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Original Research Article

Aaptos suberitoides Extract Inhibits Cell Migration in Triple Negative Breast Cancer and Decreases NF-κB and MMP-9

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ABSTRACT

Aaptos suberitoides contain *aaptamine*, which is known to have anticancer properties. This study aims to analyze the effect of *Aaptos suberitoides* extract on cell migration, and expression of NF-κB and MMP-9 in Triple-Negative Breast Cancer (TNBC); MDA-MB-231 cell lines. Cells were treated with different concentration of extract (0, 1, 2, and 5 ppm) for 24 hours. Cell Migration was assessed by wound healing/scratch assay. NF-κB and MMP-9 expression were assessed by immunohistochemistry assay. The results demonstrated that the administration of *Aaptos suberitoides* (sea sponge) extract on MDA-MB 231 TNBC cell line caused decrease in the percentage of the migration area, and reduction of NF-κB and MMP-9 expression, particularly at highest extract concentration tested; 5 ppm.

Keywords: *Aaptos suberitoides*, Migration cells, MMP-9, NF-κB.

Introduction

Deaths caused by cancer are still high and tend to increase every year. According to the World Health Organization (WHO), all malignant tumors are predicted to increase from 14 million in 2012 to 19 million in 2025 and 24 million in 2035.[1] Specifically, breast cancer is the second-highest cancer globally with an incidence rate of 11.6%.[2] Molecular classification of breast carcinoma was determined by immunohistochemical examination of estrogen receptors (ER), progesterone receptors (PR), human epidermal growth factor receptors (Her2), and Ki67. This gave rise to five subtypes, namely; Luminal A, Luminal B negative HER2, Luminal B HER2 positive, HER2 enriched, and Basal Like or better known as Triple Negative Breast Cancer (TNBC).[3] TNBC is often resistant to cytotoxic chemotherapy and creates difficulties in achieving personalized drugs because of its molecular heterogeneity.[4] Patients with TNBC tend to have a higher early relapse after diagnosis, short disease-free intervals, and reduced overall survival, mainly due to lack of targeted therapy.[4] The percentage of women with TNBC subtypes had a high recurrence rate in the first three years (34%).[5] According to research data, 19.6% of TNBC patients, metastasize to the lungs, 19.4% metastasized to bone, and 2.9% metastasized to the brain.[5] The occurrence of metastasis of TNBC is very fast, research data suggests metastasis to bone in 16.3 months, to the liver in 8.9 months, to the pleura in 7.5 months, and to the brain in 4.3 months.[6] At present, TNBC tumours are treated with several combinations, namely; surgery, radiation therapy, and chemotherapy.[7] Because the genes associated with TNBC are not well understood at this time, and there is no targeted therapy directed at TNBC.[8] Late diagnosis is a much higher risk of spreading cancer cells from the primary tumor to the surrounding tissues and organs in a process known as metastasis. Almost all breast cancer death is as a result of the associated metastasis, which accounts for 90% of tumor deaths.[9] The metastatic breast carcinoma process consists of several stages, including invasion of the extracellular matrix, cell migration, vascular propagation, homing of tumor cells, and colonization.[2]

Aaptos suberitoides is a type of marine sponge that is widely found in Indonesian waters.[10] A study revealed that *Aaptos suberitoides* contain *aaptamine*, which possesses cytotoxic effect on HeLa cells (cervical carcinoma).[11,12] This sponge has a potential anticancer effect on murine lymphoma cell line.[13,14] Sea sponge bioactive compounds have potential as a cure for various diseases.[15] Marine sponges have components that possess anticancer, antimicrobial, antihypertensive, antioxidant, anti-inflammatory, immunomodulatory, wound healing, and other therapeutic effects.

