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# Monosaccharides Composition of Fucoidan from Brown Seaweed (*Sargassum plagiophyllum*) and the Corresponding Antidengue Activity

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**Abstract**

Dengue Fever is an infectious disease caused by dengue virus infection and it poses a significant threat in tropical countries, such as Indonesia. Several studies have shown that the existing treatment options for this condition were ineffective, including the Dengvaxia vaccine released in 2015. This has led to the exploration of bioactive substances with the potential to be developed as antiviral medicines. One of the promising candidates is brown seaweed (*Sargassum plagiophyllum*), which has been reported to have anti-dengue and cytotoxic effects. Therefore, this study aimed to assess the anti-dengue activity of fucoidan from *S. plagiophyllum* and analyze its monosaccharides composition. Fucoidan extract was purified with ion exchange chromatography and characterized with H-NMR and HPLC. According to HPLC analysis, the extract consisted of various sugars, including fucose (5980 µg/g), galactose, xylose, mannose (1740 µg/g), glucose (9.34 µg/g), glucuronic, and galacturonic acid (10,06%). Furthermore, the anti-dengue activity was assessed using the viral ToxGlo test and the MTT assay. The results showed that fucoidan had an effective concentration ($EC_{50}$) of 76.49 µg/mL and a selectivity index (SI) of 3.02, indicating the presence of anti-dengue properties.

***Keywords:*** Anti-dengue activity; Brown seaweed; Fucoidan; *Sargassum plagiophyllum*

## 1. Introduction

Dengue fever, commonly referred to as dengue, is a significant health problem in Indonesia. The country frequently experiences outbreaks of this condition, particularly in tropical areas where the climate provides an ideal environment for the proliferation of *Aedes aegypti*, a known vector (Ravikumar *et al*., 2011). Furthermore, Indonesia stands among the countries with the highest incidence of dengue fever worldwide, with thousands of individuals being affected annually. According to data from the Ministry of Health of Indonesia, there were 143,266 reported cases of Dengue Hemorrhagic Fever (DHF) in 2022. This figure marks a notable 94.8% increase compared to the previous year with 73,518 cases. As of the 33rd week of 2023, a total of 57,884 individuals have been reported with a mortality rate of 422 (Kemkes, 2023). A recent report dating to 23 August 2023 shows more than 3.7 million reported cases and more than 2,000 dengue-related deaths from 70 countries and territories globally (WHO, 2023).

*Aedes aegypti* serves as the primary vector responsible for transmitting the dengue virus (DENV), a member of the Flaviviridae family, which causes dengue in humans. Furthermore, the disease poses a serious threat to public health, with symptoms ranging from high fever to life-threatening

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hemorrhage. At present, the existing treatments are considered ineffective based on clinical findings. The Dengvaxia vaccine (CYD-DTV), which was introduced in 2015, actually caused an asymptomatic natural infection in an individual who had never been infected with the Dengue virus. The limitation of this vaccine includes the occurrence of severe dengue fever in uninfected individuals who received the vaccination for the first time (Foucambert *et al.*, 2022). In response to this condition, World Health Organization (WHO) implemented a policy that only allows its usage among infected patients (WHO, 2018).

The limitations of Dengvaxia have caused a shift towards identifying compounds with the potential to act as antivirals. Sulfated polysaccharides from algae, such as fucoidan, have been shown in several studies to exhibit biological activities, including larvacidal impact against various mosquito vectors (Mahyoub, 2018) and antiviral properties (Pradhan *et al*., 2022). *Sargassum* sp., a type of seaweed, has been the subject of frequent investigation, showcasing a wide range of biological activities, including anticancer (Herawati *et al*., 2022), antibacterial, antifungal, and anti-dengue (Ahmad *et al.*, 2021). According to a recent study, sulfated polysaccharides from red seaweed and sulfated galactomannans from *Mimosa scabrella* seeds both prevented the in vitro and in vivo infection of flaviviruses, such as dengue and yellow fever viruses. Fucoidans have shown promising effects as anti-dengue drugs with no toxicity or irritancy in humans (Hidari *et al*., 2008). Therefore, this study aims to assess the anti-dengue activity of fucoidan from *S. plagiophyllum* and analyze its monosaccharides composition.

