Anticancer activity and mediation of apoptosis in human HL-60 leukaemia cells by edible sea cucumber (*Holothuria edulis*) extract

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**A B S T R A C T**

Sea cucumbers have been a dietary delicacy and important ingredient in Asian traditional medicinal over many centuries. In this study, edible sea cucumber *Holothuria edulis* was evaluated for its in vitro anticancer potential. An aqueous fraction of the edible sea cucumber (ESC-AQ) has been shown to deliver a strong cytotoxic effect against the human HL-60 leukaemia cell line. An induction effect of apoptotic body formation in response to ESC-AQ treatment was confirmed in HL-60 cells stained with Hoechst 33342 and confirmed via flow cytometry analysis. The up regulation of Bax and caspase-3 protein expression was observed while the expression of Bcl-xL protein was down regulated in ESC-AQ treated HL-60 cells. Due to the profound anticancer activity, ESC-AQ appears to be an economically important biomass fraction that can be exploited in numerous industrial applications as a source of functional ingredients. © 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Sea cucumbers, also known as Holothuroids (*Holothuroidea*), are marine invertebrates found in the benthic areas and deep seas. They are a diverse group of flexible, elongated, worm-like organisms, with a leathery skin and gelatinous body, resembling cucumber (Bordbar, Anwar, & Saari, 2011). Sea cucumbers are one of many marine animals utilised as food, particularly among the Asian population (Tajiyeb-Ali, Zainuddin, Swaminathan, & Yaacob, 2003). They are traditionally consumed raw, dried and boiled in many tropical and subtropical countries (Ozer, Mol, & Varlik, 2004). In addition, sea cucumbers have also been popular as a traditional food tonic in China, Korea and Taiwan for thousands of years (Chen et al., 2011; Wu et al., 2012). These marine invertebrates are usually processed into a dried product which are ranked as high or low in terms of their commercial value based on their species, abundance, appearance, odour, colour, thickness of the body wall and main market demand (Wen, Hu, & Fan, 2010). Sea cucumbers are believed to exert wound healing and reduce arthritis pain in humans, hence are widely used in Asian folk medicine (Aminin, 2001; Mamelona et al., 2007). In addition, recent scientific evidence supporting their importance as nutraceuticals and functional foods has attracted interest from nutritionists, pharmacologists and the general public (Zhong, Khan, & Shahidi, 2007). Therapeutic properties and medicinal benefits of sea cucumbers can be linked to the presence of a wide array of bioactives especially triterpene glycosides (saponins), chondroitin sulphates, glycosaminoglycans, sulphated polysaccharides, sterols, phenolics, cerberosides, lectins, peptides, glycoproteins, glycosphingolipids and essential fatty acids (Bordbar et al., 2011).

Alternative approaches in cancer treatment are important due to the emergence of new cases and some limited effectiveness of present treatments (Bandgar & Gawande, 2010). Much intervention in cancer cases involves surgery and chemotherapy that aims to eliminate cancer tissues (Dong et al., 2011). In addition, there is an increase in the number of cancer drugs that have been found to exhibit a relatively short clinical life span and then to become ineffective. In addition, the very potent drugs frequently exhibit serious side effects. Hence, there is an urgent need to search and develop new anticancer agents that are safe as well as being effective (Sondhi et al., 2010). Thus, possible remedies have been sought from unconventional sources, such as marine environments, and from the use of traditional information (Kong, Kim, Yoon, & Kim, 2009). This is particularly apparent in Asian countries where there has been a long-standing tradition for the use of natural products in health management and in cancer treatment (Awang et al., 2010).
This investigation examines the anticancer potential of the water-soluble extract of edible sea cucumber, Holothuria edulis, against the HL-60 leukaemia cell line. The cytotoxic effects and their induction of apoptotic body formation were investigated via Hoechst 33342 stained cells and flow cytometry analysis. Additional protein expression regulation of Bax, caspase-3 and Bcl-xL were studied on HL-60 leukaemia cells that were treated with sea cucumber extract. To the best of our knowledge, this is the first literature report that deals with an evaluation of the anticancer potential of the edible sea cucumber, H. edulis.