Aaptos has a cytotoxic effect on CEM-SS of human T-lymphoblastic leukemia cells.[12] These data open new hopes for cancer treatment, including breast carcinoma, one of the deadliest cancers. However, published data on the anticancer effects of the existing sea sponge *Aaptos suberitoides* only discuss the cytotoxic activity, and no tested has been done on its effect on cancer cell migration. Therefore, the present study is aimed at analyzing the role of *Aaptos suberitoides* sea sponge extracts in the inhibition of cell migration through NF-kB and MMP-9 on MDA-MB 231 TNBC cell lines.

Materials and Methods

Materials

The materials and instruments used were 96% ethanol, culture media RPMI1640 (Gibco, cat.no. 11875085, USA), DMEM-F12 (Gibco, cat.no. 11320033, AS), Fetal Bovine serum (FBS) (Gibco, cat.no. 26140079, USA), penicillin/Streptomycin (Sigma, Cat.no. P4333, USA), phosphate buffered Saline (PBS) (Gibco, Cat. No. 10010001, USA), trypsin (Gibco, Cat. No. 15050057, USA), MTT assay (3-4, 5-dimethylthiazol-2-YL]-2,5 diphenyl tetrazolium bromide (Cat. No. M2128, Sigma-Aldrich, USA), Dimethyl sulfoxide (DMSO) (Sigma, Cat. No. D8418, USA), Incubator (Thermo Scientific model 3429, USA, 2014), Inverted Microscope (Olympus CK40, Japan),

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Microscope camera (C-Mount kamera, China), Imaging Software (v 1.51, National Institute of Health, USA), Microsoft Excel software (v. 16.30, 2016), GraphPad Prism Software (v 7.0 a, 2016).

Sample preparation

Aaptos suberitoides marine sponges were collected in January 2019 from beaches around Tinjil Island, Banten, Indonesia, at a depth of up to 20 meters below sea level. The fresh marine sponge was chopped and then macerated with 96% ethanol for 2-3 days, followed by filtration using filter paper. The suspension was evaporated using a Rotary Evaporator to obtain a concentrated paste extract. The ethanol extract was stored in a 4°C chiller. The extract was dissolved in 100% DMSO to prepare a stock solution to of 40,000 ppm. For further experiments, the stock solution was diluted with complete culture media to the required concentration.

Cell culture condition

MDA-MB 231 cells were used as a model for triple-negative breast carcinoma. This cell was obtained from Dr. Wiemann (National Center for Tumor Diseases, Heidelberg, Germany). The status of this MDA-MB 231 cell is IHC ER/PR and Her2-. This cell was cultured using RPMI 1640 equipped with 10% FBS and 1% penicillin/streptomycin in a standard incubator maintained at 37°C and 5% CO_2. All experiments were carried out in the cell culture and Cytogenetic laboratory, Padjadjaran University teaching hospital, Bandung.

MTT assay

The toxicity effect of *Aaptos suberitoide* was tested using the MTT assay. Cells were grown on 96 well plates for 24 h. the cells were treated with serial concentrations of *Aaptos suberitoides* ethanol extract for 72 h. Thereafter, cells were treated with MTT reagent for 4 h, and the reaction was stopped using DMSO. The plate was then shaken to dilute the formazan crystals before the absorbance was read using a microplate reader at a wavelength of 550 nm. The percentage of cell death was calculated based on the absorbance value of the sample, control, and blanks. The experiment was carried out thrice with duplicate of the serial concentrations (0 ppm, 0.5 ppm, 1 ppm, 5 ppm, 10 ppm, 25 ppm, 50 ppm, and 100 ppm).

Wound healing assay

The *wound-healing assay* was performed in MDA-MB 231 cells to evaluate the inhibitory effect of *Aaptos suberitoides* ethanol extract on MDA-MB 231 cells migration. Marker lines were made with a scalpel under each plate before the cells were implanted and incubated for 24 h. After the monolayer has been formed, perpendicular cells were scratched by the tip of a 200 µL pipette across the marker line. The old medium was discarded slowly. The plate was tilted and washed with PBS to remove cell debris and then replaced with a complete culture media. The cells were treated with ethanol extract of *Aaptos suberitoides* at 0 ppm, 1 ppm, 2 ppm, and 5 ppm concentrations. Cells were incubated at a temperature of 37°C and 5% CO_2. Gaps formed periodically were captured at 0, 6, 12, 21, and 24 h. Photos were processed by ImageJ software to measure the gap area. Data were presented as mean percentage of triplicate determination of Gap Closure [(Gap area 0-Gap area n)/(Gap area 0)].

