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Original Article

Changes in rats' breast tumor ultrastructure and immune and messenger RNA responses caused by dietary Seaweed (*Kappaphycus alvarezii*) extract

Nurul'Ain Abu Bakar[a], Tengku Azmi Tengku Ibrahim[a,b], Noraijratul Asikin Mohamad Shalan[a], Suhaila Mohamed[a,*]

[a] *Institute of Bioscience, Universiti Putra Malaysia, Seri Kembangan, Malaysia*
[b] *Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Seri Kembangan, Malaysia*

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ABSTRACT

The edible red seaweed *Kappaphycus alvarezii* or *Eucheuma cottonii* is commercially cultivated in the pristine tropical seas for carrageenan production. The systemic, cellular, and molecular effects of *E. cottonii* 50% alcohol extract [seaweed *E. cottonii* ethanol extract (SECE)] on breast cancer were investigated in a rat model. Mammary tumor was induced by subcutaneously injecting LA7 cells in female rat mammary pads. After 2 weeks of cancer growth, the rats received oral administration of either SECE [150 mg/kg body weight (BW) and 300 mg/kg BW] or tamoxifen. Electron microscopy imaging results confirmed macrophage activity and hematoxylin and eosin staining indicated that tumor histopathological alterations were restored toward normal structures by the seaweed extract. The extract suppressed tumor development and modulated the immune responses. This was evidenced by the microscopic observations, the increased spleen weight, size, spleen CD19 B cells, and blood immunoglobulin G (IgG) levels. The extract also increased the circulating total white blood cells, lymphocytes, segmented neutrophils count, T cells (CD3), T-helper cells (CD4), cytotoxic T cell (CD8), and nuclear factor-kappa beta expressions. The extract enhanced cancer cell death, by upregulating the Birc5, Chk1, and p53 levels and downregulating the tumor growth cellular Mdm2 (transformed mouse 3T3 cell double minute 2) messenger RNA (mRNA) expression. The extract showed no toxicity at 150 mg/kg BW in rats. The lectin-rich SECE showed tumor suppression by enhancing immune responses and upregulating the cancer cell apoptosis mRNA expressions.

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1. Introduction

Breast cancer (BC) is the leading cause of cancer deaths (14% of all cancer deaths) in women worldwide. Populations with typical East-Asian diets have lower incidence of chronic inflammation-related cancers than populations consuming Western diets. These low rates may partly be attributed to their high intake of soy, green tea, and possibly seaweed [1]. BC survivors are the highest users of alternative, dietary, and natural remedies [2], to inhibit, delay, or reverse BC progression. Many of these remedies interfere with signal transduction regulation at different levels, and perform the

* Corresponding author. Institute of Bioscience, Lebuh Silikon, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.
E-mail address: mohamed.suhaila@gmail.com (S. Mohamed).

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following functions: decrease inflammation, modulate immune responses, inhibit oncogenes and activate tumor suppressor genes, induce terminal differentiation, activate apoptosis (cancer cell death), and inhibit angiogenesis [3].

Consumption of seaweeds may be linked to reduced BC prevalence in East-Asian countries, such as Korea, Japan, and parts of China [4]. Seaweed *Eucheuma cottonii* ethanol extract (SECE) was demonstrated to be more effective than tamoxifen in suppressing tumor growth and improving the oxidative status (malondialdehyde concentrations, superoxide dismutase activity, and erythrocyte glutathione concentrations) of various tissues (plasma, liver, and kidney). Unlike tamoxifen, SECE showed little toxicity to the liver and kidneys [5].

LA7 is a mammary adenocarcinoma cell line from 7,12-dimethylbenz[a]anthracene-induced BC rats. LA7 exhibits stem cell properties of self-renewal as well as the capacity to differentiate into all of the cell lineages of the mammary gland. Injection of cultured LA7 cells into rodent breast tissues can induce aggressive mammary carcinoma [1]. Exposure of LA7 cells to lactogenic hormones, lipids, or differentiating agent results in the formation of cells of the ductal epithelial, myoepithelial, and alveolar lineages.

E. cottonii extracts exert antioxidant, antiestrogenic, and antiproliferative properties against mammary gland tumors in rats [1,5,6]. In this study, we compared the ultrastructural changes in breast tumor tissues of LA7-induced cancer rats caused by treatment with dietary seaweed extract and tamoxifen, to evaluate the anti-BC properties of SECE, and the underlying cell death mechanisms in breast tumors. The study also demonstrates the effects of dietary SECE on immune responses and messenger RNA (mRNA) expression (cellular and molecular mechanisms) by which it fights breast tumor growth in a rat model.

2. Materials and methods

2.1. Seaweed E. cottonii extract preparation

Cultivated *E. cottonii* was obtained from the Malaysian Fishery Development Board, North East Borneo (Sabah), Malaysia, and the specimens were identified by Borneo Marine Research Institute. Algae are harvested after 45 days of planting, washed with fresh water, and oven dried. The equatorial waters do not have any season, and so seaweeds are grown all year-round. The whole cultivated seaweeds are used. The dried, milled seaweeds were weighed and soaked in 50% methanol (1:10) for 24 hours, filtered through Whatman No. 1 paper, and rotary evaporated at 40 °C to a light yellowish paste, flushed with liquid nitrogen, and stored at –20 °C in dark bottles until ready for use.

2.2. Animal study

Seven-week-old female Sprague-Dawley rats weighing between 180 g and 200 g were purchased from Chenur supplier (Serdang, Selangor, Malaysia). The animals were maintained in groups of three/cage at 22–25 °C, with 12-hour light/dark cycle, at 80% relative humidity and fed with standard chow pellets (Gold Coins, Klang, Selangor, Malaysia) and water *ad libitum*. The animals were randomly divided into the following five groups ($n = 6$/group): (1) normal control group, control; (2) BC-induced rats without treatment; (3) BC rats treated with 150 mg SECE/kg body weight (BW), BC + 150; (4) BC rats treated with 300 mg SECE/kg BW, BC + 300; and (5) BC rats treated with 10 mg/kg BW tamoxifen, BC + 10TAM. The SECE paste was dissolved in distilled water and delivered to the rats by oral gavage once a day starting from Week 6 to Week 10. The extract dose used is equivalent to a daily consumption of 10–20-g dried seaweeds for a 60-kg human based on Food and Drug Administration guidelines for "estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers" (http://www.fda.gov/downloads/Drugs/Guidances/UCM078932.pdf, date last accessed 2015). The research was approved by the University Animal Care and Use Committee (UPM/FPSK/PADS/BR-UUH/00411), Serdang, Selangor, Malaysia. The rats were exsanguinated under ketamine–ilium xylazil-20 prior to terminal blood collection by cardiac puncture and organ removal.

2.3. LA7-induced rat mammary tumor model

Roswell Park Memorial Institute-1640 (RPMI-1640) growth medium, L-glutamine, fetal bovine serum (FBS), trypsin–EDTA, streptomycin and penicillin, and phosphate-buffered saline (PBS) were obtained from Invitrogen Corporation (Burlington, ON, Canada). The LA7 cells (also called CRL 2283, a rat mammary gland tumor cell line with stem cell characteristics) were kindly provided by Dr Ahmad Bustaman Abdul (IBS-UPM), and maintained in RPMI-1640 medium supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 IU/mL penicillin [1]. Cells were inoculated subcutaneously into the mammary gland (right flank) of female Sprague Dawley rats with 200 μL of 6×10^7 cells using a 26-G needle. Rats were palpated weekly to monitor tumor development. The tumor diameters were measured with calipers and its volume was calculated as follows: [largest diameter × (smallest diameter)^2]/2.

