetroot extract when used alone or in combination with doxorubicin to mitigate the toxic side-effects of the latter. Further studies are needed to evaluate the anticancer potentials of the *S. siliquosum* extract when used alone or in combination with doxorubicin to lessen the toxic side-effects of the latter.

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