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Study on the optimal conditions for fucoidan extraction from *Kappaphycus alvarezii*

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ABSTRACT

Fucoidan, a polysaccharide composed of L-fucose with sulfate ester, has a wide range of pharmacological uses due to its massive number of biological activities. Besides, brown seaweed, fucoidan is also found from Kappaphycus alvarezii alga in the central coast of Vietnam. In the study, the influences of fucoidan extraction from Kappaphycus alvarezii alga by using HCl was assessed. The response surface methodology was applied to optimize three factors of material-to-solvent ratio, temperature, and time for the extraction. The monosaccharide composition was determined by using high-performance liquid chromatography. The fucoidan structures were studied using Fourier transform infrared (FT-IR) and ^{13}C-NMR (nuclear magnetic resonance). Antioxidant activity was determined using DPPH method. Monosaccharides of fucoidan were determined by high-performance liquid chromatography. The optimal results were HCl 1.39M at 85.1℃ in 3.64 hours; fucoidan content was 47.9 µg/mL. The FT-IR spectrum of fucoidan contains specific peaks of sulfate group in fucoidan in the axial or equatorial position. ^{13}C-NMR spectrum and monosaccharides of fucoidan were specific characteristic properties.

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1 INTRODUCTION

Fucoidans, polysaccharides containing substantial percentages of L-fucose and sulfate ester groups, are constituents of seaweeds, with many functions related to the physiological activity (Ale *et al.,* 2011; Fitton, 2011). Fucoidan has many benefits for good health, such as being used for functional foods, anti-cancer, immune, anti-inflammatory, antiviral, anticoagulant and antioxidant (Ale *et al.*, 2001; Kraan, 2012, Lakmal, 2014). Seaweeds have been known as huge fucoidan sources (Hahn *et al*., 2012). There are many methods of pre-treatment, solvents for extraction, precipitate, and anion exchange chromatography to purify fucoidan from the extract. The pretreatment is essential to remove chlorophyll, mannitol, salt, and other small compounds. Methanol–chloroform–water (MeOH-$CHCl_3$-H_2O, 4:2:1) (Ale, 2012) or ethanol 80-85% are usually used as pretreatment solvents (Yang *et al.*, 2008). Acid (Hahn *et al*., 2012) or water (60 − 100°C) (Luo *et al.*, 2009) can also be used as extraction solvents, and $CaCl_2$ is sometimes used to precipitate alginate during the extraction process (Bilan *et al.*, 2002). Extraction with acidic solutions, such as HCl, improved fucoidan yields (Kawamoto *et al.*, 2006). The addition of $CaCl_2$ to precipitate alginate can increase the purity of fucoidan but may also reduce its yield.

Kappaphycus alvarezii, a species of red algae, is one of the most important commercial sources of carrageenans. Carrageenans are grouped according to their sulfation patterns and distributions of 3,6-

anhydro-D-galactose residues into several families of carrageenan sub-types. Fayaz *et al.* (2005) analyzed *Kappaphycus alvarezii* for its chemical composition and found that this species is rich in protein (16.2% w/w), fiber (29.4% w/w) and carbohydrates (27.4% w/w), with a high proportion of unsaturated fatty acids (44.5% of the total; 11.0% oleic acid, 13.5% *cis*-heptadecenoic acid, 2.3% linoleic acid) and saturated fatty acids (37.0%, composed mainly of heptadecanoic acid). The bioactivity of sulfated polysaccharides like carrageenan depends on the degree and position of sulfation, the molecular weight, and the sugar type or glycosidic branching, among other features. The bioactivity of sulfated polysaccharides depends on several structural features such as the degree of sulfation (DS), the molecular weight, the sulfation position, type of sugar, and glycosidic branching. Chemical modification of the carbohydrates can lead to differences in their biological activities (Yuan *et al.*, 2011). Since 1993, *Kappaphycus alvarezii* has become widespread in Vietnam. However, it should be studied more about antioxidant compounds besides carrageenan in order to improve the value of this resource. The aim of this present work is to find suitable conditions for fucoidan extraction from *Kappaphycus alvarezii* on the central coast of Vietnam.