Blood samples were obtained from unfasted rats via cardiac puncture under anesthesia (50 mg ketamine/kg, 5 mg xylazine/kg) [7]. Immunofluorescence staining for estimating the percentage of leukocytes carrying the following markers was performed using monoclonal antibodies (MAbs; Becton Dickinson Biosciences, Damansara Heights, Kuala Lumpur, Malaysia): CD3, CD4, and CD8. Leukocytes were labeled with the following fluorochrome-conjugated antirat MAbs: CD3 fluorescein isothiocyanate, CD4 allophycocyanin, and CD8 peridinin chlorophyll. Red blood cells were lysed in FACS lysing buffer (Becton Dickinson Biosciences) and 10,000 events were analyzed in a FACSCalibur flow cytometer (Becton Dickinson Biosciences, Kuala Lumpur, Malaysia) with an argon source for excitation. Data acquisition was done by CellQuest Pro software (Becton Dickinson Biosciences, Kuala Lumpur, Malaysia). The plasma was collected in a heparinized tube and then centrifuged at 4000 *g* at 4 °C for 15 minutes, and stored at –80 °C. Blood serum rat immunoglobulin G (IgG) was assayed using an enzyme-linked immunosorbent assay kit

(Immunology Consultants Laboratory, Inc., Portland, OR, USA).

Tumor tissues were flash frozen in liquid nitrogen (MOX-Linde Gases Sdn. Bhd., Petaling Jaya, Selangor, Malaysia) and stored at −80 °C. RNA extraction from the tissues of each group was performed according to the standard protocol of Real Genomics Total RNA extraction kit (RBC Biosciences, Taipei, Taiwan). The tissues were excised, weighed (not more than 30 mg), and placed directly into a 1.5-mL tube with Buffer RLT Plus for disruption and homogenization. The lysates were centrifuged for 3 minutes at maximum speed. The supernatants were removed and transferred to spin columns placed in 2-mL collection tubes. The tubes were centrifuged and the flow-through was saved. To the flow-through, 600 μL of 70% ethanol was added and the constituents were mixed well by pipetting. The samples were transferred into RNeasy spin columns placed in 2-mL collection tubes and centrifuged. The flow-through was discarded. Buffer RW1 and Buffer RPE were added subsequently and centrifuged again to wash the membrane of the spin columns. The RNeasy spin columns were placed in a new 1.5-mL collection tube and RNeasy-free water was added directly to the spin columns membrane and centrifuged to elute the RNA. The extracted RNA was only used when it had absorbance readings for $A_{260/230}$ and $A_{260/280}$ falling between 1.8 and 2.0, with distinct 28 s and 18 s bands on gel electrophoresis. RNA (50 ng) samples were reverse-transcribed using multiplex universal reverse primers from Rat Multitox Plex kit purchased from Beckman Coulter Inc. (Fullerton, Ca, USA). Briefly, reverse primers of different genes were mixed together to form the reverse primer mix. RNA (300 ng) was then mixed with 1 μL of reverse transcriptase, 4000 ng of each primer, 4 μL of 5× reverse transcription buffer, and 9 μL of water. The total volume of this mixture was 20 μL. Reverse transcription-polymerase chain reaction (PCR) was run using the conditions specified in the manual. After reverse transcription, a second PCR process was carried out to amplify the amount of complementary DNA (cDNA). Magnesium chloride (4 μL) was mixed with 4 μL of 5× PCR buffer, 0.7 μL of *Taq* polymerase, 2 μL of forward primer mixture (containing 200 ng/μL of each forward primer), and 9.3 μL of cDNA. PCR was run under conditions specified in the manual. Finally, 2 μL of the PCR product was mixed with 38.5 μL of the sample loading solution and 0.5 μL of the size standard solution and genetic analysis was performed on the GeXP machine (S. Kraemer Boulevard, Brea, CA, USA) [8]. The data were initially analyzed using the Fragment Analysis module of the GeXP system software, and then imported into the analysis module of eXpress Profiler software. Data were normalized using beta actin as the reference gene. The GenomeLab GeXP genetic analysis system was used for analyzing gene expressions. It uses a combined gene-specific, universal priming strategy that converts multiplexed PCR into a two-primer process using universal primers. The gene ratio in RNA samples was maintained during the PCR process.

Rattus norvegicus gene sequences from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/nucleotide/) were used to design primers on GenomeLab eXpress Profiler software for rat gene expression studies. *Actin B* was used as housekeeping gene and *KanR* as an internal control (*KanR*). Primers were purchased from MyTACG Bioscience Enterprise (Kajang, Selangor, Malaysia). The selected genes were as follows:

- *Inflammation/cell survival: nuclear factor-kappa beta (NF-κB)* [*XM_342346*].
- *B-cell receptor signaling: Cd19 [NM_001013237*].
- *Antiapoptosis: BCL2-associated athanogene (Bag1) [NM_001106647]*; mouse double minute 2 homolog (*Mdm2*) [*NM_001108099*]; baculoviral IAP repeat-containing 5 (*Birc5*) [*NM_022274*].
- *Cell cycle arrest/Proapoptosis:* checkpoint kinase 1 homolog (*Chk1*) [*NM_080400*]; Tumor protein p53 (*p53*) [*NM_030989*].
- *Housekeeping: Actin B [NM_031144*].

2.4. Histopathology evaluation

Fresh tumor tissues were fixed with wax, deparaffinized with xylene, and rehydrated with 100%, 95%, and 80% ethanol and deionized water sequentially. While sections were in water, the surface of the slides was wiped to remove oxidized particles. The slides were stained with Harris hematoxylin, which consists of a dye (oxidized hematoxylin or hematein) and a mordant or binding agent (an aluminum salt) in solution. Initially, this stained the nuclei and some other elements reddish purple. After rinsing in tap water, samples were destained with acid ethanol to remove nonspecific background staining and to improve contrast. After this treatment, thorough rinsing was performed. The sections were then stained with eosin and dehydrated with 95% ethanol, 100% ethanol, and xylene. A drop of Permount (xylene based) was placed on the slide, and then the section was covered with a glass cover slip. Five random sections from each slide were viewed at 40× and 100× magnifications. Eosinophils and neutrophils in the tumor tissues were counted as numerical average calculated from 10 randomly selected fields from each group (400×).

