IN VITRO CYTOTOXIC AND APOPTOTIC ACTIVITIES OF SULFATED POLYSACCHARIDE FROM CODIUM EDULE P. C. SILVA AGAINST BREAST CANCER ADENOCARCINOMA

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INTRODUCTION

Sulfated polysaccharides (SPs) are used for scientific research in the discovery of chemotherapeutic drugs due to their antiproliferative effects [1]. In general, cancer is considered as one of the most lethal diseases which may threaten human life [2]. The defining characteristics of cancer are due to its capability to invade and metastasize, wherein through genetic mutation, the normal cells transform into malignant or cancerous cell, proliferate rapidly, invade its surrounding tissue, and migrate in lymphatic system [3]. Chemotherapeutic regimen is considered the main choice in treating different cases of cancer. However, the long-term use of chemotherapeutic agents may cause cumulative toxicity which may result in myelosuppression. Therefore, it is beneficial to discover a novel, effective, and non-toxic compounds from natural products that have the potential to be used as an alternative to chemotherapy to reduce the undesirable side effects [2].

SPs displayed antiproliferative activity in tumors growing cells using different cancer cell lines in vitro [4]. Furthermore, SPs were also proven to induce apoptosis, programmed cell death, and cell death through stimulation of the immune system [5]. Furthermore, SPs from different sources of marine algae showed free radical scavenging and antioxidant activities which would prevent oxidative damage [6]. Likewise, marine resources specifically green algae have relatively low toxicity and have high bioactivity. Thus, more attention has been given to search for new substances for the development of anticancer drugs [7]. Marine algae contain a high concentration of SPs with numerous health benefits, thus attracted more interest to develop and isolate novel SPs [8]. Numerous bioactive polysaccharides were found to have interesting functional properties discovered from marine algae [9]. Different biological and pharmacological activities reported from the algal polysaccharide include antitumor, anticoagulant, anti-inflammatory, and immunomodulating properties [10]. However, little attention has been given to polysaccharide from green algae compared with red (Rhodophyta) and brown (Phaeophyta) seaweeds [11]. In addition, polysaccharides which are present in the cell walls of green seaweeds were reported to boost the host immune system responsible for antitumor activity [12]. The presence of the sulfate and uronic acid in algae has been proven to influence the antioxidant and antitumor activities of SPs [2].

Genus, Codium is widely available and commonly used as food source, but there are only few studies on the structure and activities of sulfated macromolecules from this genus [10]. Green algae remain largely unexploited among the three main divisions of macroalgae [8]. Thus, marine algae-derived SPs have great potential for further development as of antineoplastic agent specifically its antiproliferative and apoptotic property. In this study, lyophilized crude SP (CSP) and SP fractions from Codium edule PC. Silva were tested for antiproliferative and apoptotic property against breast cancer cell lines (MCF-7 using Annexin V FITC and Caspase-Glo 3/7 assay).

METHODS

Standards, reagents, and chemicals
All standards, reagents, and chemicals used in this study are analytical grade which were purchased at the University of Santo

Keywords: Sulfated polysaccharide, Codium edule, Green algae, MTT assay, Annexin V, Cytotoxicity, Antiproliferative, Apoptosis.

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ABSTRACT

Objective: The primary purpose of this study is to characterize Codium edule crude sulfated polysaccharide (CSP) and its fractions and to determine its potential antiproliferative and apoptotic properties.

Methods: The CSP was obtained through hot water extraction followed by precipitation with absolute ethanol. CSP was further purified using ion-exchange chromatography, Sepharose DEAE Fact Flow column and yielded three fractions (F1, F2, and F3). The CSP and fractions were characterized for their sulfate, protein, carbohydrate, and uronic acid content. Fourier-transformed infrared spectroscopy (FT-IR) was used to determine the functional groups present in CSP and SP fractions. Antiproliferative activity against human breast adenocarcinoma (MCF-7) was analyzed using MTT assay with doxorubicin as positive control. Apoptotic activity of C. edule was analyzed using caspase 3/7 and annexin V-FITC assay.

