

Fucoidan cytotoxicity against human breast cancer T47D cell line increases with higher level of sulfate ester group

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Abstract.

The anticancer activity of different sulfate ester group content in different molecular weight was examined. The anticancer activity was achieved in vitro on human breast cancer T47D cell line. Fucoidan with lower molecular weight (5.79 kDa) tends to have lower sulfate ester group content (8.69%) and resulted in higher IC₅₀ value (184.22 µg/mL). While fucoidan with higher molecular weight (785.12 kDa) tends to have higher sulfate level (18.63%) and achieved lower IC₅₀ value (75.69 µg/mL). The result showed that in order to maintain fucoidan cytotoxic activity against human breast cancer T47D cell line, the sulfate content should be remain high.

Keywords: fucoidan, sulfate ester group, human breast cancer

1. Introduction

Marine macroalgae have been exceptionally known to produce diverse compounds and cover a wide range of biological activities. Bioactive compounds act as chemical weapons against other organisms and/or as responses to physical stress [1]. Fucoidan is a sulfated polysaccharide extracted from brown seaweed and can be regarded as a bioactive compound to various biological activities including antibacterial, antioxidant, antiviral, and antitumor. However, the biological activity behavioral is not ubiquitous, depending on its structure [2].

Many studies suggested that molecular weight is one of the most important factors determining the biological activities of fucoidan. High molecular weight fucoidan may cause low solubility and processability, thereby hampering their penetration into the cell to perform a function [3]. Conversely, low molecular weight fucoidan (LMWF) has more biological actions than native fucoidan [4]–[6]. Acid hydrolysis process is usually performed to break the glycosidic linkage and minimize the molecular size. However, acid hydrolysis can also remove the sulfate ester group. While the study on anticoagulant and antiproliferative effects in LMWF reveal that sulfate ester group content is necessary for their biological actions [7].

In this work, we attempted to determine the role of sulfate ester group and molecular weight on the anticancer effect of fucoidan extracted from *Sargassum binderi* Sonder. To that purpose, we prepared one fucoidan with hydrolysis treatment and one without hydrolysis treatment. The anticancer activity on both fucoidans was examined on human breast cancer T47D cell line.

2. Material and Method

2.1. Material

The raw material used in this work was brown seaweed *Sargassum binderi* Sonder which collected from Lampung, Indonesia. Chemical reagents used were mostly analytical grades and some technical grades.



2.2. Extraction, purification, and hydrolysis of fucoidan

Dried seaweed *S. binderi* (100 g) was grinded into small pieces and macerated into a mixture solvent MeOH – CH₂Cl₂ – H₂O (4:2:1) (500 mL) at room temperature for 3 hours to remove the lipophilic pigment (chlorophyll, fucoxanthin, carotene) and fats. The *S. binderi* then treated with 0.1 N HCl (1:20) (w/v) (2 L) at pH 4 and stirred for 6 hours at room temperature. The filtrate then collected and centrifuged at 5.000 rpm for 15 minutes. The filtrate was neutralized with 0.5 M NaOH, added with 2% CaCl₂ solution and incubated for 3 hours then centrifuged. The filtrate was collected and added to ethanol (1:2) and incubated overnight. The precipitate in the ethanol solution was separated by 5000 rpm centrifugation for 15 min at 15°C. The precipitate was dried with freeze-dry to obtain crude fucoidan. Fucoidan was purified using dialysis membrane (Thermo Scientific Slide-A-Lyzer 3,5 kDa) in a 0.5 M NaCl solution for 24 hours and continued with aquabidest for 24 hours. The purified fucoidan (50 mg) was hydrolyzed in 1 M TFA solution (5 mL) for 1.5 hours at 121°C in an autoclave. Finally, the fucoidan solution was neutralized with NaOH at the same concentration and then freeze-dried to obtain a hydrolyzed fucoidan.