2 MATERIALS AND METHODS

2.1 Materials

Fresh alga *Kappaphycus alvarezii* was collected in Dam Mon area (Khanh Hoa province, Vietnam). Salt, sand, and epiphytes were removed with tap water. The samples were then rinsed carefully with fresh water and stored in a plastic bag at -5°C. To prepare for the experiment, the alga was dried to about 10% moisture, ground and sieved to one mm inhomogeneous size.

Dried alga and salted alga were bought at Dai Hai Foods Company (Ho Chi Minh city). Salted seaweed has rinsed the impurities.

2.2 Methods

2.2.1 Fucoidan extraction protocol

The algal powder was mixed with ethanol 80% and stirred for at least 12 hours at room temperature to remove lipids and pigments. Then the solutions were centrifuged to remove the supernatant. The remaining sediment was then dried at 50°C to remove the remaining alcohol prior to fucoidan extraction with solvent. After centrifugation, TCA (trichloroacetic acid) was added to the supernatant to precipitate protein (4°C, 30 minutes). The solution was then centrifuged, and the supernatant was harvested.

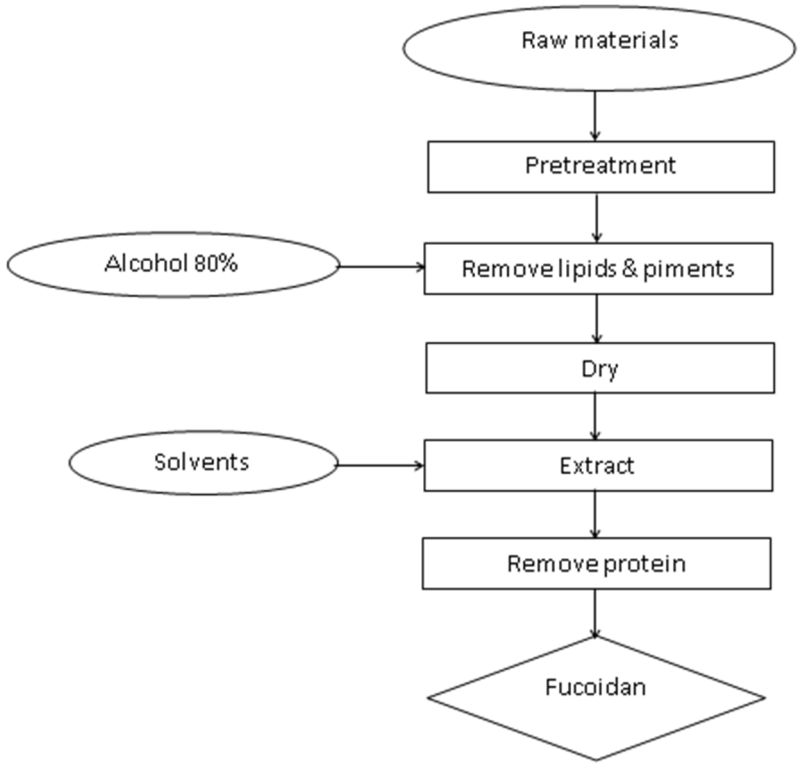

Fig. 1: Fucoidan extraction protocol

2.2.2 Investigation of fucoidan extraction conditions

The initial step of the preliminary experiment was to select appropriate seaweed type of *Kappaphycus alvarezii* alga for fucoidan extraction. Three different seaweed types, namely fresh alga, salted alga, and dry alga were examined. Based on fucoidan content, the best type was chosen. The second step of the preliminary experiment was to determine the medium extraction. Three selected media were 1 M $BaCl_2$, 1 M HCl and distilled water. The next step was to examine the solid-to-solvent ratio at different ratios (1/10, 1/20, 1/30, 1/40, 1/50, and 1/60). The final step of the preliminary experiment was to select the appropriate extraction time course for extraction of fucoidan during various extraction times (1 hour, 2 hours, 3 hours, 4 hours, and 5 hours).

2.2.3 Optimal conditions for fucoidan extraction

Optimization of fucoidan extraction from *Kappaphycus alvarezii* was carried out using the Response Surface Method (RSM). A central composite rotatable design (CCRD), five levels (±α, 0, ±1) consisting of 20 experimental runs were employed including six replicates at the center point.

All the runs were carried out in duplicate. The design variables were the solvent concentration (X1, %), extraction temperature (X2, °C) and the time (X3, min) while the response variable was fucoidan content.