Results: CSP afforded 6.3% sulfate, 4.1% protein, and 68.7% carbohydrate. F1 has the highest content of sulfate, protein, carbohydrate, and uronic acid among the fractions. FT-IR shows a broadband around 3400 cm−1 suggests the presence of hydroxyl stretching vibration of polysaccharide (-OH) and a band at 2922 cm−1 suggests a C-H stretch (alkane). 1658 cm−1 may be attributed to the C=O stretches of amide C=N group. Peak around 1259 cm−1 is a characteristic band for 5-O sulfate ester. The antiproliferative activity of C. edule against MCF-7 showed significant difference in the mean percent inhibition between CSP and F3 (p=0.001), F1 and F3 (p<0.001), F2 and doxorubicin (p=0.025), and F3 and doxorubicin (p<0.000). F1 of C. edule has the lowest IC50 of 5.54 µg/ml and displayed apoptotic phase and caspase 3/7 activity.

Conclusion: The investigation revealed that SP from green seaweed, C. edule, could be used as potential anticancer treatment against breast cancer adenocarcinoma.
Tomas – Laboratory Equipment and Supplies Office. Doxorubicin HCl, human breast cancer adenocarcinoma cell lines (ATCC HTB-22) (ATCC, USA) were provided by the UST Mammalian Tissue Culture Laboratory. Human Dermal Fibroblast (CC0045C) was purchased from Invitrogen. Annexin V FITC was purchased from Biovision Laboratory. Caspase-Glo 3/7 assay kit was purchased from Promega through a local distributor, Golden Bat Laboratory Inc.

**Algal sample collection and identification**

Fifty kilograms of the fresh thalli of *C. edule* were collected from the coast of Brgy. San Vicente in Santa Ana, Cagayan Province (18°31’11’’N 121°09’6’’E) in May 2016 with permit from the Bureau of Fisheries and Aquatic Resources-Region 2. The fresh algal materials were thoroughly washed with seawater and distilled water to remove extraneous matter and debris.

Fresh and herbarium specimens were identified by the Botany Division of the National Museum of the Philippines in Manila City and given identification control no. 16-06552.

**Plant sample extraction and fractionation**

The cleaned thalli were air-dried in shade for 5 d, milled using Wiley mill, sieved to have uniform particle size of <250 mm, and were stored in a tightly sealed amber bottle.

The algal powder (80 g) was treated with 85% ethanol (800 ml) under constant mechanical stirring at room temperature. The residual part was separated using a Whatman filter paper, collected and rinsed with acetone, and dried at room temperature inside the fume hood. The dried biomass was further extracted by hot water extraction method with stirring for 2 h. The extracts were centrifuged at 3500 rpm for 15 min at room temperature, and the supernatants were collected. Absolute ethanol (1:1) was added into the collected supernatant and was placed at 4°C for 24 h. The polysaccharide was obtained through filtration using a Whatman filter paper and was washed with absolute ethanol and then dried at room temperature inside the fume hood.

The precipitated polysaccharide was referred to as the CSP and the yield was calculated based on the weight of dried biomass obtained after treating the milled sample with 85% ethanol using the formula:

\[
\text{Yield} = \frac{\text{Weight of lyophilized extract of } C. \text{ edule}}{\text{Weight of dried } C. \text{ edule}} \times 100
\]

**Fractionations of SP**

CSP (250 mg) was dissolved in distilled water at 70°C with stirring for 4 h. The solution was cooled to room temperature, sonicated for 10 min, and then centrifuged at 3500 rpm for 15 min. The obtained supernatant was added into the DEAE Sepharose Fast Flow column (DFF100) (GE Healthcare Bio-Sciences AB). The SP fraction polysaccharides were eluted with distilled water and with a stepwise NaCl gradient (0.5 M–2 M). Three fractions were obtained and were referred to as F1, F2, and F3. These fractions were lyophilized and stored in tightly sealed bottle at 2–8°C until use.

**Characterization of CSP**

Sulfate content of CSP was determined by ashing-water digestion ion chromatography. The amount of sulfate was computed from standard curve prepared from the peak area reading of standard sulfates. Protein content of the CSP of *C. edule* was obtained by Kjeldahl Titrimetric method while the carbohydrate content of the CSP was calculated using the obtained amount of protein, fat, moisture, and ash content.