2. Materials and methods

2.1. Materials and reagents

RPMI-1640 medium, FBS and trypsin–EDTA were purchased from Gibco/BRL (Burlington, Ont, Canada). MTI, RNase A, dihydro-ethidium (DE), propidium iodide (PI), ethidium bromide (EtBr), DMSO and Hoechst 33342 were purchased from Sigma (St. Louis, MO, USA). Antibodies against Bax, Bcl-xL, cleaved caspase-3 and β-actin were purchased from Cell Signaling Technology (Bedford, Massachusetts, USA). All chemicals and reagents used were of analytical grade.

2.2. Preparation of the sea cucumber extract and fractionation

Fresh edible sea cucumbers (H. edulis) were collected, cleaned and freeze dried. Freeze dried tissues were extracted in 80% methanol for 3 days at room temperature. The mixture was further homogenised and extracted for another 3 days. The homogenate was separated and the resulting extract was concentrated via vacuum evaporation to obtain the concentrated edible sea cucumber methanolic extract. The resulting crude extract was re-dissolved in ethyl acetate and partitioned with double distilled water (DDW) in a 3:1 ratio. The ethyl acetate fraction was dehydrated over anhydrous sodium sulfate, and concentrated to leave an oily red crude extract (1.1 g). The viscous aqueous fraction (ESC-AQ) was freeze-dried to produce aqueous extract (54 g). During the cell assays, samples were dissolved in dimethyl sulfoxide and further diluted in culture media.

2.3. Determination of approximate chemical composition

Approximate chemical composition of the aqueous extract (ESC-AQ) was determined according to the AOAC method (1990). Crude carbohydrate was determined by phenol–sulfuric acid reaction (absorbance at 480 nm; using glucose as the calibration standard), crude lipid was quantified by Soxhlet extraction and crude ash was measured by drying at 550 °C in a dry-type furnace. Moisture content was determined by oven drying and crude protein content was determined by the Kjeldahl method. The content of the each component was expressed as a percentage.

2.4. Cell culture

Human promyelocytic leukaemia cell line (HL-60) was grown on RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL). The culture was maintained at 37 °C in a 5% CO2 incubator. Vero cell line was grown on Dulbecco’s modified Eagle medium (DMEM), supplemented with 10% heat-inactivated foetal bovine serum (FBS). The culture was maintained at 37 °C in a 5% CO2 incubator. Cell lines were monitored daily and media changes were made every 3–4 days.

2.5. Cell growth inhibitory assay

The cytotoxicity of the ESC-AQ against the tumor cells was assessed via a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HL-60 cells were seeded (1.0 × 105 cells/mL) together with various concentrations of the ESC-AQ sample (25, 50 and 100 μg/mL) and incubated for up to 48 h prior to MTT treatment. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide stock solution (50 μL; 2 mg/mL in PBS) was added to each well to achieve a total reaction volume of 250 μL. After 3 h of incubation, the plates were centrifuged for 10 min at 2000 rpm and the supernatants were aspirated. The formazan crystals in each well were dissolved in dimethyl sulfoxide. The amount of purple formazan was assessed by measuring the absorbance at 540 nm using an ELISA reader (Sunrise; Tecan Co. Ltd., Australia).

2.6. Nuclear staining with Hoechst 33342

The nuclear morphology of the cells was evaluated using the cell-permeable DNA dye, Hoechst 33342. Cells with homogeneously stained nuclei were considered viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis (Gschwind & Huber, 1995; Lizard et al., 1995). The HL-60 cells were seeded in 24-well plates at a concentration of 1.0 × 105 cells/mL. Sixteen hours after seeding, the cells were treated with various concentrations of the ESC-AQ sample (25, 50 and 100 μg/mL), and further incubated for 12 h at 37 °C in a humidified atmosphere. Then, 1.5 μL of Hoechst 33342, a DNA-specific fluorescent dye, was added at a final concentration of 10 μg/mL, followed by 10 min of incubation at 37 °C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro colour digital camera, in order to examine the degree of nuclear condensation.