2.3 Analytical methods

2.3.1 Fucoidan determination by spectrophotometer

Standard curve setting: Standard fucoidan (Sigma) was used as the standard. Three replicates of the standard were prepared in different concentrations with distilled water ranging from 10-100 µg/ml. Sample solution of each concentrations (1.0 mL) was added to a standard test tube. The test tubes were cooled on the ice at 4^0C (2-3 minutes), 4.5 mL of sulfuric acid (85%) is added and the samples are homogenized with the help of glass stirrer. Tubes were then cooled under running tap water, and 0.3% cysteine hydrochloric acid was added to the tubes and mix. Tubes were placed in darkness for 2 hours, then the absorbance was measured on a spectrophotometer at 396 nm and 430 nm. A blank with distilled water treated under the same manner was used for the zero. The two absorbance values were subtracted through the following equation: Absorbance = (A396 nm – A427 nm).

Determination of fucoidan content: The same above protocol for fucoidan determination from the samples, in which the sample is replaced by standard fucoidan.

2.3.2 Monosaccharide composition of Fucoidan

Determination of the monosaccharide composition of fucoidan was the first important stage in the investigation of fucoidan structure. The general protocol is known as hydrolysis of fucoidan into monosaccharides before analyzing each monosaccharide by HPLC. Fucoidan sample (5 mg) in tube 5 mL, was added 1mL TFA (trifluoroacetic acid) 2 M and shaken. Fucoidan was hydrolyzed at 100^0C in 6 hours. Then they were vacuum evaporated prior washed three times with MeOH to remove remained TFA. The resin was dissolved with 1 mL deionized water to the solution. This solution was used to analyze monosaccharides on the machine of IC-500 Biotrongik (Germany), Shim-pack ISA- 07/S2504 column (0.4 x 25 cm), a mobile phase of borat kali buffer, the flow rate at 0.6 mL/min. Monosaccharides were analyzed by HPLC. The purified form of fucose, glucose, galactose, mannose, xylose, rhamnose were used as standards.

2.3.3 Determination of fucoidan spectra by the IR method

Objectives: The specific peak of sulfate in fucoidan was determined by infrared spectra (IR).

The FT-IR infrared spectra of fucoidan were recorded by the machine of Tensor 37 Brucker (at the Center for Critical Analysis, Ho Chi Minh City University of Technology) with KBr beam detector in an absorbance mode 400-4000 cm^{-1}. The trembling was recorded as a graphic representation.

2.3.4 Determination of fucoidan structure by NMR spectra

The application of nuclear magnetic resonance (NMR) spectroscopy would also be very useful to obtain more structural information on fucoidan by identifying the present residues and how this polysaccharide is linked together. The side chains can also be determined and should lead to a much better understanding of the various biological properties that fucoidan have. The spectra were obtained on a spectrometer provided with a 5 mm probe at room temperature. The solution of polysaccharide samples in H_2O was sonicated at 20 kHz, and then D_2O was added to produce a solution containing calcium 40 mg in 0.4 mL of 1:1 H_2O – D_2O. Acetone was added as internal standard

(referred to Me_4Si by calibrating the acetone methyl group to 31.1 ppm). Typical parameters were as follows: maximum acquisition time, no relaxation delay, 90°-pulse angle, and 40,000 scans.

2.4 Data analysis

Statistically significant differences between samples were analyzed using a one-way analysis of variance (ANOVA) test with Microsoft Excel 2013 and IBM SPSS Statistics 20 software. P values less than 0.05 were considered statistically significant. Optimal data were handled by using JMP 10 software.

3 RESULTS AND DISCUSSION

3.1 The effects of material types and moisture on fucoidan extraction

The effects of types and moisture of materials on fucoidan extraction are shown in Table 1. The amount of fucoidan extracted from fresh alga (26.7 µg/mL) was higher than that in salted alga (19.7 µg/mL) and dry alga (12.8 µg/mL). Thus, fresh alga was selected as the raw material for fucoidan extraction. In addition, at different moistures of 82.5% (fresh alga), 47.3% (medium moisture) and 9.8% (dried alga), the value of fucoidan content obtained was not significantly different (Table 1b). In fresh seaweeds, various microorganisms can decompose plant structure, alginate, and polysaccharide compounds. The drying process can reduce the material moisture, it is facilitated for the storage. In addition, the fucoidan content in algae is dependent on harvest time (Park *et al.*, 1997; Usov *et al.*, 2001). In this study, overmuch fresh alga was harvested, then dried and stored for all experiments.