2.3. Characterization of hydrolyzed and non-hydrolyzed fucoidan

2.3.1. Determination of total carbohydrate

The determination of total carbohydrate content was performed by Phenol - Sulfuric acid method. Each hydrolyzed and non- hydrolyzed fucoidan samples (5 mg) was dissolved in aquadest (1 mL) and added to concentrated H₂SO₄ (2.5 mL) and incubated in an ice bath for 30 minutes. Then, 5% phenol (0.5 mL) was added to the sample and also incubated in the cold ice tub for 30 minutes. Fucose, mannose, and xylose were used as standards in various concentrations (200-1000 ppm). Sample, standard, and blank were measured at 480 nm and 490 nm with a UV-VIS spectrophotometer.

2.3.2. Determination of sulfate ester group content

The determination of sulfate ester content in fucoidan was performed with BaCl₂ – Gelatin method. In 3.5 N HCl (2 mL), each hydrolyzed and non- hydrolyzed fucoidan samples (6 mg) were heated for 17-18 hours at 105-110°C. Then, each sample (10 µL) was placed in the 96-well plate, added with 3% TCA (190 µL) and 0.5% BaCl₂ – gelatin (50 µL). The plate was left for 20 minutes until the barium sulfate ready to be scanned. Na₂SO₄ was used as a standard with various concentrations (100 – 500 ppm) while aquadest used as a blank. The samples, standards and the blank were scanned with Microplate Reader Spectrophotometer at $\lambda = 360$ nm.

2.3.3. Determination of weight average molecular weight

The average molecular weights of hydrolyzed and non- hydrolyzed fucoidan were measured by the Size Exclusion Chromatography (SEC) method. Each fucoidan sample and pullulan standard (5 mg) were dissolved separately in aquabidest (1 mL) and left overnight in cold storage. Then, sample and standard were eluted on TSK-Gel G6000 PW column using RID detector with flow rate 0.8 mL/min for 25 minutes and water pure grade HPLC as the eluent.

2.4. Anticancer activity assay

2.4.1. Cell line and culture

The human breast cancer T47D cell line was cultured in RPMI medium supplemented with 10 % FBS, 2 % penicillin-streptomycin, and 0.5% fungison in an incubator (ESCO CelCulture) with a humidified 4.2% CO₂ at 37 °C.

2.4.2. Growth inhibition assay

The effect of hydrolyzed and non-hydrolyzed fucoidan on the inhibition of cancer cells was assessed in vitro by MTT assay [8]. The hydrolyzed and non-hydrolyzed fucoidan were respectively dissolved in aquabidest, DMSO, and medium growth into different dosages (non-hydrolyzed fucoidan: 50; 100; 200; 400; and 800 µg/mL, while hydrolyzed fucoidan: 20; 40; 80; 160; and 320 µg/mL). Three types of control were also prepared, which are cancer cell control, media control, and sample control.

Briefly, human breast cancer T47D cells lines were incubated in 96-well plate (1.5×10^4 cells/well) until reach a confluent phase (12 – 24 hours). After incubation, each fucoidan samples (100 µL) were added to the medium growth and incubated for 24 hours in a CO₂ incubator. Cells were then added to MTT reagent and incubated for 4 hours. The reaction stopped with the addition of SDS 10% and

incubated for 12 hours. The cell growth was observed with microscope inverted and detected with Spectrophotometer Microplate Reader at wavelength 570 nm. The inhibition rate was calculated according to the formula:

$$\text{Inhibition rate (\%)} = \frac{(C-D)-(A-B)}{(C-D)} \times 100\%$$

where each absorbance stands for: A = fucoidan samples; B = control sample; C = control cells; D = control media. The IC₅₀ value was calculated in probit statistic.

