Extraction Optimization by Response Surface Methodology and Characterization of Fucoidan from Brown Seaweed *Sargassum polycystum*

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**Abstract:** Fucoidan were recovered from *Sargassum polycystum* by single-step extraction with ultrasonic wave pretreatment (amplitude 80%, 15 minute). Extraction were optimized using central composite design of response surface method. Ultrasonic wave pretreatment conditions were 80% amplitude, for 15 minute. Alga solution in 0.03 M HCl ratio 1:20 (b/v) at 70-90°C for 3-5 hour were evaluated during this process to establish a condition to maximize the extraction. The yield (%), total carbohydrate (mg/g), fucose (mg/g) and sulfate contents (mg/g) were also determined for each experimental condition. The result showed that all extraction factors had significant effects (p<0.05) on fucoidan yield, fucose, total carbohydrate and sulfate contents. The optimum conditions were 81°C for 4.04 hours. The validated experimental crude fucoidan yield were 7.15±5.58%, 131.15±1.92 mg/g fucose, 396.8±65.58 mg/g total carbohydrate and 94.67±1.37 mg/g sulfate contents. Characterization of crude fucoidan by FTIR and HPLC showed that crude fucoidan (*Sargassum polycystum*) is composed by fucose, xylose, galactose, rhamnose, glucose, mannose and sulfate.

**Keywords:** fucoidan, fucose, sulfate, *Sargassum polycystum*, extraction method.

**Introduction**

Fucoidan, a cell-wall matrix polysaccharide in brown seaweed, is composed by L-fucose and sulfate including minor amounts of galactose, xylose, glucose and mannose. Fucoidan exert various biological activities such as antitumor and anticancer. Fucoidan can be extracted from *Sargassum polycystum*. Extraction of fucoidan from brown seaweeds generally involves multiple, extended aqueous extractions, usually with hot acid (hydrochloric acid), and may include addition of CaCl₂ to promote alginate precipitation. Each step lasting several hours and resulted lower fucose contents.

http://www.sphinxsai.com/framesphinxsaichemtech.htm
Ultrasonic wave extraction techniques have been applied for extracting fucoidan from the brown seaweed *Laminaria japonica* and *Eucheuma denticulatum*. The extraction process were fast but produced a low yield. Extraction combination using degradation by ultrasonic waves followed by water bath heating can produce more optimal fucoidan yield and fucose content. However, little attention devoted to the combination of extraction method followed by water bath heating and characterization of fucoidan (*Sargassum polycystum*). Herein, we report the detail of combination extraction, ultrasonic degradation and single-step optimization extraction by using response surface methodology and characterization of fucoidan *Sargassum polycystum*.

**Material and Method**

**Material and Reagents**

Brown seaweed *Sargassum polycystum* was obtained from Madura island, crude fucoidan commercial was obtained from PT. SOHO IndustriPharmasi Jakarta, chloroform, methanol, aquades, hydrochloric acid (HCl) 37%, etanol 99.8%, trifluoroacetic acid (TFA) 99%, trichloroacetic acid (TCA) 99%, HSO₄, fenol, cisteinhidrochloride, NaOH, CaCl₂, perchlorate acid (HClO₃), BaCl₂, K₂SO₄, D-glucose and D-xylose, L-rhamnose, D-galaktose, D-mannose (Sigma-Aldrich) dan L-fucose were purchased from Santa Cruz. All chemicals used were analytical grade.

**Single-step extraction of fucoidan**

General extraction process *Sargassum polycystum*. Algal were washed with fresh water to remove salt, sand and epiphytes, dried at sun rice and milled using an Coffee Mill 100 watts to pass through a 500-µm sieve, pretreated with a MeOH-CHCl₃-H₂O (4:2:1), and mixed at room temperature to remove colored matter and phenol compounds prior to extraction.