## 2. Experimental

### *2.1. Microwave Assisted Extraction of Sulfated Polysaccharides (Fucoidan)*

*S. plagiophyllum* was harvested in Gunung Kidul, Yogyakarta, Indonesia. Approximately 50 g of *S. plagiophyllum* was extracted in 300 mL absolute ethanol at ambient temperature for 24 hours, and then at 70°C for an additional hour. Centrifugation was carried out to recover any potential residue after the extraction process was complete, and the result was dried overnight. Subsequently, the residue was suspended in 300 mL of 0.1 M HCl solution, microwaved at 700 W for 5 minutes, subjected to an irradiation treatment, and the suspension obtained was centrifuged for 15 minutes at 3000 rpm. It was crucial to note that the filtrate and absolute ethanol were combined at a volume ratio of 1:1, while the brown precipitated extract was collected. The extract was then held at room temperature and dried for a week (Fig. I) (Herawati *et al*., 2022).

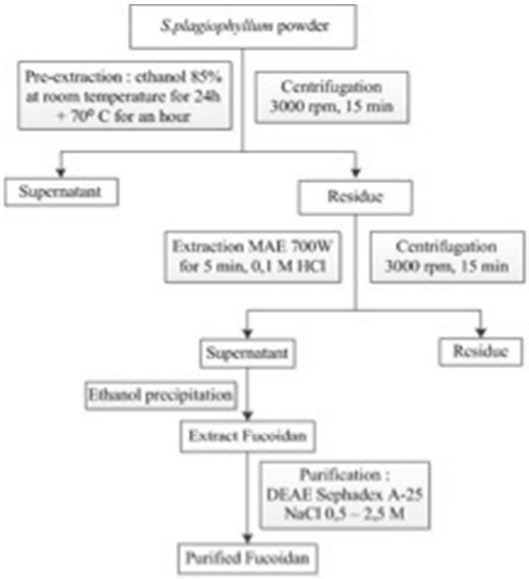


**Fig. I.** The scheme of extraction and purification fucoidan

### *2.2. Fucoidan Purification*

Fucoidan was purified using DEAE Sephadex A-25 and then eluted with gradient NaCl. A column of DEAE Sephadex A-25 was loaded with crude fucoidan that had been dissolved in 0.1 M *buffer* Tris-Cl pH 7.0, and step-by-step elution was carried out using 50 ml of NaCl solutions (0.5 – 2.5 M) flowing at a rate of 1 ml/min. The eluents (5 ml/tube) obtained separately were combined with 1:2 ethanol. All fractions were filtered, frozen dried, and kept at 4°C until they were used (Rafi and Veerichetty, 2020). The amount of carbohydrates in each eluent was calculated using the phenol-sulfuric acid method with fucose as the reference material.

### *2.3. Evaluation using HPLC*

Fucoidan's monosaccharide content was assessed using Shimadzu, SIL 10AD High-Performance Liquid Chromatography (HPLC), a refractive index detector (RID), and an Agilent Hi-Plex H Column with ligand exchange for carbohydrates. The elution was carried out using 0.005 M $H_2SO_4$ at a flow rate of 0.7 mL/min with a separation time of 30 minutes, followed by the addition of a 20 µL sample. Furthermore, Fucose, rhamnose, glucose, mannose, galactose, and xylose were among the common monosaccharides used in this study (Lutfia *et al*., 2020).

### *2.4. Structural Characterisation of Purified Fucoidan by H-NMR Spectroscopy*

A total of 20 mg of Fucoidan dried powder was dissolved in 1.0 mL Deuterium Oxide ($D_2O$). On a JEOL 400 MHz at 65°C, $^1$H-Nuclear Magnetic

Resonance (NMR) data were collected. D2O internal reference value is 4.7 ppm (Artemisia *et al.*, 2019).

*2.5. Screening Examination for Anti-DENV Activity* (Elias *et al.*, 2021)

*1. Medium and Sample Preparation*

The medium used was Minimum Essential Medium Eagle (MEM) with 10% Fetal Bovine Serum (FBS). The sample was dissolved in 1 mL of a suitable solvent, which was then used as a stock solution. The maximum sample concentration was 10 µg/mL and it was used as a stock solution. Furthermore, the maximum sample concentration was 10 µg/mL with half dilution to the smallest concentration of 1.56 mg/mL on a 96 U bottom microplate.

*2. Cell Preparation*

The cells to be used were 80-100% confluent, and the medium in the flask was discarded. The cells were then added with 2 ml trypsin-EDTA and incubated for 5 minutes at 73°C 5% $CO_2$, leading to the dispersal of the cell layer (observation with an inverted microscope, the cells will appear to float). The samples were neutralized with 2 mL MEM 10% FBS and resuspended until they became a single cell. The cells were then transferred, suspended in a sterile tube, and centrifuged at 3500 rpm for 5 minutes. The supernatant obtained was removed by decantation and the pellet (cell sediment) was suspended in MEM 10% FBS.