Table 1a: The effects of material types

Material types	Fucoidan content (µg/mL)
Fresh alga	26.7 ± 0.48^{a}
Salted alga	19.7 ± 0.47^{b}
Dried alga	12.8 ± 0.40^{c}

The numbers with different superscript letters in the same column were significantly different by LSD test (P < 0.05).

Table 1b: The effects of moisture

Material moisture	Fucoidan content (µg/mL)
82.5%	26.7 ± 0.48^{ns}
47.3%	26.4 ± 0.56^{ns}
9.8%	27.2 ± 0.35^{ns}

ns: not significant (P < 0.05).

3.2 The effects of solvents on fucoidan extraction

The appropriate medium extraction ensures fucoidan extracted without denaturation. According to earlier studies (Ponce *et al.,* 2003; Synytsya *et al.,* 2010; Sethi, 2012; Wang and Chen, 2016), 1 M HCl, 1 M $BaCl_2$ and distilled water were chosen to examine the extraction. The results were shown in Figure 2.

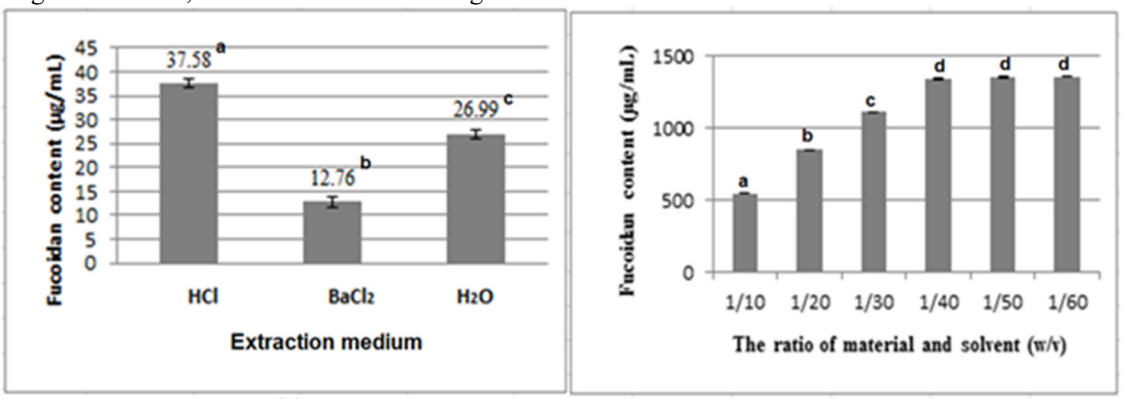

Fig. 2: The effects of solvents (a), solvent ratio (b) on fucoidan extraction

The numbers with different superscript letters in the same column were significantly different by LSD test (P < 0.05).

The yield of fucoidan extraction with 1 M HCl was higher than that of distilled water and $BaCl_2$. HCl is a proper solvent to extract fucoidan in *Kappaphycus alvarezii* algae (Fig. 2a). Besides, fucoidan extraction in HCl lower concentration is more stable than other solvents in case of structure guarantee. Also, using water at high temperature, carrageenan is extracted to increase a viscosity that impedes further stages. Thus, HCl was chosen as the extraction solvent for the following experiments. This result is similar to the published procedure by Black *et al*. 1952.

Figure 2b showed that the value of fucoidan content increased with the increase of solvent volume at the

initial stage. This is due to the fact that higher solvent volume resulted in increasing the capacity extraction of fucoidan. Higher volume of solvent used resulted in a higher amount of fucoidan (the ratio of solvent and material in the range of 1/10, 1/20, 1/30, 1/40). It helps the solvent to penetrate into the material and extract soluble constituents because of the increase of concentration gradient. However, it is not significant at the certain limit which leads to the cost of the solvent and unwanted compounds extracted like carrageenan. It is a cause of viscosity increase and obstacle for later stages. Thus, the suitable ratio of material and solvent was 1/40 (w/v).

3.3 The effects of temperature and time on fucoidan extraction

The effects of temperature and time extraction on the fucoidan content were shown in Figure 3.