*Sargassum polycystum* pretreatment diluted in 0.03 M HCl(1:20 w/v), degraded by ultrasonic extraction (amplitude 80%, 15 minute) and than extracted with water bath at 70-90°C for 3-5 hours. The suspensions were filtrated (supernatant 1) through nylon fiber to separate the residual alga. The residual alga were washed with aquades (1:5 w/v) and filtrated (supernatant 2). The first and second supernatant were combined (Extract A). A liquid fraction from Extract A was precipitated with 1 M CaCl₂ and the mixture was maintained overnight at 4°C to release and precipitate alginate. The fraction obtained by ionization of CaCl₂ was separated by filtration. Three volume of ethanol absolute were added to the resultant filtrate and the mixture stored at 4°C for 8 hours. Ethanol-precipitated fucoidan was recovered by centrifugation (8,500 rpm, 15 minute, 4°C), dried at 50°C overnight, milled and stored for further analysis.

**Experimental Design of RSM**

Second-order experiment were the extraction process optimization using central composite design of RSM with 2 variables: temperature(x₁) and time(x₂). Optimization extraction step has 13 randomly ordered treatment with 5 replicates center point (9-13 treatments), each condition following the extraction process central composite rotatable esign(CCRD) of RSMare presented in Table 1 below. Based on experimental data, regression analysis and fit models polynomial equation second-order:

\[
y = \beta_0 + \sum_{i=1}^{2} \beta_i x_i + \sum_{i=1}^{2} \beta_i x_i^2 + \sum_{i<j} \beta_{ij} x_i x_j
\]

Where Y is the response variable, \(\beta_0\) is the intercept coefficient; \(\beta_i\), \(\beta_{ij}\) are the regression coefficients of each linear, quadratic, interaction, and \(x_i\), \(x_j\) code from two independent variable amplitude and time. The accuracy of the data analysis performed with polynomial equations model of Design-Expert software version 7 to determine the correlation coefficient \(R\) and coefficient of determination \(R^2\) of data extraction, total carbohydrate, fucose and sulfate contents. Correlation coefficient \(R\) and coefficient determination \(R^2\) were tested for statistical significance by F-test at the probability \(p=0.05\).
Analysis of fucoidan characterization

The fucose content were analyzed by cistein hydrochloride method, sulfate content by barium chloride-gelatin method, total carbohydrate content by phenol-sulfuric acid method using L-fucose as standard.

Functional group was analyzed by Infra Red Spectroscopy (FT-IR) spectrometer (shimadzumodel 8400S) using 16 scans and 400-4000 cm\(^{-1}\) frequency range. The crude extract fucoidan was conducted using potassium bromide pellets (KBr), fucoidan were smoothed by potassium bromide powder and then pressed to 1 mm pellets. Vibrational transition frequency of each spectrum corrected absorbance at baseline and normalized between 0 and 1.

Monosaccharide composition of crude fucoidan extract were analyzed using High Performance Liquid Chromatography (HPLC), 10-15 mg crude extract fucoidan were hydrolyzed with 2 MT rfluoroacetic acid (0.5 ml) at 121°C for 2 h in a glass tube closed with N\(_2\) gas. The glass tube was cooled with ice-water, centrifuged at 5000 rpm for 5 minute, the liquid fraction was neutralized to pH 7 with 2 M NaOH. The sample is injected in the HPLC system with two column. System HPLC Knauerman 5000, pump 1000, 4050 columnoven, refractive index detector S2300, aminex HPX87P column, temperature 85°C, aqubide mobile phase. Aminex HPX 87H column, temperature 65°C, H\(_2\)SO\(_4\) 0.005 M mobile phase, flow rate of 0.6 ml/min, injection volume of 20 µl.

Result and Discussion

Effect temperature and time extraction on the yield fucoidan

Research the effects of temperature and time extraction different on the yield of crude fucoidan *Sargassum polycystum*, extractions were performed at 70-90°C temperature and extraction time of 3-5 h. The yield of crude fucoidan increased the extraction temperature 60-80°C and 2-4 h, decreased the extraction temperature of 90°C and 3-5 h (Figure 1b). This is consistent with reports of extracting fucoidan and other polysaccharides of brown algae. Statistical analysis showed significant differences between 70°C, 80°C and 90°C (p<0.05).