**Characterization of SPs fractions**

Sulfate content of SP fractions was estimated by turbidimetric method of barium chloride using anhydrous sodium sulfate as a standard. Bradford assay was used to determine the protein content of the of SP fractions using bovine serum albumin (BSA) as a standard. Phenol-sulfuric acid method by Dubois (1956) was adopted to determine the carbohydrate content of the SP fractions using glucose as a standard while uronic acid content was determined by m-hydroxybiphens method using galacturonic acid as standard [13].

**Functional group identification using Fourier-transform infrared spectroscopy (FT-IR) of CSP and SP fractions**

Lyophilized CSP and SP fractions were subjected to FT-IR analysis to determine the functional groups present in the polysaccharides. The CSP and SP fractions were triturated with KBr and were placed in a pan. The spectrum used was between 400 cm⁻¹ and 4000 cm⁻¹ using Shimadzu IRPrestige-21 spectrophotometer.

**In vitro cytotoxic activity assay**

The cytotoxic effect of CSP and SP fractions (F1, F2, and F3) against MCF-7 and human dermal fibroblast (HDF) was determined by MTT assay following the manufacturer’s protocol. MCF-7 and HDF cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum and were maintained at 37°C with a humidified atmosphere containing 5% carbon dioxide (CO₂). The cultures were maintained in aseptic condition. Cells were seeded into 96-well plates at 10,000 cells/well and were incubated for 24 h inside cell culture incubator. The temperature was maintained at 37°C with a humidified atmosphere containing 5% CO₂. Fifty microliters of each sample: CSP, SP fractions (F1, F2, and F3), and doxorubicin and 100 µl of DMEM were added per well and incubated for another 24 h in cell culture incubator. After 24 h, the media were removed and 10 µl of 5 mg/ml of MTT reagent was added into each well. The plate was incubated for 4 h inside the cell culture incubator and the MTT reagent from each well was removed and replaced with 100 µl of DMSO to dissolve the formazan crystals. The absorbance was read at 570 nm using a microplate reader.

**Annexin V FITC**

Apoptotic activity of F1 against MCF-7 was determined by Annexin V FITC by fluorescence microscopy (EVOS FL Imaging System). Cells were directly seeded into 96-well plate at 100,000 cells/well and were incubated for 24 h inside a cell culture incubator. Temperature was maintained at 37°C with a humidified atmosphere of 5% CO₂. Apoptosis was induced for 24 h and 48 h. One hundred microliters of 1.563 µg/ml F1 was added per well and incubated for another 24 h and 48 h in cell culture incubator. The cells were collected and subjected to centrifuge for 15 min. The cells were resuspended in 500 µl of binding buffer then 5 µl of Annexin V-FITC was added into the resuspended cells and incubated in the dark at room temperature for 5 min. After incubation, the apoptosis was detected using fluorescence microscopy. Cells that showed a green fluorescence in the plasma membrane, due to bound Annexin V FITC, underwent apoptosis. The assay was performed in duplicate. Procedure was adopted from Annexin V-FITC Apoptosis Detection Kit Protocol, BioVision.

**Caspase-Glo 3/7 assay**

The detection of apoptotic cells by the activation of caspase 3/7 was performed following the manufacturer’s protocol (Promega, WI, USA). The Caspase-Glo 3/7 reagent and the lyophilized Caspase-Glo 3/7 substrate were equilibrated to room temperature before use. Cells were directly seeded into 96-well plates at 10,000 cells/well and were incubated for 24 h inside a cell culture incubator. Temperature was maintained at 37°C with a humidified atmosphere of 5% CO₂. F1 of *C. edule* was used as the treatment group. After treatment, the cell medium in the 96-well plate was replaced with 50 µl of serum-free cell DMEM and 50 µl of Caspase-Glo 3/7 reagent was added to each well. The luminescence was measured at 30 min, 1 h, 2 h, and 3 h using GloMax™ Discover Multimode Microplate Reader (Promega).