2.5. Immunohistochemistry evaluation

The immunohistochemistry kits (ChemMate DAKO EnVision Detection Kit, peroxidase/3,3′-diaminobenzidine, rabbit/mouse) were purchased from DAKO (Denmark). The tumor and liver tissues were embedded in paraffin blocks and cut into 4-μm sections using a microtome, deparaffinized, and successively incubated in xylene, absolute ethanol, and 90%, 70%, and 50% ethanol (for 3 min in each solvent). They were then treated with sodium citrate (pH 6) and boiled for 8 minutes for demasking. The slides were rinsed with washing buffer (prepared by dissolving 5 PBS tablets in 1 L distilled water and then adding 1 mL of Tween-20 to the prepared solution), followed by blocking with 300 μL of H_2O_2 for 5 minutes. Tissue sections were washed two times in PBS and then immunostained with primary antibodies for evaluating interleukin-4 (IL-4), interferon-γ (IFN-γ), and anti-integrin β1 (CD29) antibody EP1041Y expression (Novus; Genomax

Technologies Sdn Bhd, Petaling Jaya, Malaysia) overnight at 4 °C. Subsequently, slides were treated for 1 hour with secondary, goat antimouse/antirabbit antibodies conjugated with horseradish peroxidase, and developed with 3,39-diaminobenzidine. Finally, the tissue sections were rinsed in distilled water, counterstained with Mayer's hematoxylin, and mounted with DPX mounting medium for microscopic examination. The expression of the aforementioned products were evaluated under a microscope (Nikon Eclipse Ti-S, Amsterdam, Netherlands). Immunohistograms were taken with a phase contrast microscope (Leica DM-LB2, Solms, Germany).

Morphological alterations induced by SECE were examined using transmission electron microscope. Tumor samples (about 1–1.5 mm^3) were washed in phosphate buffer and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 16 hours. After three buffer washes, the samples were postfixed in 1% osmium tetroxide and dehydrated in graded series of dilutions of acetone (30%, 50%, 70%, 90%, 95%, and 100%). Samples were infiltrated overnight with a 50:50 mixture of acetone and resin, then infiltrated for 10 hours with 100% resin, and subsequently dropped into prefilled resin, labeled beam capsules, and polymerized at 60 °C in an oven for 16 hours. Tissues blocks were sectioned using the ultramicrotome to obtain 1-Å thick sections. Ultrathin sections on copper grids were stained with uranyl acetate and lead citrate and examined under a Hitachi H-7100 transmission electron microscope, Tokyo, Japan (TEM).

2.6. Statistical analysis

All data were expressed as means ± standard deviation of at least three independent experiments. One-way analysis of variance, using Statistical Package for the Social Sciences software version 22.0 for Windows (SPSS Inc., Chicago, IL, USA), was used to test for differences between the groups. Differences at a $p < 0.05$ significance level were identified by the Duncan *post hoc* test.

3. Results

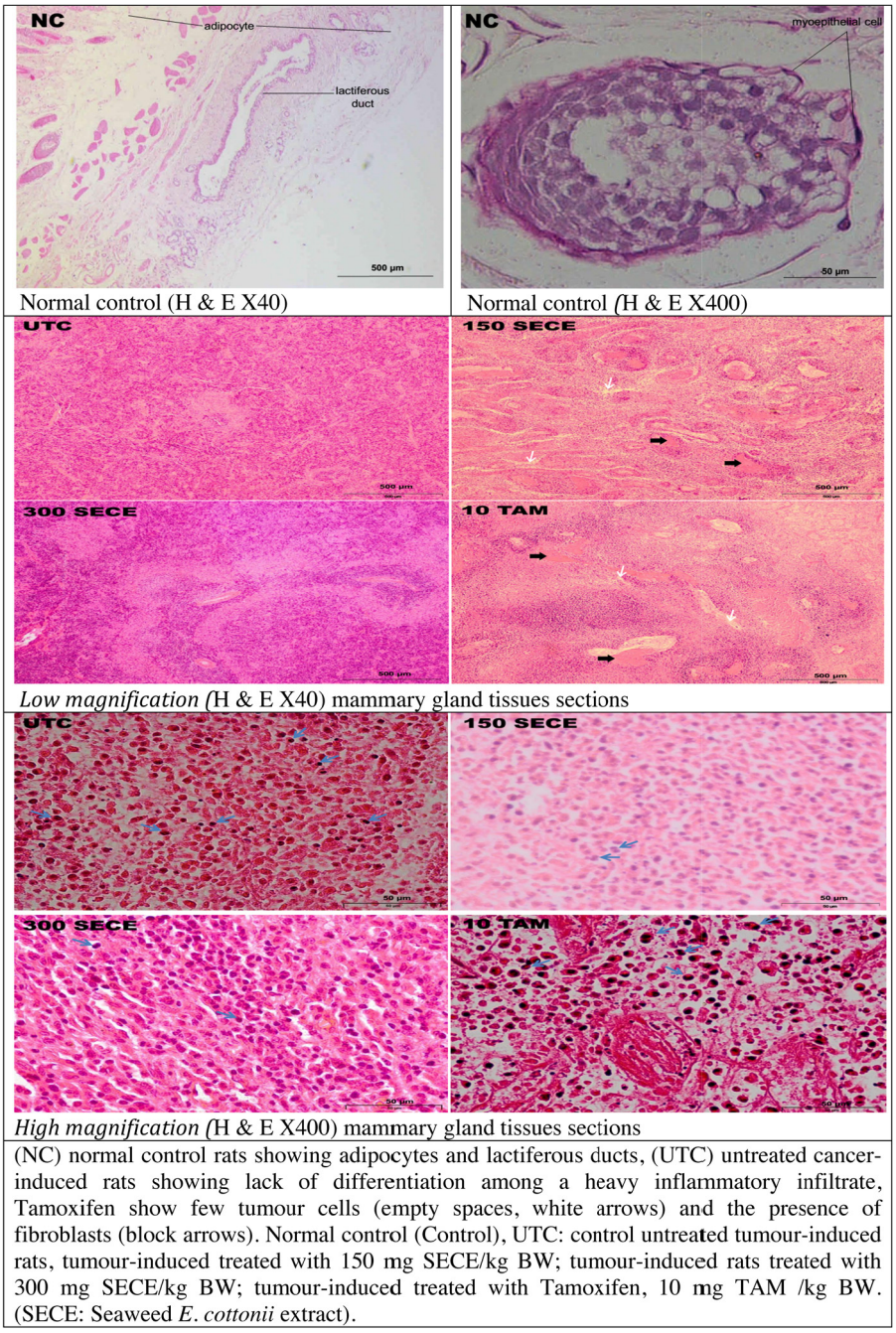
While there were no visible mammary tumors in the normal control group, macroscopic tumors of various sizes were visible in the mammary glands tissues of the tumor-induced groups. The tumor induction by LA7 produced fibro-adenocarcinoma characterized by heterogeneous tumor, typical appearance of neoplastic cell with enlarged nucleus, high mitotic activity with diversity in size and shape of the cell nucleus (nuclear pleomorphic), and hyperchromatin structures (excessive pigmentation) as evidenced from hematoxylin and eosin staining of tumor tissue (Figure 1). A low dose (150 mg/kg BW) of SECE substantially improved the cellular tumor tissue architecture together with the presence of fibroblasts (normal mammary stroma) and significant empty space, which was better than that noted in the tamoxifen (10 mg/kg)-treated group (Figure 1).