The results showed that the treatment temperature (80°C) and extraction for 4 h had positive effect on the yield of fucoidan, meanwhile extraction at 90°C within 3-5 h decreased the yield of fucoidan. Cell walls of brown algae *Sargassum polycystum* more porous with increasing extraction temperature (70-80°C) and time (3-4 h) brown algae increased degraded, which is out intercellular tissue of more soluble in the solvent HCl 0.03 M, whereas at 90°C and 5 hours in a 0.03 M HCl solution partially degraded fucoidan. Cell wall matrix *Sargassum henslowianum* in a mild acid solution (0.2 M HCl) tend to be porous and wrinkled due to increased temperature and extraction time, the yield increased at 30-60°C and 1-3 hours, at 90°C and 5 h extraction, fucoidan were partially degraded.

Effect of temperature and extraction time on the fucose contents

Treatment at 60-90°C for 2-5 hours obtained 62.60 mg/g-145.28 mg/g fucoidan *Sargassum polycystum* fucose contents. Figure 1b' showed that the results of treatment at 70-90°C for 3-5 h had positive effect on the increased fucose contents of crude fucoidan *Sargassum polycystum*. Fucose contents increased at 60-80°C and 3-4 hours, and then declined at 90°C, 3-5 hours. Statistical analysis showed that treatment temperature and time extraction and interaction significantly affect fucose contents of fucoidan *Sargassum polycystum* (p<0.05).

Fucose contents at 70°C, 3 h were 81.13 mg/g, the highest fucose content (145.28 mg/g) achieved at 80°C and 4 hours extraction, whereas the extraction temperature of 90°C and 5 h fucose content decreased 84.91 mg/g. Low fucose content were obtained because the damage of Molecules integrity in 0.03 M HCl at 90°C and 5 hours extraction. Higher temperature, time and acid solvent of fucoidan extraction broke the cell wall matrix contract followed by acid penetration into the intercellular tissue resulting in partial degradation of fucoidan fucose damage.

Effect temperature and time extraction on the total carbohydrate contents

To investigate the effects of temperature and time extraction difference on the total carbohydrate content of fucoidan *Sargassum polycystum*, the extraction process were carried out at 70-90°C for 3-5 hours. The results figure 1b'' showed the treatment temperature and time extraction on the total carbohydrate contents. Total carbohydrate content of fucoidan tends to increase with increasing temperature and extraction time, after reaching the optimal point (80°C, 4 h) the total carbohydrate contents decline. Total carbohydrate contents
increased at 70-80°C for 3-4 h extraction, and decline at 90°C, 3-5 h. Statistical analysis showed that temperature and extraction time significantly affect the total carbohydrate content \((p<0.05)\). Total carbohydrate contents were 312.34 mg/g at 70°C and 3 h extraction, the highest total carbohydrate content were 408.74 mg/g obtained at 80°C for 4 h extraction, and at 90°C, 5 h extraction the total carbohydrate content was decreased (353.21 mg/g). The state alleged integrity polysaccharide molecules are relatively stable at 70-80°C and 3-4 h extraction in 0.03 M HCl solution, whereas at 90°C and 3-5h, polysaccharides undergo depolymerization into free sugars and solubility was decreased in 0.03 M HCl solution. Higher temperature and extraction time causes depolymerization of polysaccharide into free sugars and decreased solubility.\(^{14}\)

**Effect of temperature and time extraction on the sulfate content**

Temperature and time extraction has negatively significant effect on the sulfate contents of crude extract fucoidan *Sargassum polycystum*, sulfate contents tend to decrease with increasing temperature (70-90°C) and extraction time 3-5 h (Figure 1b'''). Statistical analysis showed that treatment temperature and extraction time significantly affect the sulfate contents \((p<0.05)\). The sulfate content at 70°C and 3 hours were 124.46 mg/g, sulfate content at 80°C and 4 h were 98.93 mg/g, sulfate content decreased rapidly at 90°C and 5 hours were 82.55 mg/g. Fucoidan has a sulfate contents that were relatively unstable in acidic solvent (0.03 M HCl), ultrasonic wave, increasing temperature (70-90°C) and time (3-5 h). Sulfate bond severed sulfate contents were decreased. High-temperature treatment in 0.03 M HCl solvent causes partial degradation of fucoidan *Sargassum henslowianum* severed bond sulfate, sulfate contents decreased rapidly at a temperature extraction 90°C and extraction time of 5-10 h\(^{1}\).