*3. Seeding Cells into Microplate 96 Flat bottom Luminescence*

The number of cells with trypan blue exclusion was determined with a ratio of 1:1 and resuspended with a final cell density of 5 x $10^4$ cells/mL in the medium.

a. Preparing 10 µL trypan blue in a sterile microtube.
b. Adding 10 ml cell suspension to the trypan blue solution and then homogenize.
c. Cleaning the counting chamber (hemocytometer) with 70% alcohol and dry.
d. Using a micropipette, slowly inject 10 µL trypan blue-cell suspension solution into one of the counting chambers.
e. Counting the number of cells/mL. The cells were seeded in a 96 flat bottom luminescence microplate and incubated for 42 hours at 37°C with 5% $CO_2$.

$$Cell\ concentration\ (cells/mL) = \frac{cell\ number\ x\ 10000}{number\ of\ boxes}\ x\ dilution\ factor$$

*4. Treatment of Cells with Samples*

The microplate 96 flat-bottom luminescence wells were labeled as required (medium, control, cell control, DENV control, and sample), and the microplates were removed. Furthermore, the process continued with the addition of 50 µL MEM 10% FBS in the medium and cell control wells. A total of 25 mL of the sample with varying concentrations was added into each well corresponding to the previous labeling and each concentration was replicated 3 times. Approximately 25 mL of DENV stock with a concentration of 2 x $10^3$ FFU/mL was added, followed by 50 µL of DENV control. The samples obtained were incubated for 48-144 hours at 37°C in 5% $CO_2$.

*5. Administration of Viral ToxGlo Assay and Luminescence Measurement*

A total of 100 µL Viral ToxGlo assay reagent was added to each well and then incubated for 10 minutes at 37°C and 5% $CO_2$. Subsequently, the results were read with the GloMax reader in the luminescence menu.

*6. Calculation*

The data obtained in the form of luminescence (RLU) for each well was converted into percent cell CPE and calculated using the formula:

$$Cell\ CPE\ (\%) = \frac{Luminescence\ Treatment - Luminescence\ Control\ Medium}{Luminescence\ (K.DENV + Control\ Cell) - Luminescence\ Control\ Medium}\ x\ 100\%$$

Based on the % CPE cell data and concentration, the linear regression equation y = ax + b was used to determine $EC_{50}$ by substituting the number 50 for Y, to obtain the value x (concentration). The x value was the CPE cell concentration of 50% or $EC_{50}$.

## 3. Results and Discussion

*3.1. Extraction and Purification of Fucoidan*

The isolated and dried crude fucoidan weighed approximately 6.73 g. For the initial extraction, 50 g powdered seaweed was used and the yield was 13.4%. Furthermore, anion exchange chromatography was used in purifying the extract fucoidan with gradient elution of NaCl (0.5 M – 2.5 M) in the DEAE Sephadex A-25 column. To identify the fractions that were positive for sugar, the collected samples were examined using fucose reagent. Furthermore, the samples were measured by comparing them to the reference fucose. Based on Fig. 1, it could be observed that the purification results obtained were 30 fractions. In this study, fraction 17 had the highest absorbance at 0.552 with a fucose content of 0.54%. The results showed that the higher the absorbance value, the higher the fucose content (Rafi and Veerichetty, 2020). Fraction 17 was

obtained from the elution of 2 M NaCl with a yield of 19.56%.

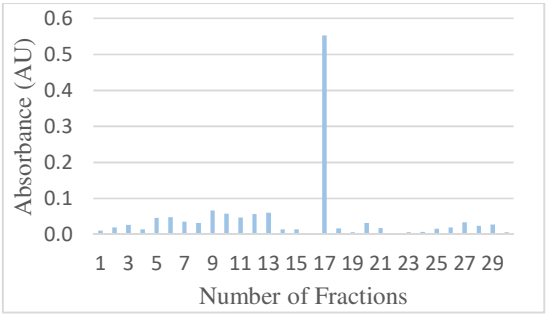


**Fig. 1.** The fraction number (0 – 30) was plotted as the absorbance value that was acquired for each fraction that was eluted in anion exchange chromatography

Fucoidan had a high anion content due to the presence of sulfate groups, hence, the purification process used anion exchange chromatography. DEAE Sephadex A-25 was a resin containing a positively charged diethylaminoethyl (quaternary ammonium) group and an anionic exchange group. These groups could interact with the sulfate on fucoidan, which was negatively charged. The resin had been reported to have the ability to retain and separate fucoidan from the sample mixture based on differences in anionic charge. Other components in the sample that were not negatively charged passed through the column quickly. Furthermore, fucoidan bound to the resin could be removed using NaCl eluent. The $Cl^-$ charge of the eluent was exchanged with the negative charge of the sulfate group to ensure the elution of fucoidan from the resin matrix. The pure fucoidan was then collected as an eluate fraction. The lower the sulfate content, the lower the NaCl concentration required to elute from the resin (Hahn *et al*., 2012).