3.1. Electron microscopy observations

The normal mammary gland cells (majority) were characterized by large centrally located nuclei (Figure 2, "N"). The cell cytoplasm was largely filled with rough endoplasmic reticulum (rER), a few mitochondria, and electron-dense granules interspersed in between the rER. Three cell types were identified in the breast tumors: (1) mammary gland-like cancer cells, (2) cancer cells, and (3) macrophages. Some of the mammary gland-like cancer cells were normal as in the negative control samples, whereas others were at different stages of degeneration. There were cancer cells (Figure 2A) and cells with swollen rER (Figure 2B), whereas others exhibited shrunken, irregular shaped nucleus with marked peripheral nuclear chromatin condensation. Apart from mitochondria, which appeared lysed (no cristae), a number of electron-dense granules of different sizes were present in the cytoplasm (Figure 2C). An advanced stage cell degeneration appeared in the form of a cell with completely lysed organelles containing nuclear remnants (Figure 2D). The breast tumor contained cancer cells, which exhibited large, centrally located nuclei, whereas their cytoplasm appeared pale being devoid of the normal cytoplasmic organelles except for ribosomes and a few mitochondria (Figure 2E). The macrophages present in the breast tumor exhibited the typical horseshoe-shaped, eccentrically located nuclei with extensive cytoplasmic projections on their cell surface (Figure 2F). The cytoplasmic organelles observed included the Golgi apparatus, mitochondria, rER, and electron-dense granules.

After treatment with SECE, the tumor-induced rats showed active immune cell activity. Besides, monocytes and macrophages were observable in these tumors under a TEM (Figure 3). Breast tumors from rats treated with 150 mg extract/kg BW showed mammary gland-like cancer cells, cancer cells, and macrophages, which exhibited some degenerative changes such as marked peripheral nuclear chromatin condensation (Figure 4A). Other degenerated cancer cells displayed electron-dense bodies and nuclear remnants in the cytoplasm (Figure 4B). The degenerating nuclei of cancer cells in the seaweed-treated groups were surrounded by highly vacuolated cytoplasm, which was not present in the other groups (Figure 4C). Electron opaque granules were present among the vacuoles and bordered each vacuole. The macrophages in these tissues have vacuolated cytoplasm and were degenerated (Figure 4D). The degree of degeneration was dose dependent (Figures 4E and 4F). However, at 300 mg/kg dose, no macrophage was detected. tamoxifen also caused cancer cell degeneration in the rats' breast tumors; the earliest changes were characterized by the electron opaque granules and mitochondrial cristae lysis before any alterations in the cell nucleus (Figure 4G). Further cell degeneration revealed increased large irregular shaped electron-dense/opaque granules (Figure 4H), without significant degenerative changes in the cells nuclei. Advanced degenerated cancer cells have marked peripheral nuclear condensation with the absence of macrophage.

In the extract-treated groups, a dose dependent increase in size and weight of the spleen ($p < 0.05$) was observed



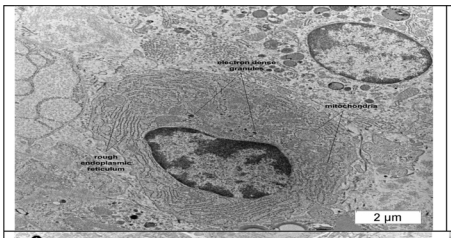
(NC) normal control rats showing adipocytes and lactiferous ducts, (UTC) untreated cancer-induced rats showing lack of differentiation among a heavy inflammatory infiltrate, Tamoxifen show few tumour cells (empty spaces, white arrows) and the presence of fibroblasts (block arrows). Normal control (Control), UTC: control untreated tumour-induced rats, tumour-induced treated with 150 mg SECE/kg BW; tumour-induced rats treated with 300 mg SECE/kg BW; tumour-induced treated with Tamoxifen, 10 mg TAM /kg BW. (SECE: Seaweed *E. cottonii* extract).

Fig. 1. Low (H&E 40×) and high magnification (H&E 400×) images of rats' breast tissues. H&E = hematoxylin and eosin.

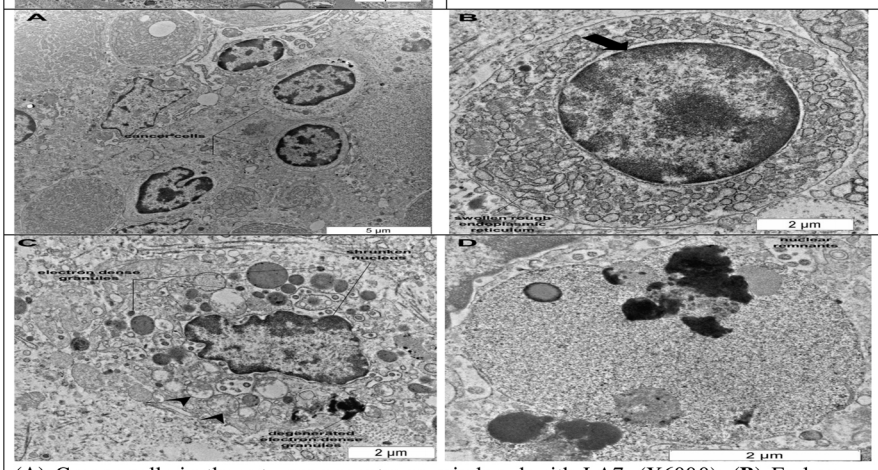
when compared with the untreated breast tumor group (Figures 5A and 5B). There was also a significant increase of NF-κB and CD19 expression ($p < 0.05$) following treatment with both SECE and tamoxifen (Figure 5C).

The total white blood cells (TWBCs); lymphocytes; segmented neutrophils; percentages of CD3, CD4, and CD8; and serum IgG levels were minimal in the untreated cancer control, and SECE dose dependently increased them. Interestingly, the TWBC, lymphocytes, and segmented neutrophils from the high-dose (300 mg/kg) SECE-treated LA7 tumor-induced group showed strikingly greater number of cells as compared with those from the normal control group (Figure 5). The CD19 expressions were also significantly ($p < 0.05$) increased by both SECE and tamoxifen administration.

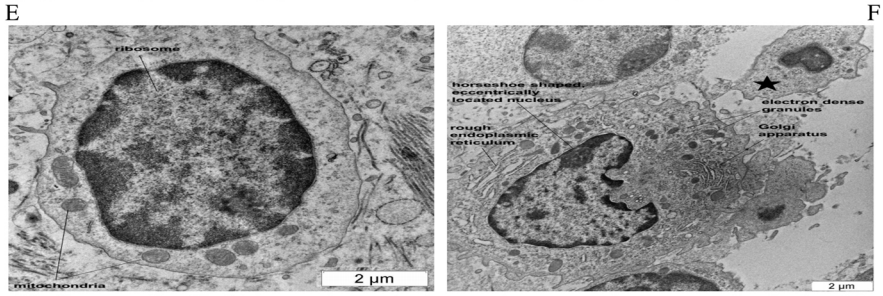
The expressions of cell death-related mRNA, namely, *Birc5, Chk1, p53, Bag1*, and *Mdm2*, in mammary tumor sections were analyzed for the possible mechanisms triggered by SECE. The mRNA levels of *Bag1* and *Chk1* were not significantly different between the tumor-induced animals



(N) Electron micrograph of Control Normal rat's mammary gland showing nuclei with marginated heterochromatin appearing as dense line along the nuclear envelope of rat. Note also a few spherical mitochondria, rough endoplasmic reticulum dispersed within the cytoplasm. (X8000).