3. Results and Discussion

3.1. Extraction, purification, and hydrolysis of fucoidan

First, fucoidan extraction by acid method was carried out. The yield of crude fucoidan from brown seaweed *Sargassum binderi* Sonder was 6.64%. It is essential to be noted that the yield of crude fucoidan may vary from each species and the extraction techniques can also significantly improve the yield [9]. There are various methods in fucoidan extraction, includes dilute acid, hot water, or calcium chloride. Acid extraction was used as the major steps in fucoidan extraction [10]. Though it causes degradation and affects the integrity of overall fucoidan make-up, the advantage of using acid extraction will help the precipitation of alginic acid from alginates. The protons or hydroxide ions will interfere the hydrogen bonds between the various polysaccharides in brown seaweed (alginate, laminarin, and fucoidan) and then release them into the solution [11].

In order to remove the unnecessary compounds other than fucoidan, purification was conducted using dialysis membrane (3.5 kDa MWCO) with NaCl solution. After purification, the overall fucoidan yield decrease to 1.67%. Dialysis process separate small impurities attached to fucoidan by diffusion rate in a semipermeable membrane. The following process then split into two major in which one underwent hydrolysis and one without hydrolysis. In this work, mild acid hydrolysis was used to depolymerize fucoidan into low molecular weight fucoidan (LMWF). Though mild acid hydrolysis works in a nonspecific method [12], a reduction in molecular size is promising.

3.2. Characterization of hydrolyzed and non-hydrolyzed fucoidan

General characterization of fucoidan can be seen by reviewing the carbohydrate, molecular weight, and sulfate content [13]. Schweiger first suggested that fucoidan is a heteropolymer type of sulfated polysaccharide. Thus, fucoidan characterization on each species of brown seaweed needs to be clearly determined in order to see the effect to its bioactivity [14]. When fucoidan was hydrolyzed in mild acid solution, most of the chemical and physical characteristics in fucoidan have shown some modification [15]. From Table 1, we see that all aspect in fucoidan shows some decrement after hydrolysis.

Table 1. Characterization of hydrolyzed and non-hydrolyzed fucoidan

Fucoidan	Carbohydrate (%)	Sulfate (%)	MW (kDa)
Non-Hydrolyzed	82,79	18,63	785,12
Hydrolyzed	55,86	8,69	5,79

As a sulfated polysaccharide, the total carbohydrate in fucoidan is closely related to the amount of sulfate attached in carbohydrate. From the data showed in Table 1, the non-hydrolyzed fucoidan gave higher carbohydrate content than modified fucoidan (hydrolyzed). Mild acid hydrolysis at high temprature also reduces the sulfate ester group bond. The sulfate content in Table 1 showed that hydrolyzed fucoidan has lower sulfate content than the non-hydrolyzed. It confirmed that hydrolysis affect the total carbohydrate and therefore decrease the sulfate content.

The initial goal of hydrolysis is to depolymerize fucoidan into low molecule. Apart from the other effect of hydrolysis in fucoidan, the decrease in molecular weight can help better penetration into cells and initiate higher bioactivity. In Table 1, the molecular weight of hydrolyzed fucoidan was

successfully modified became low molecular weight fucoidan (LMWF). It proved that mild acid hydrolysis is suit to depolymerize fucoidan molecule, yet did cause a significant change in sulfate and carbohydrate content.

3.3. Cytotoxic fucoidan of human breast T47D cancer cell lines

To evaluate the biological activity in fucoidan against human breast cancer T47D cell line, cytotoxic activity was conducted with MTT assay. The cytotoxic of fucoidan from *Sargassum binderi* Sonder was compared over the hydrolysis treatment and to standard fucoidan commercial from *Fucus vesiculosus*. From Table 2, the result showed that standard fucoidan exhibit the highest cytotoxic activity against human breast cancer. Although the non-hydrolyzed fucoidan has slightly lower IC_{50} than the standard fucoidan, it can be rounded that non-hydrolyzed fucoidan was also achieved high cytotoxic activity. While the hydrolyzed fucoidan was obtained the lowest cytotoxic activity among the other fucoidan (Figure 1).