**RESULTS AND DISCUSSION**

**Extraction yield and physical characteristics**

Eighty grams of dried powder of *C. edule* yielded 15.8% (12.64 g). The obtained CSP is a light green color powder with fishy-like odor. The obtained three SP fractions which were labeled as F1, F2, and F3 are light green substance with fibrous texture and without a distinct odor.
Sulfate content determination of crude SP and SP fractions
Determination of the sulfate content of the CSP of *C. edule* was obtained by ashing-water digestion ion chromatography method. Sulfate was successfully separated from the crude SP after acid treatment. Sulfate was successfully eluted at the retention time of 15 min with an area of 89,403 mAu (Fig. 1).

Sulfate content of the SP fractions using anhydrous sodium sulfate as a standard was found to be 6.21%±0.030, 0.91%±0.0125, and 0.021%±0.002 for F1, F2, and F3, respectively. This indicates that the CSP and SP fractions (F1, F2, and F3) of *C. edule* contain a sulfated group which may contribute to its biological activity as anticancer property specifically antiproliferative and apoptotic activities. According to Wang et al., the presence of sulfated groups may increase the negative charges on the surface of polysaccharides, which can promote the antioxidant, antitumor, and immune activity of polysaccharides [8]. Likewise, many researches have reported that the presence of sulfate in polysaccharides tends to increase its activity.

Protein content determination of crude SP and SP fractions
The concentration of protein in the CSP using BSA as a standard was found to be 4.10% (w/w). The protein content of SP fractions using BSA as a standard was found to be 1.10%±0.043, 1.07%±0.063, and 0.067%±0.002 for F1, F2, and F3, respectively. The protein content in seaweeds varies from about 10% to 40% per dry weight, and it differs according to the seasons and the species. In general, the highest protein values in seaweed are found during the period of winter-early spring and the lowest during summer-early autumn [14].

Carbohydrate content determination of crude SP and SP fractions
Determination of carbohydrate content of the CSP was calculated using the obtained amount of protein, fat, moisture, and ash content; based on the protein content of 4.13% (w/w), fat content of 1.20% (w/w), moisture content of 15.10% (w/w), and ash content of 10.90% (w/w). The total carbohydrate content of 24.93%±0.941, 16.66%±1.099, and 0.72%±0.040 for F1, F2, and F3, respectively, was determined using glucose as standard.

Seaweeds contain high levels of polysaccharides, most of which constitute dietary fiber [15] and made up of carbohydrate monomers which are normally hexoses that are linked by glycosidic bonds. Relative to several studies, total polysaccharide contents in macroalgae range from 4% to 76% of dried weight of seaweed, wherein the highest contents in green seaweed, *Ulva*, can have a high content of carbohydrate for up to 65% of dried weight of green seaweed [16]. The high carbohydrate content in *C. edule* was in agreement with the reported contents in most green algae.

Uronic acid content determination of SP fractions
The uronic acid content 0.96%±0.024, 0.71%±0.031, and 0.036%±0.001 uronic acid was determined for F1, F2, and F3, respectively, using galacturonic acid as standard. Uronic acid in the form of galacturonic acid is a major component of polysaccharide which is present in the cell wall of marine alga. The green algae, Chlorophyceae, contain mostly ulvans [17], wherein its matrices are composed of highly sulfated heteropolysaccharide with several major sugars such as galacturonic acids and glucuronic acid [18].

It has been suggested that algal polysaccharide with higher uronic acid content positively showed antioxidant activity [19]. Even a small uronic acid content of 1.1–1.42% reported in *Codium fragile* enhanced its immunomodulatory activity [10]. The presence of uronic acid in the SP fractions of *C. edule* may have contributed to the observed antioxidant effect.

Functional group identification using FT-IR of crude SP and SP fractions
FT-IR was conducted to determine the functional groups present in *C. edule* CSP and SP fractions (F1, F2, and F3). The spectra of CSP and the overlay spectra of SP fractions (F1, F2, and F3) between the ranges of 400 cm⁻¹ and 4000 cm⁻¹ are shown in Figs. 2 and 3. The broadband around 3400 cm⁻¹ indicates the presence of hydroxyl stretching vibration of polysaccharide (–OH) and a band at 2922 cm⁻¹ suggests a C-H stretch. 1658 cm⁻¹ may be attributed to the C=N group. Peak around 1259 cm⁻¹ is a characteristic band for S=O sulfate ester. The band around 1022 cm⁻¹ indicates D-glucose while 1259 cm⁻¹ corresponds to ester sulfate groups [20]. These results suggest that CSP of *C. edule* has glucose and ester sulfate group, which are characteristic components of SPs in seaweeds.