(A) Cancer cells in the rat mammary tumour induced with LA7, (X6000). **(B)** Early cancer cells degeneration showing peripheral chromatin condensation (big arrow) & numerous swollen rough endoplasmic reticulum, (X10000). **(C)** Degenerated cancer cell with lysed cytoplasm; shrunken, irregular shaped nucleus with marked peripheral chromatin condensation; presence of electron dense granules and lysed cristae of mitochondria (arrowhead), (X9000). **(D)** Advanced stage completely degenerated cancer cell with lysed cytoplasmic organelles & apoptotic nucleus (nuclear remnants), (X17000).



(E) Cancer cell with high protein synthesis contains many ribosomes and mitochondria, (X14000); **(F)** Macrophage characterised by horseshoe shaped nucleus, extensive cytoplasmic projections containing an engulfed dense body (star), marked endoplasmic reticulum and Golgi apparatus, (X8000).

Fig. 2. Transmission electron microscopy photomicrograph of rats' normal (O) and breast tumor cancer cells induced by LA7 at various degeneration stages showing lysed cytoplasmic organelles.

and those in the normal control group. Tumor induction caused an increase in the expressions of *Birc5, p53*, and *Mdm2* in the tumor tissues. The two doses of SECE dose dependently ($p < 0.05$) increased the *Chk1* and *p53* expression levels in the tumor-induced rats. High dose of SECE additionally increased *Birc5* and *Bag1* with relatively low *Mdm2* expressions. Tamoxifen significantly increased *p53* expression but not the *Chk1* level. There was a significant ($p < 0.05$) reduction in Mdm2 levels in the tumors from rats treated with a low dose of SECE (Figure 6). The *NF-κB* expressions were also significantly ($p < 0.05$) increased by doses of both SECE and tamoxifen. Immunohistochemical analysis showed that the untreated control tumors exhibited significant IL-4 increase that attracts the macrophages (Figure 6A). The seaweed treatment dose-dependently reduced IL-4 (Figure 6B) and increased IFN-γ levels.

4. Discussion

The yield extract was 10.1% of dried sample; 100 g of dried sample contained 49 mg of total phenolic content (gallic acid equivalent) and 15 mg of total flavonoid content (catechin) equivalents [9], and the sample was used

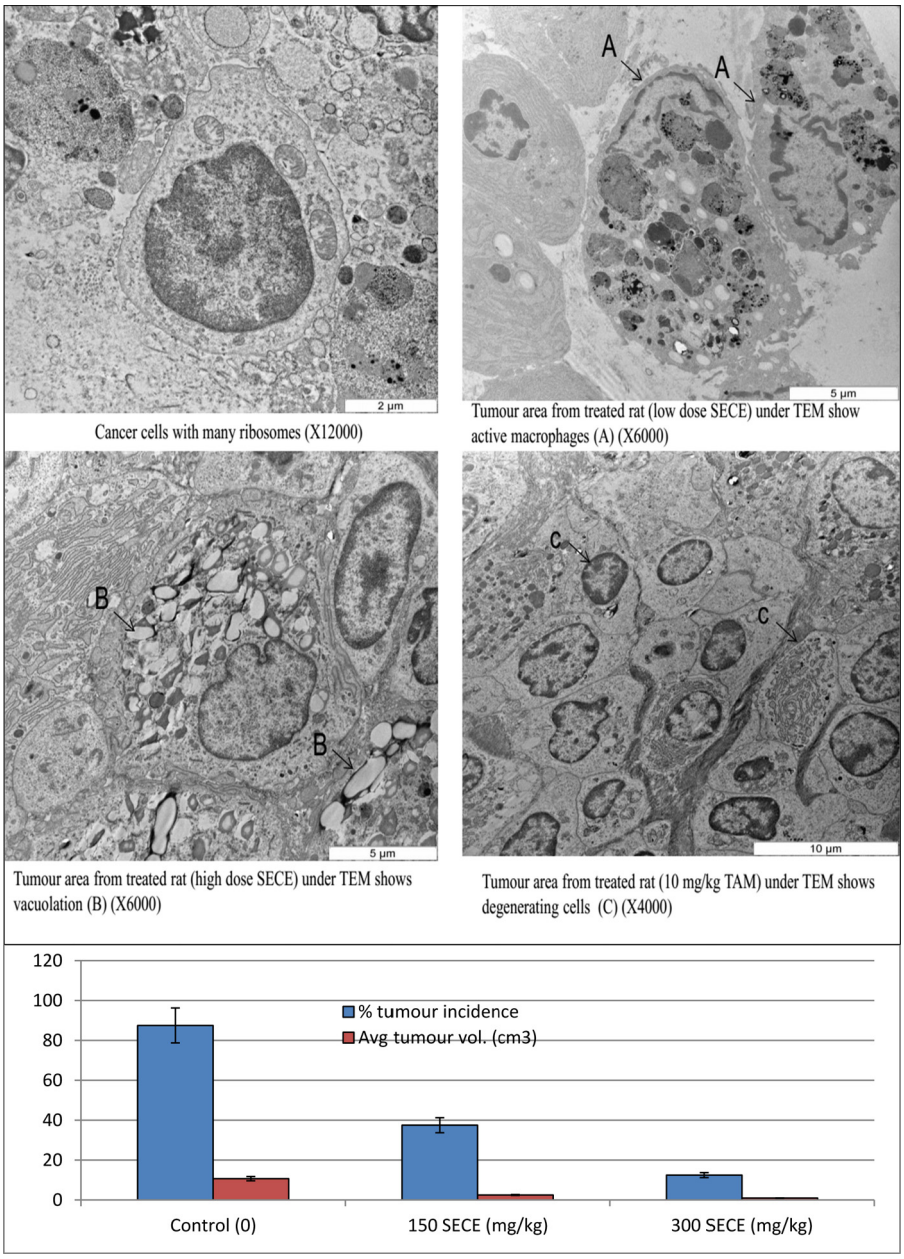


Fig. 3. Transmission electron microscopy (TEM) photomicrograph of the macrophage activities in the rat breast tumor tissues after 4-week treatment and percentage of tumor incidence and average tumor volumes. SECE = seaweed *Eucheuma cottonii* ethanol extract.

within a month. Methanol extract demonstrated a higher level of antioxidant activity, and may contain more polar, semipolar, and nonpolar compounds compared with aqueous and ethanol extracts [10]. The aqueous methanolic extract was found to be practically nontoxic and its LD_{50} could not be calculated because the treatment did not cause any death response [11,12]. Fourier transform infrared spectroscopy (Perkin Elmer Spectrum 100 FTIR) identification of the functional group indicated that the main bioactive compound in the extract is lectin peptides [7]. The immunomodulatory effects of the seaweeds are most likely due to lectins or simple peptides [7]. Other seaweed and plant lectins reportedly have good immunomodulatory properties [13].