Immunocytochemical assay

The method used for immunocytochemical staining was labelled streptavidin-biotin immunoperoxide complex, using One Step Neopoly Detection Kit (Biogear Scientific). The primary antibody used was NfkB p65 rabbit monoclonal antibody (Bioenzy) with 1:200 dilution and MMP-9 rabbit polyclonal antibody (Bioenzy) with 1:200 dilution. Cultured cells were planted on round coverslips (Thermo Fisher), fixed with 96% alcohol overnight. The epitope's opening was performed with an antigen retrieval method using ethylenediamine tetraacetic acid (EDTA) solution inside a decloaking chamber at a temperature of 100°C for 20 min. Hematoxylin Mayer's *w*as used as the counterstain.

Assessment of both biomarkers' immunoexpression was done with histoscore, in the form of observations of the intensity and colour distribution. NF-κB was rated as positive if the tumour cell's nucleus and/or cytoplasm were stained brown, while MMP-9 was rated as positive if the tumour cell membrane and/or cytoplasm were stained brown. Staining intensity ranged from 0 (negative), +1 (weak), +2 (intermediate), or +3 (strong). Percentages of tumour cell that were positive for staining was given 0 (negative), 1 (10-25%), 2 (26-75%), and 4 (>75%) for NF-κB. While, for MMP-9 was given 0 (<10%), 1 (10–25%), 2 (26–75%), 3 (>75%). Histoscore was obtained from the multiplication of intensity value and staining distribution, with the score of 0-12 for NF-κB and 0-9 for MMP-9, then sorted into categories of low (histo score of 0-4 for NF-κB and 0-3 for MMP-9) and high (histo score 6-12 for NF-κB and 4-9 for MMP-9). The assessment of immunocytochemistry NF-κB and MMP-9's result was conducted blindly by two pathologists.

Statistical analysis

Data were presented as percentages. The statistical analysis begins with tests of normality and homogeneity. The data then analyzed by One Way Annova, Kruskal Wallis and Generalized Linear Model repeated measures. The significance of the statistical test results was determined based on the value of $p < 0.05$. Data were recorded in Microsoft excel form and then processed with the SPSS program version 24.0.

Results and Discussion

Data showed that the extract of *Aaptos suberiotides* induced cell death in triple-negative breast cancer cell lines. Besides, the cytotoxic activity of extract of *Aaptos suberiotides* was in a dose-dependent manner. The IC_{50} value was 6 ppm (Rsqr 0.9860).

In this study, evaluating the effects of *Aaptos suberitoides* extract on the migration of MDA-MB 231 cells was analyzed. The data was collected in three replicates of the migration tests between the control group and the treatment group (*Aaptos suberitoides* sea sponge extract at concentrations of 1 ppm, 2 ppm, and 5 ppm). The concentrations were derived from the previous MTT test result (IC_{50} = 6ppm).

Figures 1 and 2 show the effect of *Aaptos suberitoides* sea sponge extracts on migration of MDA-MB 231 cells. The higher the concentration of *Aaptos suberitoides* sea sponge extract, the lower the migration area percentage.

The wound-healing assay test showed that the treatment with *Aaptos suberitoides* sea sponge extract affected the inhibition of the MDA-MB 231 TNBC cell line migration area, especially at a concentration of 5 ppm (81.79 ± 1.525%), which had the most potent effect. Aaptamine, a component of the sea sponge *Aaptos suberitoides* has been shown to have α-adrenoreceptor blocking activity. Aaptamine and its derivatives have antitumor, antimicrobial, and antiviral activities.[16,17] Aaptamines potent cytotoxicity is attributed to their ability to intercalate DNA.[11]

Figure 3 shows the effect of *Aaptos suberitoides* sea sponge extract on NF-κB immunoexpression (histoscore). In general, there was a decrease in NF-κB immunoexpression in MDA-MB 231 cell lines at various concentrations of *Aaptos suberitoides* sea sponge extracts compared to the controls. At the 24th hour, there was a tendency for the curve to rise at a concentration of 1 ppm (3.00 ± 1.155) and 2 ppm (1.50 ± 0.577). while at a concentration of 5 ppm (1.50 ± 0.577), the curve tended to fall.