**Prediction models and statistical analysis**

Predictive models of quadratic polynomial equations response yield fucoidan, fucose contents, total carbohydrate contents and linear regression the response sulfate content result analysis regression of experimental data fucoidan *Sargassum polycystum*. Results of analysis of variance experimental data model polynomial multiple responses fucoidan has value coefficient determination \(R^2\) between 0.9055 and 0.9840 fit between the experimental results with the predicted value of the program, a second-order polynomial equation multiple responses: yield, fucose, total carbohydrate and sulfate contents (variable code) as follows:

\[
\begin{align*}
y &= 7.01 + 0.37x_1 + 0.29x_2 - 1.03x_1^2 - 0.40x_2^2 - 0.022x_1x_2 \\
y &= 132.45 + 2.24x_1 - 4.20x_2 - 29.32x_1^2 - 12.18x_2^2 - 14.15x_1x_2 \\
y &= 97.36 - 11.65x_1 - 8.23x_2
\end{align*}
\]

\(R^2=0.984\)

\(R^2=0.905\)

\(R^2=0.939\)

\(R^2=0.919\)

Examined lack of fit test multiple responses fucoidan *Sargassum polycystum* has yield as a value \(p = 0.0567\), fucose contents \((p=0.1646)\), total carbohydrate contents \((p=0.0522)\), sulfate contents \((p=0.0522)\), each response has \(p\) value higher than 0.05 indicates that the model is not significant. This result in accordance to the result of Liu et al.\(^{15}\) lack of fit test has a \(p\)-value higher than 0.05 indicates that the inaccuracy model of the pure error were not significant mean equation polynomial models was accurate.

**Respon surface and contour plot**

The 3D curve response surface and 2 D contour plots representative of the regression equations response yield, fucose content and total carbohydrate contents, whereas the sulfate contents has linear regression equation. Response surface and contour plot showing the relationship between variable experiment with the response and the type of interaction between the two variables tested. Circular shape or contour plot indicating elliptical quality of interaction between the independent variables significant or not. Circular contour plot indicates that the interaction between the corresponding variable are negligible, while elliptical contour plot sindicate that the quality interaction between the corresponding variables are significant on the response\(^{16}\). Response surface plot sand contour plots multiple response fucoidan using Design-Expert software is presented in Figure 1. Effect of interaction temperature and time extraction is not significant on the yield (Figure 1a) and total carbohydrate
content (Fig. 1a'') the shaped contourplot are circular. Interaction of temperature and extraction time are significant on the fucose contents (Fig. 1a') evident from the shaped contourplots are elliptical.

Figure 1. Contour plot (a, a', a'' and a''') and response surface plot (b, b', b'' and b''') showing the effect of temperature and time extraction on the yield, fucose, carbohydrate total and sulfate contents of crude fucoidan Sargassum polycystum.
Optimization Extraction and Validation

Optimization extraction

The optimum extraction point fucoidan, Sargassum polycystum initially degradation ultrasonic waves (amplitude 80%, 15 minute) the calculation Design Expert software was the extraction temperature of 81.00°C and extraction time 4.04h. At the optimum conditions, the maximum predicted yield fucoidan were 7.03%, 131.94 mg/g fucose contents, 406.10mg/g total carbohydrate content and 96.50mg/g sulfate contents. The optimal extraction point of fucoidan Sargassum polycystum initia ldegraded ultrasonic waves (amplitude 80%, 15 minute) occurred at a temperature(81°C), this were lower than the optimal point extraction fucoidan Sargassumhenslowianum results by Aleetal.1 which obtained at 90°C,and 4 h, but faster than Qiaoetal (4.46h) 18. This suggests that the combination extraction of pretreatment ultrasonic wave degradation and extraction of water bath heating is more effective than manually extracting water bath heating. This condition were caused by vibration of the ultrasonic waves on the initial degradation break down the cell walls of Sargassum polycystum and increase the penetration of the solvent into the cell so that fucoidan is present in the cell can extracted easily. The effect of ultrasonic cavitation produces broken power will mechanically break down the cell wall, increasing penetration of fluids towards the cell membrane and increase the transfer of material so that the components in the cell extracted easily 20.