Table 2. IC_{50} of standard fucoidan from *Fucus vesiculosus*, hydrolyzed and non-hydrolyzed fucoidan from *Sargassum binderi* Sonder against human breast cancer

Fucoidan	Standard	Non-Hydrolyzed	Hydrolyzed
IC_{50} ($\mu\text{g/mL}$)	72.13	75.69	184.22

There are two main factors to increase the bioactivity of fucoidan, which is sulfate ester group content and molecular weight. Sulfate ester group carries a negative charge in the molecule which can facilitate the formation of fucoidan-protein complexes involved in cell proliferation [16]. On the other hand, low molecular weight fucoidan will increase the solubility and penetration into the cell. Generally, the optimum bioactivity of fucoidan has molecular weight range at 50 – 100 kDa, while large molecular weight (>850 kDa) showed a low activity [17]. However, the optimum molecular weight range for anticancer activity have not yet been reported to date.

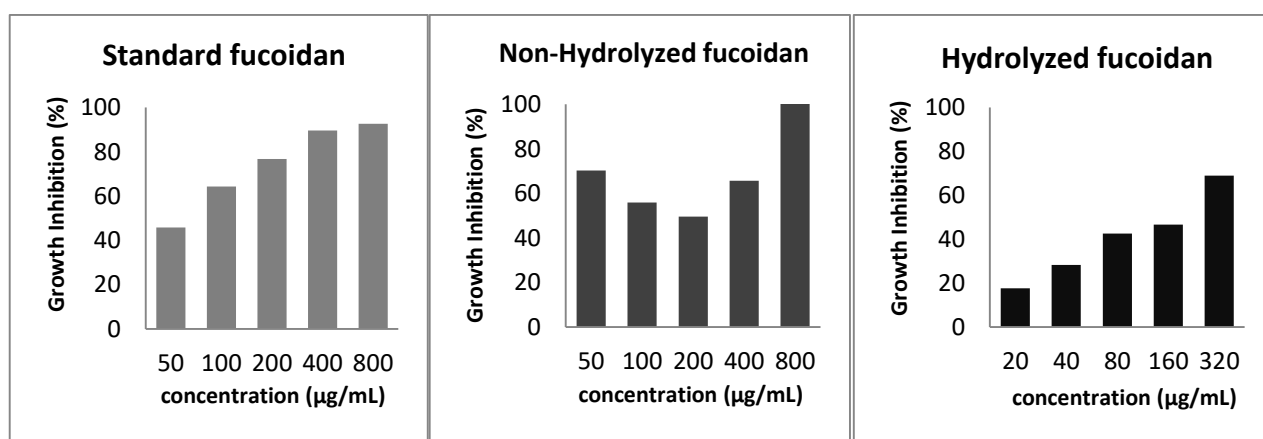


Figure 1. Growth inhibition diagram of standard fucoidan from *Fucus vesiculosus*, hydrolyzed and non-hydrolyzed fucoidan from *Sargassum binderi* Sonder against human breast cancer

The result of the cytotoxic test also implies that sulfate ester group content has played more important role than molecular weight. Non-hydrolyzed fucoidan with higher sulfate content (18,63%) achieved higher cytotoxic activity (75.69 $\mu\text{g/mL}$) than the hydrolyzed fucoidan (184.22 $\mu\text{g/mL}$). Compared to the molecular weight of non-hydrolyzed fucoidan (785,12 kDa), the hydrolyzed fucoidan has successfully achieved low molecular size (5,79 kDa), yet the sulfate content was not high enough

to inhibit the cancer cell. Hence, in order to maintain fucoidan cytotoxic activity against human breast cancer T47D cell line, the sulfate content should be remain high.

4. Conclusion

This study describes the role of molecular weight and sulfate ester group to increase fucoidan cytotoxicity against human breast cancer T47D cell line. It was found that higher sulfate content is more significant to inhibit cancer activity rather than to lower the molecular weight of fucoidan. Hence, in order to maintain fucoidan cytotoxic activity against human breast cancer T47D cell line, the sulfate content should be remain high.

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