According to Dyshlovoy *et al*.,[16] treatment with *Aaptos suberitoides* sea sponges also reduced NF-κB immunoexpression, especially at a concentration of 5 ppm. The study stated that the sea sponge *Aaptos suberitoides* has aaptamine compounds which have proapoptotic and anti-proliferative effects.[16] It modulates the transcription factor AP-1, NF-κB in monocytic leukemia, colon cancer, cervical cancer, and breast cancer.[16]

As shown in Figure 4, we observed the effect of *Aaptos suberitoides* sea sponge extract on MMP-9 immunoexpression (histoscore). In general, there was a reduction in MMP-9 immunoexpression in MDA-MB 231 cell lines at various concentrations of *Aaptos suberitoides* sea

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sponge extracts compared to the controls. In the 24th hour, there was a tendency for the curve to rise at a concentration of 1 ppm (3.25 ± 1.50) and 2 ppm (2.00 ± 1.414). However, at a concentration of 5 ppm (1 ± 1), the curve tended to fall.

Based on the hypothesis test using the Generalized linear test, the model shows that there was an influence of the treatment with *Aaptos suberitoides* on the area of migration, NFkB and MMP-9 immunoexpression in MDA-MB 231 cell lines, and this was statistically significant (p value < 0.05).

The decrease of NF-κB immunoexpression and MMP-9 immunoexpression due to the effect of *Aaptos suberitoides* sea sponge extract in this study shows that the inhibition of cell migration by *Aaptos suberitoides* affects the decrease of NF-κB and MMP-9 immunoexpression. Cell migration and invasion are complex processes involving extracellular matrix proteolytic degradation. It is also known that matrix metalloproteinases (MMPs) can reduce ECM and membrane basement to facilitate migration and invasion of cancer cells.

This study concluded that the treatment of MDA-MB 231 TNBC cells with *Aaptos suberitoides* marine sponge extract affected: (1) percentage of migration area; (2) decreased immunoexpression of NF-κB at a concentration of 5 ppm after 24 hours; and (3) decreased immunoexpression of MMP-9 at a concentration of 5 ppm after 24 hours.

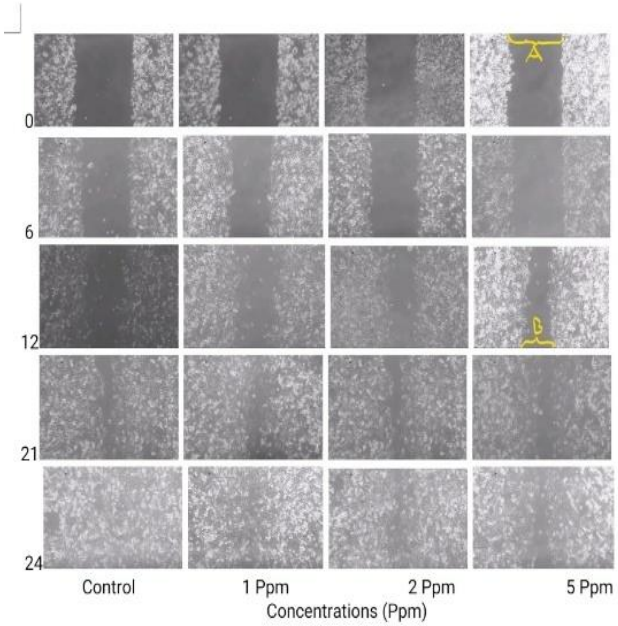

Figure 1: The effect of *Aaptos suberitoides* marine sponge's extract on the migration area.
Note: The migration area is a 0-hour gap (A) minus treatment hour gap (B).