2.7. Cell cycle analysis

Flow cytometry analyses were conducted in order to determine the proportion of apoptotic sub-G1 hypodiploid cells (Lizard et al., 1995). The HL-60 cells were placed in 6-well plates at a concentration of 4.0 × 104 cells/mL and, 16 h after plating, the cells were treated with various concentrations of the ESC-AQ sample (25, 50 and 100 μg/mL). Then, after 12 h, the cells were harvested at the indicated time and fixed for 30 min in 1 mL of 70% ethanol at 4 °C. The cells were then washed twice with phosphate buffer solution (PBS), and incubated for 30 min in darkness in 1 mL of PBS containing 100 μg PI and 100 μg RNase A, at 37 °C. Flow cytometric analysis was conducted using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, USA). Effects on the cell cycle were determined by measuring changes in the percentage of cell distribution at each phase of the cell cycle, and were assessed by histograms generated by the Cell Quest and Mod-Fit computer programs.

2.8. Western blot analysis

Cells (2.0 × 105 cells/mL) were treated with different concentrations of ESC-AQ and then harvested. The cell lysates were prepared with lysis buffer (50 mmol/L Tris–HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS and 1 mmol/L EDTA). Cell lysates were washed via centrifugation, and the protein concentrations were determined using a BCA™ protein assay kit (Thermo Scientific Pierce, CA, USA). The lysates containing 30 μg of protein were subjected to electrophoresis on 10% or 12% sodium dodecyl sulfate–polyacrylamide gels, and the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated with primary antibody against Bax, Bcl-xL, cleaved...
caspase-3, and β-actin in TTBS (25 mmol/L Tris–HCl, 137 mmol/L NaCl, 0.1% Tween 20, pH 7.4) containing 0.5% nonfat dry milk for 1 h. The membranes were then washed with TTBS and incubated with secondary antibodies. Signals were developed using an ECL Western blotting detection kit and exposed to X-ray films.

2.9. Statistical analysis

All the data are expressed as mean ± standard deviation of three determinations. Statistical comparison was performed via a one-way analysis of variance followed by Duncan’s multiple range test (DMRT). P values of less than 0.05 (P < 0.05) were considered as significant.

3. Results

3.1. Approximate chemical composition of ESC-AQ

The extraction of *H. edulis* described gave two final extracts; an aqueous extract (ESC-AQ) and an ethyl acetate extract (ESC-EA). Upon quantification, the ESC-AQ yield was much higher compared to the ESC-EA. Total phenolic contents of ESC-AQ and ESC-EA were 10.32% and 4.54%, respectively. This trend is expected since polyphenols are readily soluble in an aqueous solvent, compared to non-polar solvents. In addition, the approximate chemical composition of the ESC-AQ was determined. Moisture, carbohydrate, crude protein, crude lipid contents were 23.3%, 12.2%, 5.4% and 3.4%, respectively. Ash content accounted for 55.7% of the total dry weight after the elimination of the moisture via drying. This aqueous extract was clear and viscous in nature.

3.2. Inhibitory effect of ESC-AQ on the growth of HL-60 cells

Evaluations of sea cucumber *H. edulis* extracts for potential anti-cancer activity on HL-60 cells were carried out by studying the growth inhibitory effects of both ESC-AQ and ESC-EA fractions using the MTT assay. Preliminary test of extracts at 250 μg/mL resulted in the 20% and 100% survival of HL 60 cells for ESC-AQ and ESC-EA, respectively. Hence, the aqueous fraction of the edible sea cucumber (ESC-AQ) was identified as exhibiting profound anticancer effects on HL-60 cells. In addition, ESC-AQ strongly inhibited HL-60 cell proliferation in a dose-dependent manner (Fig. 1). Moreover, ESC-AQ showed more than 82% cell proliferation inhibition at the concentration of 100 μg/mL. Then, the effect of ESC-AQ on cell viability in normal (Vero) cells was tested. Toxicity evaluation in Vero cells revealed only limited cell deaths at the tested concentration (Fig. 2).