Cytotoxicity test using MTT assay against MCF-7
The highest inhibition against MCF-7 is 81.33% by CSP followed by 68.74% by F1 at 20.8 µg/ml (Fig. 4). The computed IC₅₀ values of 11.13 µg/ml.
5.54 µg/ml, 9.52 µg/ml, and 10.01 µg/ml were established for *C. edule* CSP, F1, F2, and F3, respectively, after 24 h exposure time.

There is a significant difference in the mean percent inhibition against MCF-7 between CSP and F3 (p≤0.001), F1 and F3 (p<0.001), F2 and doxorubicin (p<0.025), and F3 and doxorubicin (p≤0.000). In addition, there is no significant difference in the mean percent inhibition between the doxorubicin and the CSP and F1 (p>0.05). Hence, they have comparable cancer cell growth inhibition activity with the standard drug, doxorubicin. The decrease in cell viability shows that the CSP and SP fractions (F1, F2, and F3) are toxic to the cancer cell, thereby impeding its growth. Thus, *C. edule* CSP and SP fractions (F1, F2, and F3) have a cytotoxic activity against MCF-7 cancer cell.

**Cytotoxicity test using MTT assay against HDF**

The activity of *C. edule* CSP and SP fractions (F1, F2, and F3) against normal cells, HDF, is illustrated in Fig. 5, wherein *C. edule* CSP and SP fractions exhibited proliferation of HDF. Doxorubicin causes cytotoxicity against the normal cell, HDF, while the CSP has a proliferative effect on HDF as it increases the growth of normal cells. The trend for the activity on HDF in increasing percent cell viability is doxorubicin < SP F3 < SP F2 < SP F1 < crude SP.

**Caspase-Glo 3/7 assay**

F1 was evaluated for apoptotic assay because it has the lowest IC<sub>50</sub> among the polysaccharide fractions. A significantly lower mean luminescence was observed for cells treated with 250 µg/ml which may be due to possible necrosis or death of some cells. At 125 µg/ml and 62.5 µg/ml, consecutively, there was a significant increase in the mean luminescence which may indicate an increase in apoptotic activity (Fig. 6). There is, however, a significant decrease in the activity at 31.25 µg/ml and 15.625 µg/ml that may indicate that at lower concentration, the extract is not cytotoxic, thus a decrease in apoptotic activity. Furthermore, the maximum apoptotic activity of the F1 of *C. edule* CSP and SP fractions (F1, F2, and F3) have a cytotoxic activity against MCF-7 cancer cell.
**edule** was achieved at 62.5 µg/ml concentration. There was a significant decrease in the mean luminescence of the sample for each time period. Hence, the apoptotic activity of the sample is time dependent (Fig. 7).

**Annexin V FITC fluorescence microscopy assay**

Treatment of MCF-7 with F1 (15.625 µg/ml) showed an intense green fluorescence by fluorescent microscope after 24 h and 48 h of treatment. An increase number of fluorescent cells after 48 h of treatment were observed compared with 24 h of untreated time (Fig. 8). F1 undergoes the initial phase of apoptosis which is the translocation of PS. Since during apoptosis, the plasma membrane integrity is lost; thus, the Annexin V could bind to the exposed PS and would emit fluorescence using the FITC dye.

**CONCLUSION**

The study revealed that the SP from green seaweed, *C. edule* showed chemical contents that are in good agreement with SP extracted from other green algae. CSP and SP fractions displayed antiproliferative effect on cancer cell line, human breast adenocarcinoma (MCF-7) but not with HDF cells. F1 of *C. edule* has the lowest IC50 of 5.54 µg/ml and induces apoptosis of MCF-7 by altering its membrane integrity and at 62.5 µg/ml concentration promoted maximum caspase 3/7 activity. These results strongly suggest the potential of *C. edule* as sources of bioactive polysaccharides with notable cytotoxic and apoptotic activities.

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**CONFLICTS OF INTEREST**

All authors have none to declare.

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