The ability of the extract to dose-dependently reduce LA7-induced breast tumor incidence and size has been well demonstrated [1,5] and visualized in this study via light and transmission electron microscopy studies. SECE was more effective than tamoxifen for suppressing mammary tumor growth (27%), improving tissues (plasma, liver, and kidney) malondialdehyde levels, superoxide dismutase activity, and erythrocyte glutathione concentration ($p < 0.05$) [5]. SECE displayed little toxicity to the liver and kidneys as compared with tamoxifen. *E. cottonii* contains natural

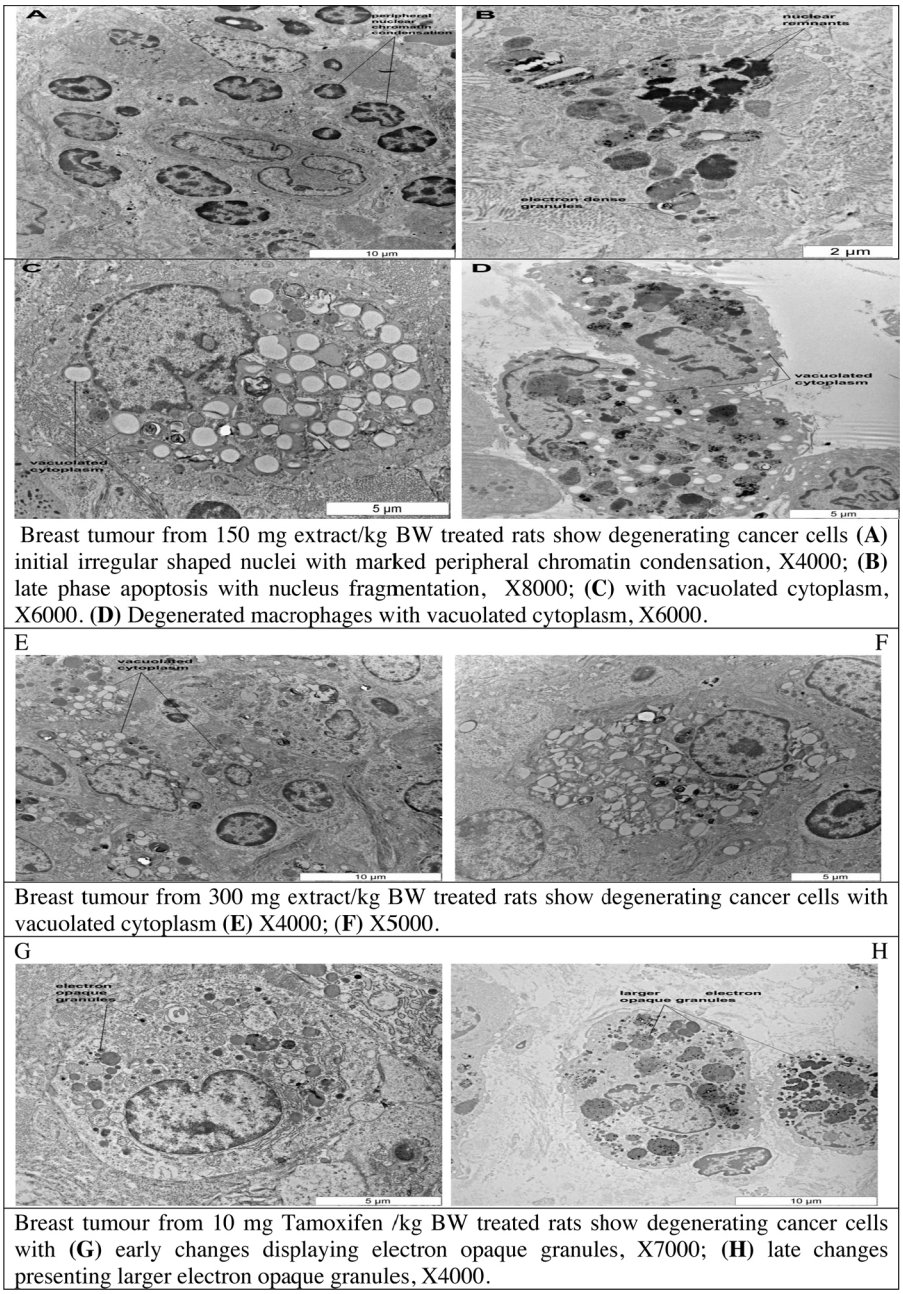
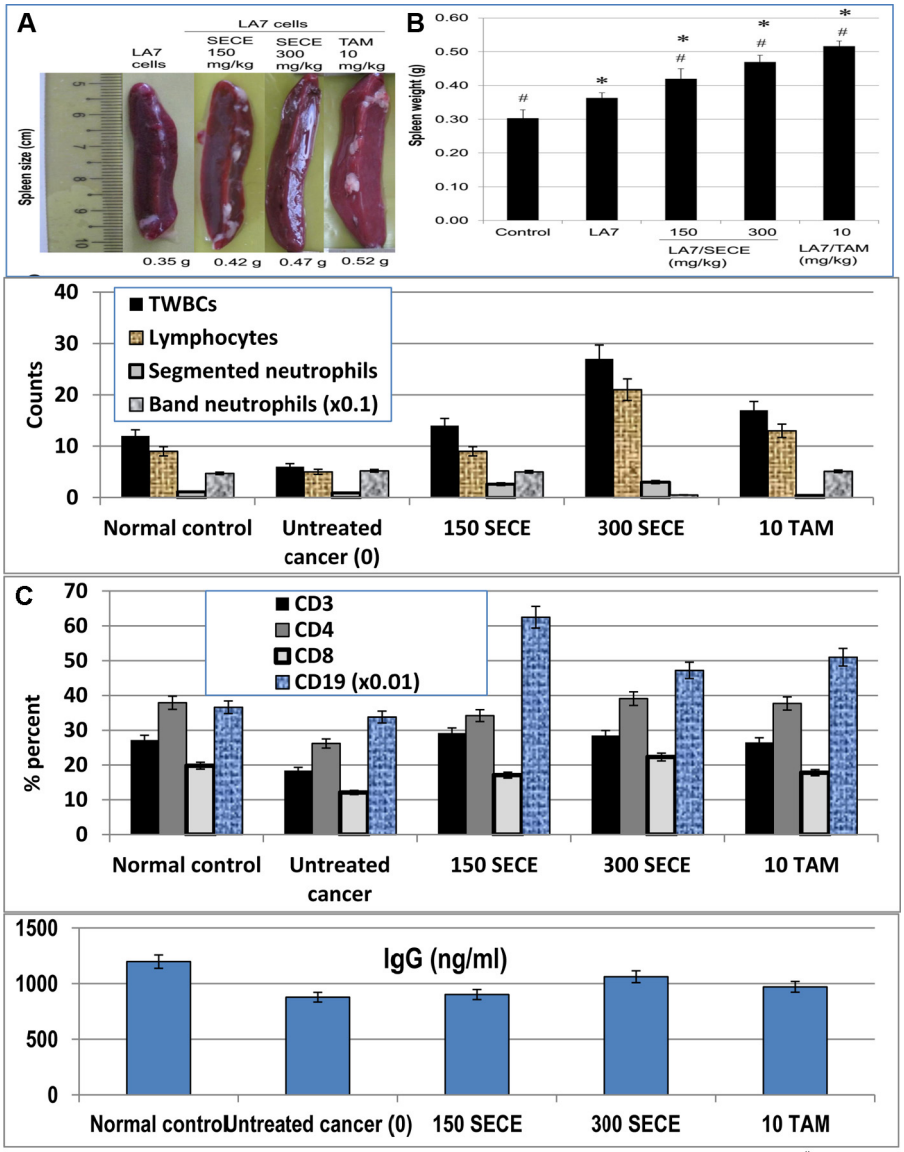


Fig. 4. Transmission electron microscopy ultrastructure of breast tumors in seaweed extract- and tamoxifen-treated rats.

antioxidants, which include flavonoids, carotenoids, and sulfated polysaccharides [9].