*3.2 Structural Characterization of Fucoidan*

Characterization using 1H-NMR (Fig. 2) showed that the signal at 1.2-1.9 ppm was confirmed as a proton signal from H-6 methylation of fucopyranose. This signal could appear as a single peak or as a more complex cluster of peaks depending on the number of methyl groups in the fucoidan molecule (Artemisia *et al.*, 2019). The signal at 2.758 ppm was from protons bound to the sulfate groups and tended to appear as a sharp peak (Lutfia et al., 2020). The 3.5-4 ppm signal was the resonance of the hydroxyl group of the fucoidan monosaccharide (H-2 to H-5 from the proton ring). Meanwhile, the variant at 4.9 ppm was the anomeric proton signal from sugar (α-L-fucosa) and β-sugar (H1β) in the anomeric proton H1α (Marudhupandi and Kumar, 2013).

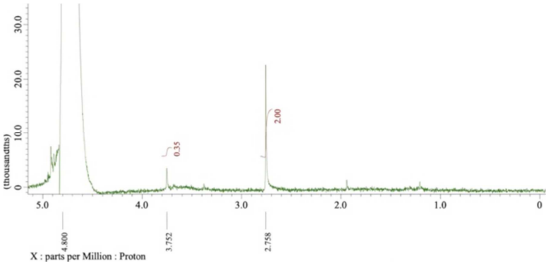


**Fig. 2.** $^1$H-NMR spectrum of fucoidan

The monosaccharide content of fucoidan was determined by HPLC using the standards, namely fucose, glucose, rhamnose, xylose, mannose, glucuronic acid, and galacturonic acid. Based on the results of HPLC analysis (Fig. 3), the constituent monosaccharides of fucoidan included fucose (15.71 minutes), glucose (12.59 minutes), mannose, xylose and galactose (13.86 minutes), glucuronic acid and galacturonic acid (7.95 minutes). The retention times of mannose, xylose, and galactose were very close, leading to their appearance in one peak with the same retention time. Furthermore, there were limitations to the column used, causing its inability to separate compounds with close retention times.

The fucose content from the HPLC results was 5.98 mg/g, with 9.34 µg/g glucose, 1.74 mg/g mannose, xylose, and galactose, as well as 10.06% glucuronic acid and galacturonic acid. The monosaccharide content obtained was almost the same as the monosaccharide content of *S. plagiophyllum* from India, which was extracted using HCl solvent for 24 hours to produce 70.8 mol % fucose, 13.5% galactose, 2.5% xylose, and 11.2% mannose (Suresh *et al.*, 2013).

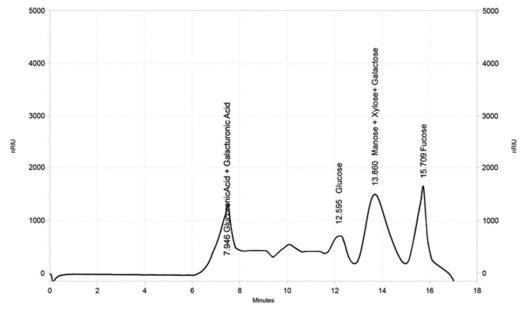


**Fig. 3.** HPLC chromatogram of fucoidan

*Sargassum* sp. which is extracted with HCl solvent for 24 hours also contains fucose, mannose, xylose, and galactose (Lutfia *et al.*, 2020). Extraction of *F. vesiculosus* using microwaves at a pressure of 120 psi with water as a solvent produces fucoidan containing fucose, galactose, and xylose (Rodriguez-Jasso *et al*., 2011). Meanwhile, *A. nodosum* which is extracted with HCl solvent using microwaves with temperature variations produces a fucoidan composition, namely fucose, rhamnose, galactose, glucose, xylose, mannose, and glucuronic acid (Yuan and Macquarrie, 2015). The extraction method and type of seaweed could influence the sugar composition of fucoidan extract (Wang *et al*., 2021).