Validation optimum point

In order to validate the adequacy of the the model equation, five verification experiment validation were carried out the optimum extraction point under these variable extraction condition: degradated ultrasonic wave (amplitude 80%, 15 minute), temperature extraction 81.00°C and time extraction 4.04 h. The Result validate experiment the optimum extraction point fucoidan (Table 2) was obtained: yield 7.15±0.07%, 131.15±1.92 mg/g fucose content, 396.86±5.58mg/g carbohydrate total content and 94.67±1.37 mg/g sulfate content. The difference between experiment validate and predicted Design-Expert software: 1.45%yield, 0.89%fucose content, 2.27%total carbohydrate content and 1.27% sulfate content. Levels error at less than 5% between experimentwere validated and predicted by software design Expert, indicated that independent variable of the optimum extraction point was satisfactory and accurate. The experiment validation and predicted value result has levels error less than 5% showed that optimum variable point value is accurate 22.

Table 1. Central Composite Rotatable Design (CCRD) matrix and respon fucoidan Sargassum polycystum

<table>
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<tr>
<th>Temperature (°C)</th>
<th>Time (hour)</th>
<th>x₁</th>
<th>x₂</th>
<th>Yield (%)</th>
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<th>Fucose content (mg/g)</th>
<th>Sulfate content (mg/g)</th>
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Table 2. Validation experimental and predicted

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<th>Variable Extraction</th>
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<th>Fucose contents (mg/g)</th>
<th>Carbohydrate total contents (mg/g)</th>
<th>Sulfate contents (mg/g)</th>
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<td>Time (h)</td>
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<td>Predicted</td>
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Functional group characterization

Functional group characterization of fucoidan *Sargassum polycystum* analyzed by using fourier transfer infrared (FTIR) in 4000-400 cm⁻¹ wavelength. The analysis of functional groups fucoidan *Sargassum polycystum* compared with fucose standards and commercial fucoidan *Fucusvesiculosus* is presented in Figure 2.

Absorption band at 3409.10 cm⁻¹ and 3533.35 cm⁻¹ in fucoidan *Sargassum polycystum*, 3425.34 cm⁻¹ in commercial fucoidan standart *Fucusvesiculosus* and 3407.02 cm⁻¹ in fucoidan showed the stretching vibrations of OH groups of carbohydrates⁸,¹⁷. Peaks at 2935.46 cm⁻¹ in fucoidan *Sargassum polycystum*, 2989.46 cm⁻¹ and 2939.31 cm⁻¹ in commercial fucoidan *Fucusvesiculosus*, 2987.53 cm⁻¹ and 2920 cm⁻¹ of fucoidan showed CH vibration. Peak 1622.02 cm⁻¹ in fucoidan *Sargassum polycystum*, 1645.17 cm⁻¹ in commercial fucoidan and 1645.17 cm⁻¹ in fucoidanstandart showed vibration C=C indicated absorbance for uronat acid.

Absorption band at 1610 cm⁻¹ in fucoidan *Sargassum henslowianum* and 1620 cm⁻¹ in fucoidan commercial *Fucusvesiculosus* indicates the absorbance of uronat acid². Peak at 1417.58 cm⁻¹ in fucoidan *Sargassum polycystum*, 1421.44 cm⁻¹ in commercial fucoidan *Fucusvesiculosus* showed the CH stretching fucose and indications of sulfate groups attached to fucoset C2 and C4 as well as a variety of vibration shadow of polysaccharide consisting of glucose, mannose, xylose and rhamnose. Fucose has a CH group absorption in wave number 1452.30 and 1414.69 cm⁻¹. Peak at 1452.30-1384.4 cm⁻¹ shows a variety of CH vibration of the polysaccharide composed of D-glucose, D-mannose, D-xylose and acid galakturonat²⁴. Peak in the range 1470-1400 cm⁻¹ indicates the scissoring vibration of CH₂ (galactose and mannose). Peak at 1139.85 cm⁻¹ and 1118.64 cm⁻¹ in fucoidan *Sargassum polycystum* and absorption band at 1080.06 and 1053.06 cm⁻¹ in commercial fucoidan *Fucusvesiculosus* showed the CH stretching vibration of fucose and S=O bound to the axial position of C-4⁸,¹³. Fucose has a strong absorbance at wave number 1200-1050 cm⁻¹. Peak at 988.77 cm⁻¹ in fucoidan *Sargassum polycystum* and 904.55 cm⁻¹ in commercial fucoidan *Fucusvesiculosus* showed CH bend vibration of polysaccharide composed of galactose, rhamnose, mannose, galactose, glucose.³⁴, Peak 850 cm⁻¹ and 820 cm⁻¹ is a sulfate group COS, sulfate bound at the equatorial position of C-2 and C-3 of L-fucose, and the axial position of C-4². Aleet al.², reported that sulfate groups of fucoidan *Sargassum henslowianum* and *Fucusvesiculosus* contained in the wave number 817 cm⁻¹ and 822 cm⁻¹, sulfate bound at C-2 and C-3 of L-fucose, absorption band 840-850 cm⁻¹ sulfate bound to fucose axial position C-4¹⁰. Commercial fucoidan *Fucusvesiculosus* sulfate groups contained in wave number 848.62 cm⁻¹ bound to the fucose axial C-4 position. Sulfate groups of the fucoidan *Sargassum polycystum* at wave number 817.76 cm⁻¹ bound to the L-fucose equatorial positions C-2 and C-3. Peak 669.25 cm⁻¹ fucoidan, *Sargassum polycystum* and commercial fucoidan *Fucusvesiculosus* at wavenumber 690.47, 669.26 showed CH₂-S vibration sulfate bound to fucose and indicated xylose²⁷. Peak 599.82 cm⁻¹ fucoidan *Sargassum polycystum* and commercial fucoidan *Fucusvesiculosus* at wave number 576.68 cm⁻¹ showed the vibration CH₂-S, fucose has a strong absorption the wave number 610.43 cm⁻¹.