TEM studies showed the presence of tissue monocytes and macrophages that are associated with resident dendritic cells (DCs) activation, stimulating their migration to the lymphoid tissue bearing tumor antigens. TEM studies demonstrated phagocytosis by macrophages, which is a major immune response after exposure to tumor-associated antigens on the tumor cells surface. The DCs and macrophages present antigens to the CD4 and CD8 T cells in the lymphoid tissues to activate them. Activated CD4 and CD8 T cells then enter the circulation to destroy the tumor cells [14].

Malignant development of cancer cells is characterized by their independence to growth signals, insensitivity to growth-inhibitory (antigrowth) signals, avoidance of programmed cell death (apoptosis), unrestrained replicative potential, persistent angiogenesis, metastasis, and tissue invasion. The extract restored apoptotic responses of the cells as well as cellular homeostasis to help suppress tumor. SECE caused irreversible cancer cell damage by increasing the expression of p53. In mammalian cells, replication is

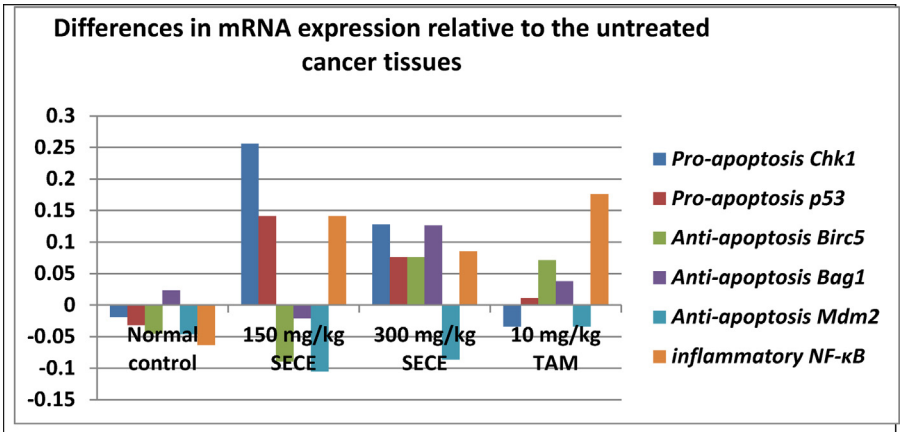


Values are mean ± SD of $n = 3$. $^{*}p < 0.05$ significantly different from Control. $^{\#}p < 0.05$ significantly different from untreated cancer group (Duncan test). Normal control (Control), control untreated tumour-induced rats, tumour-induced treated with 150 mg SECE/kg BW; tumour-induced rats treated with 300 mg SECE/kg BW; tumour-induced treated with Tamoxifen, 10 mg TAM /kg BW. (SECE: Seaweed *E. cottonii* extract).

Fig. 5. Effects of seaweed extract administration on spleen (A) size and (B) weight; circulating white blood cells count and CD3, CD4, CD8, and serum immunoglobulin G (IgG) levels in rats treated with seaweed extract, compared with tamoxifen (TAM). TWBC = total white blood cell.

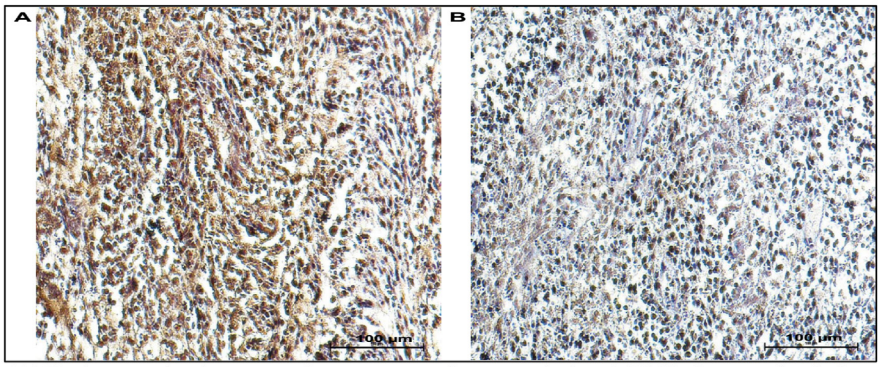
regulated in an orderly fashion from G_1 to S to mitosis by phase-specific oscillations in the level of cyclins, cyclin-dependent protein kinases (CDKs), and CDK inhibitors. Cell cycle progression is accelerated by cyclins and CDKs, and decelerated by CDK inhibitors (such as p16, p21, p27), p53, retinoblastoma tumor suppressor protein (Rb), and by ARF (also known as **p16, cyclin-dependent kinase inhibitor 2A**, **multiple tumor suppressor 1** and as several other synonyms and functions as a tumor suppressor protein). Cell-cycle arrest, apoptosis, and senescence are widely accepted as the major mechanisms by which *p53* inhibits tumor formation. Chk1 can activate the tumor suppressor *p53* directly to induce apoptotic response during various stress [15].

The extract showed dose-dependent response against the tumor growth for some parameters but not for others, possibly due to biphasic antiestrogenic effect at the 300 mg/kg dose. The biphasic (estrogenic and/or antiestrogenic) effects of dietary phytoestrogens (such as kaempferol and other polyphenols) and estrogens in BC cells or other cells have been reported previously [16,17].



Genes mRNA	Pro-cell death		Anti-cell death			inflammatory
Group	*Chk1*	*p53*	*Birc5*	*Bag1*	*Mdm2*	*NF-κB*
Normal control	0.179	$0.015^{\#}$	$0.083^{\#}$	0.224	$0.092^{\#}$	0.059
Untreated	0.198	0.047^{*}	0.128^{*}	0.201	0.138^{*}	**0.122**
150 mg/kg SECE	$0.454^{*\#}$	$0.188^{*\#}$	$0.039^{*\#}$	0.180	$0.033^{*\#}$	**0.263**
300 mg/kg SECE	$0.326^{*\#}$	0.123^{*}	$0.204^{*\#}$	$0.327^{*\#}$	$0.052^{\#}$	**0.207**
10 mg/kg tamoxifen	$0.164^{\#}$	$0.058^{*\#}$	$0.199^{*\#}$	0.239	$0.104^{\#}$	**0.298**

Values are mean ± SD of $n = 3$. $^{*}p < 0.05$ significantly different from Control. $^{\#}p < 0.05$ significantly different from untreated cancer group (Duncan test). Normal control (Control), control untreated tumour-induced rats, tumour-induced treated with 150 mg SECE/kg BW; tumour-induced rats treated with 300 mg SECE/kg BW; tumour-induced treated with Tamoxifen, 10 mg TAM /kg BW. (SECE: Seaweed *E. cottonii* extract).



(A) IL-4 expression in untreated cancer group (brown stains) and **(B)** IL-4 expression in low dose seaweed treated sample, IHC X200.

Fig. 6. Messenger RNA and cytokines expressions in rats' mammary tissues.