*3.3 Antidengue Activity*

The anti-dengue activity of fucoidan from *S. plagiophyllum* was shown to be maximum on *Aedes aegypti* ($EC_{50}$ = 76.49 µg/mL). Furthermore, the non-toxic effects were defined as a $CC_{50}$ value greater than 50 µg/mL. The cytotoxicity of fucoidan was 219.56 µg/mL, indicating the absence of a cytotoxic effect on the Vero cells. The selectivity index (SI) value was 2.87, which indicated the ability of the compound to selectively kill plasmodium compared to Vero cells. An extract was classified as having low selectivity when the SI value ≥ 2.0 and < 5 (Trujillo-Correa *et al*., 2019). Although the SI of fucoidan was classified as weak, it was not toxic because it only killed the dengue virus without affecting the host (Hidari et al., 2008). The anti-dengue fucoidan activity of *S. plagiophyllum* was lower when compared to polysaccharide sulfate (ulvan) from *Caulerpa cupressoides* (green seaweed) with 0.35 µg/mL $EC_{50}$ value against DENV-1 Vero cells, > 714 selectivity index, and > 1000 µg/mL $CC_{50}$ (Rodrigues *et al.*, 2017). The results showed that sulfate polysaccharides isolated from *Gymogongrus griffithsiae* and *Cryptonemia crenulate* (red seaweed) showed an inhibitory effect *in vitro* against DENV-2 Vero cells with IC50 values of 1 µg/mL, > 1000 µg/mL CC50, and > 1000 µg/mL selectivity index (Freile-Pelegrín and Tasdemir, 2019).

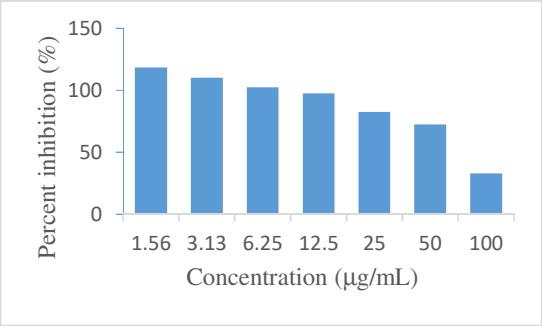


**Fig. 4.** Cytotoxicity test of fucoidan from *S. plagiophyllum*

The cytotoxic test results (Fig. 4) showed the impact of fucoidan on the survival or viability of Vero cells. The cells were often used in biological and health studies due to the stability and ease of cultivation (Ammerman *et al.*, 2008). The higher the concentration of fucoidan applied to Vero cells, the greater the potential of toxic effects

Fucoidan had been reported to have the ability to enhance the host antiviral response by preventing viral attachment, adsorption, and reproduction. This compound could also inhibit several phases of the viral life cycle by inactivating virions directly before contamination began or inhibiting their reproduction within host cells. Viral attachment started from an ionic interface reaction between a positively charged glycoprotein and a negatively charged host cell surface component. The sulfate group of fucoidan reacted with areas of positively charged viral glycoproteins, causing the cell surface to have a higher negative charge density, leading to disrupted viral cell interactions (Pradhan *et al.*, 2022).

The compound could also prevent virus binding to the cell surface, thereby avoiding virus entry into the host. Viral receptors on the host cell and electrostatic interactions between them generated irreversible adsorption when the virus attached to the host cell. Another interaction mechanism comprised direct interaction with virions, directly with viral receptors, or by inhibiting virus contact with the host cell surface (Wang *et al.,* 2012).

Dengue fever was often accompanied by strong inflammation in the body. According to previous studies, fucoidan could reduce this inflammation, but its mechanism of action remained unknown. Several studies showed that it also had potential antiviral activity, specifically on the dengue virus. Furthermore, it interfered with viral replication or activated the body immune response against the

virus. Fucoidan had antioxidant properties, which could protect body cells from oxidative damage during infection. It was hypothesized that the beneficial antiviral impact of fucoidans was related to its antioxidant, anti-inflammatory, and immunomodulatory capabilities as well as the selective effects on various stages of viral infection (Krylova *et al.*, 2020).

## 5. Conclusion

In conclusion, fucoidan showed potential as an anti-dengue agent in experimental studies. Cytotoxic reports indicated that its ability to desensitize dengue virus-infected cells was possibly through antiviral and anti-inflammatory activities. However, it was important to note that this study was still in its early stages, and more investigation was needed to understand the reaction mechanisms and effective dosage as well as to validate these findings in animal models and humans. The development of fucoidan as a potential adjuvant therapy for dengue fever required further studies and clinical trials to ensure its safety and effectiveness in patients affected by this disease.

## 6. Conflicts of Interest

There are no conflicts to declare.

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