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Wave number (cm$^{-1}$)

**Figure 2.** Spectrum FT-IR fucose standard (a). Spectrum FT-IR crude fucoidan commercial *Fucus vesiculosus* (b). Spectrum FT-IR crude fucoidan *Sargassum polycystum* (c)

**Monosaccharide composition**

HPLC analysis of fucoidan *Sargassum polycystum* showed that the polysaccharide consist mainly of fucose, galactose, rhamnose, xylose, mannose and glucose.

Concentration of each component monosaccharides fucoidan *Sargassum polycystum* as follows: 48.64 mg/g fucose, 35.29 mg/g xylose, 34.33 mg/g galactose, 21.93 mg/g rhamnose, 5.72 mg/g glucose and 5.72 mg/g mannose. Relative percentage monosaccharide composition of fucoidan *Sargassum polycystum* HPLC analysis is the highest of fucose 32.08%, 22.64% galactose, xylose 23.27%, 14.47% rhamnose, glucose 3.77% and 3.77% mannose. Monosaccharide composition and retention time fucoidan *Sargassum polycystum* HPLC analysis results are presented in Table 3.

**Tabel 3.** Monosaccharide composition of crude fucoidan *Sargassum polycystum* after hidrolysis with 2M TFA

<table>
<thead>
<tr>
<th>Peak</th>
<th>Composition monosaccharide</th>
<th>Retention time (minute)</th>
<th>Contents (mg/g)</th>
<th>Relative percentage (%)</th>
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</thead>
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<td>Glucose</td>
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<td>4</td>
<td>Fucose</td>
<td>10.27</td>
<td>48.64</td>
<td>32.08</td>
</tr>
<tr>
<td>5</td>
<td>Galactose</td>
<td>14.10</td>
<td>34.33</td>
<td>22.64</td>
</tr>
<tr>
<td>6</td>
<td>Mannose</td>
<td>17.35</td>
<td>5.72</td>
<td>3.77</td>
</tr>
</tbody>
</table>
Figure 3. Chromatogram of glucose, xylose, rhamnose, fucose(a) galactose, mannose (a’), (b) from hydrolyzate fucoidan *Sargassum polycystum* by AMINEX HPX 87H Column, (b’) by AMINEX HPX 87P Column
Conclusion

The optimal extraction point fucoidan *Sargassum polycystum* occurred at 81.00°C for 4.04 h. Experimental validated results showed the optimal point extraction of crude fucoidan were 7.23%, 131.15 mg/g fucose, 403.34 mg/g total carbohydrate and 94.67 mg/g sulfate content. FT-IR and HPLC analysis showed that crude fucoidan *Sargassum polycystum* were composed by fucose, galactose, xylose, glucose, mannose and sulfate.

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References


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