The cytoplasm of mammary gland cells is largely occupied by rER, a few ribosomes, and small electron-dense granules. The electron-dense granules are due to free plasma amino acids derived from hydrolysis of circulating short-chain peptides for protein synthesis [18]. The cancer cells were identified by their pale cytoplasm due to the lack of cytoplasmic organelles except for ribosomes and a few mitochondria [19]. Ultrastructure of the cancer cells showed the following characteristics: generally round cells with a centrally placed nucleus, showing indentation and usually containing one or two prominent nucleoli. The large nucleus is about similar in size to the cytoplasm [20]. There were numerous mitochondria, ribosomes, and microfilaments, but the rough and smooth ER showed poor development [21].

The LA7 cells have self-renewal and gland-reconstituting abilities [22], thus numerous mammary gland-like cancer cells were observed. A sequence of degenerative changes could be identified during the process with the earliest change manifested by swelling of the rER followed by nuclear shrinkage and lysis of mitochondrial cristae. In a more advanced degenerated cell, there was nuclear fragmentation with complete lysis of cytoplasmic organelles.

Cytoplasmic vacuolation and electron-opaque granules were the earliest degenerative changes that occurred

before any changes in the cancer cells nuclei. The TEM studies showed how seaweed extracts dose-dependently affect both the standard cancer cells and the mammary gland-like cancer cells in the breast tumor, evidenced by marked peripheral nuclear chromatin condensation, even while the mitochondria remained intact initially.

The emergence of macrophages in the tumor tissues indicated a response to help eliminate the cell debris as a result of cancer cells degeneration. They are known as tumor-associated macrophages (TAMs). They can be differentiated into either proinflammatory M1 macrophages or anti-inflammatory M2 macrophages. M1 macrophages activate and support proinflammatory type 1 helper T cells (Th1), and have the ability to kill pathogens besides being tumoricidal. The M1 phenotypes respond to cytokines such as IFN-γ. The M2 macrophages are weakly tumoricidal, are generally driven by IL-4, and are involved in wound healing where they downregulate the inflammatory reactions, promote angiogenesis, recruit fibroblasts, and regulate connective tissue remodeling [23]. A good correlation exists between high TAM infiltration and poor patient outcome in BC [24]. TAMs in untreated cancer cells are driven by IL-4, whereas in the low-dose seaweed-treated group, it is probably mediated by IFN-γ. The 300-mg/kg seaweed dose and 10-mg/kg tamoxifen dose groups showed no macrophage in the tumor tissues, indicating attenuated macrophage activation and polarization. Macrophages are activated and polarized via specific cytokines and chemokines. TAMs are derived from circulating monocytes or resident tissue macrophages, which form the major leukocyte infiltrate found within the BC stroma. TAMs enhanced tumor progression by promoting tumor invasion, migration, and angiogenesis [23]. Apparently, ingesting a moderate dose of seaweed extract helped attenuate TAM M2 and increased TAM M1.

The seaweed extract not only induced degenerative cytoplasmic vacuolation in the cancer cells but also cytoplasmic vacuolation in the macrophages (potentially M1 TAM). The presence of highly vacuolated cells suggests the occurrence of Type II nonapoptotic programmed cell death [25]. The tamoxifen treatment also produced degenerative changes in the tumor cells, the most prominent being the occurrence of slightly more electron-dense lipid droplets that did not become vacuolated but instead coalesced to form large irregular shaped droplets [26].

SECE administration significantly ameliorated cancer-related tissue oxidative stress, demonstrated by the improved tumor tissue levels of malondialdehyde and erythrocyte glutathione and superoxide dismutase activity [5]. Seaweed consumption has been shown to decrease estradiol levels and increase the endogenous antioxidant enzymes, superoxide dismutase, glutathione peroxidase, and sometimes catalase activities *in vivo*, while prolonging the estrous cycle [1].

Tamoxifen was a good positive control because of its antibreast tumor, antiproliferative, proapoptosis, and antiestrogenic properties (blocking the intracellular estrogen receptor, blocking the action of steroid hormone, and inhibiting protein kinase C activity and its binding to calmodulin) [27]. However, LA7 cells may not respond to tamoxifen well because they are cancer stem cells that are highly tumorigenic, multidrug resistance, and can differentiate into various cell phenotypes of the parental tumor. Tamoxifen treatment significantly increased the spleen-reactive follicular hyperplasia and spleen weight.

The ability of the extract to promote immune responses was evidenced by the increase in TWBC, lymphocytes, segmented neutrophils, CD3, CD4, CD8, and IgG levels in tumor-induced rats (Figure 5), influencing the innate immunity, and thus behaving as an immune-stimulating agent. The extract increased the systemic cellular immune responses in the breast tumor-induced rats, suggesting an additional antitumor response mechanism.

The extract also promoted NF-κB and CD19 expression in the spleen, possibly for B-cell proliferation but did not ameliorate the spleen hypertrophy, because the tumor-bearing rats developed splenomegaly. NF-κB activation can enhance B-cell responses to pathogens. B-cell differentiation requires the interaction of various cytokines secreted from macrophages or T cells. The CD19 functions as the main signaling component on the mature B-cell surface, for the development of B cells from their early differentiation in the bone marrow to late maturation steps in the spleen [28].

In an open-label combined Phase I and II study, the intake of seaweed nutrient complex containing a blend of extracts from three different brown algae, *Fucus vesiculosis*, *Macrocystis pyrifera*, and *Laminaria japonica*, produced significant immunomodulation, decreased B cells and IL-6 levels, increased phagocytosis by monocytes, and increased cytotoxic T cells [29]. Some seaweed extracts (e.g., *Hizikia fusiformis* and *Meristotheca papulosa*) stimulated normal spleen B-cell proliferation, enhanced B-cell IgG production, and macrophages tumor necrosis factor production [30]. Many natural polysaccharides are biological response modifiers and modulated various immune responses. Kappa-carrageenan oligosaccharides from *Kappaphycus striatum* inhibited cancer metastasis and tumor growth by modulating the immune system [31].

The 150 mg SECE/kg BW administration upregulated the proapoptotic genes (*Chk1* and *p53*), and downregulated the antiapoptotic gene expressions (*Bag1, Birc5*, and *Mdm2*) that help encourage cancer cell death. BC cells with wild-type *p53* often have high *Mdm2* oncogenic expressions, which may block the *p53* function. *Mdm2* expression is increased in the presence of estrogen and enhances the estrogen receptor alpha positive (ERα+) function [32]. This study supported the *E. cottonii* breast tumor inhibitory properties by demonstrating its ability to enhance selective immune responses and restore apoptotic and antiproliferation gene expressions to favor cancer cell death.

E. cottonii is a potential dietary supplement for the prevention and management of BC and possibly other cancers, in general. This study provides some evidence that SECE prevents BC by enhancing immune responses and enhancing or restoring apoptosis-related gene expression responses in the cancer tissues. Ultrastructure studies of rats' breast tumors indicated that the orally administered seaweed *E. cottonii* aqueous alcoholic extract could induce BC cell degeneration at 150 mg/g dose against the highly invasive rat breast adenocarcinoma *in vivo*.

Conflicts of interest

All authors declare we have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmau.2016.08.001.

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