3.3. Pro-apoptotic effect of ESC-AQ on HL-60 cells

In order to examine the nuclear morphological changes in response to ESC-AQ treatment, HL-60 cells were stained with the cell-permeable DNA dye Hoechst 33342 and visualised by fluorescent microscopy. The determination of apoptosis in different concentrations of ESC-AQ-treated HL-60 cells was carried out. As shown in Fig. 3A, a typical image of untreated cells featuring round intact nuclei was observed. In contrast, the ESC-AQ-treated cells showed morphological changes characteristic of apoptosis, including chromatin condensation, bleb formation around the nucleus, and phase bright nuclear fragmentation (Fig. 3B–D). In addition, Fig. 3B–D also showed the gradual increment of apoptotic cells in a dose-dependent manner and showed typical morphological changes, including reductions in cellular volume, bright staining and condensed or fragmented nuclei.

3.4. Effect of ESC-AQ on sub-G1 DNA contents in cell cycle analysis

In order to measure quantitatively the apoptosis induction by ESC-AQ treatment, flow cytometry analysis was carried out. As shown in Fig. 4A, the sub-G1 DNA content was 7.0% in control cells. However, as demonstrated in Fig. 4B–D, ESC-AQ treatment in the HL-60 cells at different concentrations (25, 50 and 100 μg/mL) induced dose-dependent increases in the proportion of apoptotic cells, which was reflected by the sub-G1 populations (7.8%, 59.9% and 69.0%). These results demonstrate that ESC-AQ-induced cell death was caused principally by apoptosis induction.

3.5. Effect of ESC-AQ on apoptosis-related protein expressions in HL-60 cells

Expressions of the intracellular proteins related to apoptosis, such as Bax, Bcl-xL and caspase-3, were investigated to understand the mechanisms by which ESC-AQ induces apoptosis in HL-60 cells. As shown in Fig. 5, the level of the pro-apoptotic protein Bax was increased markedly and the level of an anti-apoptotic protein Bcl-xL was decreased markedly in ESC-AQ treated HL-60 cells. In contrast, expression of Bcl-xL was completely suppressed by ESC-AQ at the concentration of 100 μg/mL. The expression of the active form of caspase-3 was increased with the presence of 100 μg/mL of ESC-AQ in HL-60 cells.
4. Discussion

Sea cucumber is an important ingredient in traditional Chinese medicine and has been considered to be a functional food for health management among the Asian communities since ancient times. This traditional knowledge has been further endorsed by scientific reports of its biological potential with biomedical importance (Bordbar et al., 2011). In an effort to search for new lead natural products with potent biological activities, we have reported for the first time an anticancer evaluation of the water-soluble extract of *H. edulis*. The findings in this research are important due to the emergence of cancer as the second leading cause of death after cardiovascular diseases (Bandgar & Gawande, 2010; Sondhi et al., 2010). Although a great deal of effort has been taken to treat various forms of cancer, until recently, chemotherapy of cancer seems to be the best option.

Many chemotherapeutic agents are reported to exert their anticancer effects by inducing apoptosis of cancer cells (Kamesaki, 1998). Apoptosis, also known as programmed cell death, is characterised by typical cellular morphology and biochemical features including cell shrinkage, cytoplasm vacuolization, chromatin condensation, DNA fragmentation and, finally, cellular breakdown into apoptotic bodies (Heo et al., 2011). Apoptosis is an important biological mechanism that contributes to the maintenance of the integrity of multi-cellular organisms that is dependent on the expression of cell-intrinsic suicide machinery. The mitochondrial permeability transition is an important step in the induction of cellular apoptosis (Kang et al., 2012).

In this study, the cytotoxic effect of ESC-AQ was investigated in the human HL-60 leukaemia cell line. Due to the promising cytotoxic effect showed by ESC-AQ, it was further evaluated on cell apoptosis, cell cycle analysis and apoptosis related protein expressions. The results of this study showed typical morphological characteristics of apoptosis, such as nuclear condensation and apoptotic body formation, in HL-60 cells treated with ESC-AQ. In addition, the apoptosis of HL-60 cells induced by ESC-AQ was also confirmed by the sub-G1 DNA accumulation. These results suggest that ESC-AQ might mediate its growth inhibitory effects on HL-60 cells by mechanisms involving the induction of apoptosis.