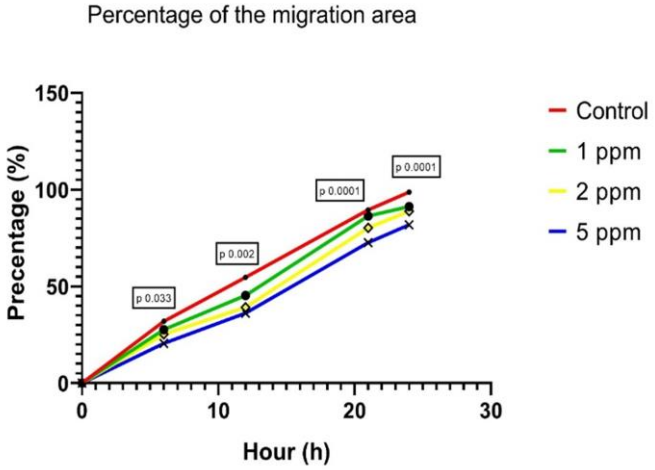

Figure 2: Percentage of the area of migration

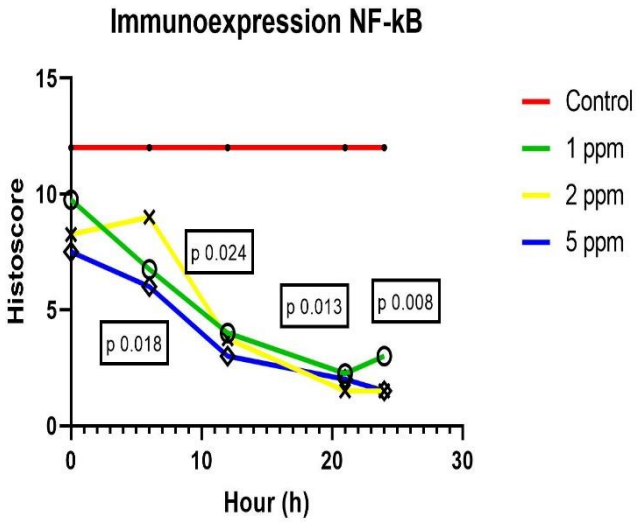

Figure 3: Distribution of NF-κB immunoexpression

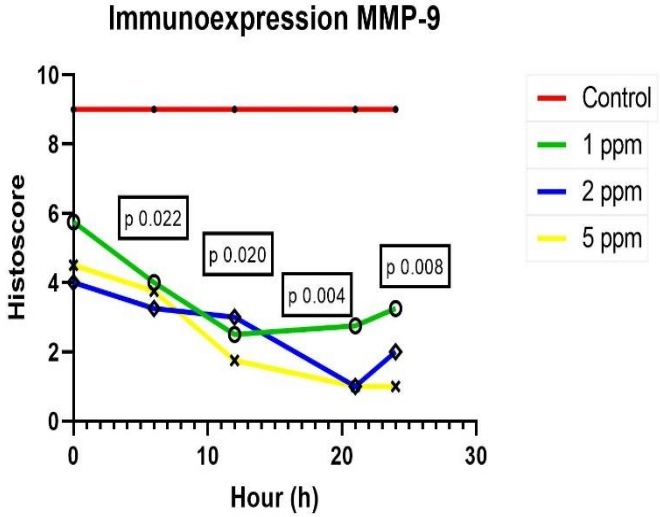

Figure 4: Distribution of MMP-9 immunoexpression

Conclusion

The treatment of MDA-MB 231 cell lines with *Aaptos suberitoides* sea sponge extract can significantly influence cell migration, decreased NF-κB immunoexpression, and decreased MMP-9 immunoexpression. Thus, the sea sponge *Aaptos suberitoides* is a potential source of complementary therapy in triple negative breast cancer.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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