Fig. 2. Effect of ESC-AQ of *H. edulis* on cell viability in normal (Vero) cells. Cells were treated with ESC-AQ and cell viability was measured by MTT assay at 48 h. Values are mean ± SD of three determinations.

Fig. 3. Induction of ESC-AQ on apoptotic body formation in HL-60 cells. The cells were treated with different doses of ESC-AQ for 12 h and visualised for apoptotic bodies under fluorescent microscope (400×) using a blue filter after staining with Hoechst 33342. (A) Control, (B) 25 μg/mL, (C) 50 μg/mL and (D) 100 μg/mL.
Fig. 4. Effect of ESC-AQ on cell cycle pattern and apoptotic cell proportion in HL-60 cells. Apoptotic sub-G1 DNA content was detected by flow cytometry after propidium iodide staining. (A) Control, (B) 25 μg/mL, (C) 50 μg/mL, (D) 100 μg/mL, and (E) bar graph for sub-G1 peak patterns of HL-60 cells treated with different concentrations of ESC-AQ.

Fig. 5. Effect of ESC-AQ on apoptosis-related protein expressions in HL-60 cells. Cells were treated with ESC-AQ at the indicated concentration for 12 h. Whole cell lysates were subjected to Western blot analysis of anti-Bax, -Bcl-xL, and cleaved caspase-3 monoclonal antibodies. β-actin was used as internal control.
As indicated in many other studies, the regulation of apoptosis involves a host of molecules, in particular, the expression of proteins such as Bcl-xL and Bax are altered with the induction of apoptosis (Ren, Zhao, Yang, & Fu, 2008). Bcl-xL is a transmembrane molecule in the mitochondria. It is one of several anti-apoptotic proteins, which are members of the Bcl-2 family of proteins. It has been implicated in the survival of cancer cells. Bax is a pro-apoptotic Bcl-2 protein and it promotes apoptosis by competing with Bcl-2 proper. In addition, sequential activation of caspases plays a central role in the execution-phase of cell apoptosis (Heo et al., 2011). Chemopreventive agents help to interrupt or reverse the progress of cancer and to prevent carcinogenesis and reverse precancerous (Namvar et al., 2012). The ESC-AQ suppressed the growth of human HL-60 leukaemia cells via apoptosis induction through modulation of apoptosis related protein expression. According to the results observed, ESC-AQ could inhibit the proliferation of HL-60 cells by mechanisms involving the induction of apoptosis. Furthermore, the apoptosis of HL-60 cells induced by ESC-AQ is associated with the up regulation of Bax, down regulation of Bcl-xL and activation of caspase-3 (Fig. 5).

The potent biological activities seen in this investigation may be attributed to the water-soluble chemicals inherently available sea cucumbers. In general, these organisms are known to contain a high amount of good-quality protein that has been associated with beneficial effects (Bordbar et al., 2011). The proteins produced from its body wall are rich in glycine, glutamic acid and arginine. These amino acids are known to stimulate production of IL-2 and B-cell antibodies. In addition, arginine can enhance cell immunity by promoting activation and proliferation of T-cells. They are also known to contain considerable amount of phenolics and free radical scavengers. Some of these bioactive phenolics were identified as chlorogenic acid, pyrogallol, rutin and coumaric acid (Esmat, Said, Soliman, El-Masry, & Badiea, 2013). In addition, bioactive secondary metabolites such as triterpene glycosides, glycosaminoglycan, sulfated polysaccharides, sterols and glycosphingolipids have been reported to be present in sea cucumbers (Bordbar et al., 2011).

5. Conclusion

In conclusion, these findings revealed for the first time the anticancer potential of edible sea cucumber, *H. edulis*. The presence of biological activity in raw *H. edulis* extract indicates that their consumption could be beneficial to health. In addition, the findings of this study may facilitate awareness about the potential anticancer properties of *H. edulis* and help future developments of anticancer therapeutics on an industrial scale. In addition, the water-soluble fraction of raw *H. edulis* could be further developed for its pharmacological potential as a complementary cancer remedy and also promoted as a functional food and nutraceutical.

References