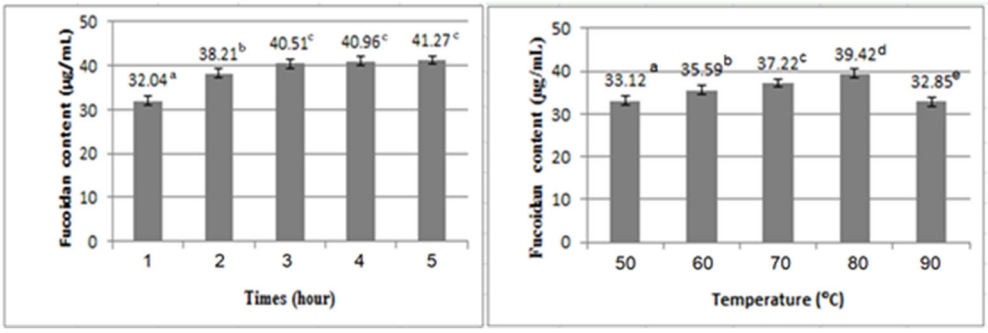

Fig. 3: The effects of temperature (a) and time extraction (b) on fucoidan content

The numbers with different superscript letters in the same column were significantly different by LSD test (P < 0.05).

The results showed that the highest fucoidan content was recorded at 80°C (39.4 µg/mL) (Fig. 3a). In the case of temperature rise, it accelerates the extraction stage, and more fucoidan is extracted. However, at the higher temperature (90°C), carrageenan, agar in algae begins to be extracted (Dunstan *et al.*, 2001; May, 2002). The high viscosity of these compounds inhibits the extraction of fucoidan. Moreover, high temperature leads to degradation in the chemical structure of ingredients.

As shown in Figure 3b, the amount of fucoidan increased over time. However, after three hours, the increase was not significant because the fucoidan content does not increase despite longer time extraction at the threshold limit yield. It also released lots of unwanted compounds (such as alginate, agar), which impede filtration, waste energy and time. Therefore, 80°C and time extraction three hours were chosen for fucoidan extraction from *Kappaphycus alvarezii* by HCl solvent.

3.4 Optimizing fucoidan extraction

Thanks to the above single factor investigation, it was found that solvent concentration, time and temperature extraction had significant effects on fucoidan extraction yield. The results of optimizing three above factors of X_1 (solvent concentration, %), X_2 (temperature, °C), X_3 (time, minute) with a central composite rotatable design CCRD were shown in Table 2.

Table 2: Results of optimizing fucoidan extraction

No.	Factors			Fucoidan content (µg/mL)	No.	Factors*			Fucoidan content (µg/mL)
	X_1	X_2	X_3			X_1	X_2	X_3	
1	-1	-1	-1	25.61	11	0	-1.68	0	35.43
2	-1	-1	1	30.21	12	0	1.68	0	42.47
3	-1	1	-1	35.67	13	0	0	-1.68	36.78
4	-1	1	1	38.09	14	0	0	1.68	42.56
5	1	-1	-1	35.83	15	0	0	0	46.23
6	1	-1	1	38.71	16	0	0	0	45.41
7	1	1	-1	37.42	17	0	0	0	42.35
8	1	1	1	49.19	18	0	0	0	47.28
9	-1.68	0	0	40.46	19	0	0	0	42.46
10	1.68	0	0	43.57	20	0	0	0	47.62

**Factors: X1 (solvent concentration), X2 (temperature), X3 (time)*

The adequacy of the model was checked accounting for R^2 and adjusted-R^2 (Caporaso, 2016). RSM models with correlation coefficient R^2 values higher than 0.80 are considered as valid ones (Joglekar *et al*., 1987). The parameter lack-of-fit is an indication of the adequacy of a model to describe the experimental factors and the response variable, considering the data not included in the regression or some variations that cannot be accounted for random error. The lack of fit illustrated in Table 3 did not result in a significant p-value for the studied variables, therefore meaning that these models were sufficiently accurate for predicting the relevant responses. The regression equation demonstrates the relationship between the fucoidan (Y) content and the influencing factors as follows:

$$Y = 45.33 + 2.69X_1 + 3.06X_2 + 2.30X_3 - 1.89X_1^2 - 2.97X_2^2 - 2.72X_3^2 \quad (Eq.1)$$

Table 3: ANOVA analysis

Source	Degrees of freedom	Sum of squares	Mean square	F value	P value > F
Regression					
Model	9	558.18	62.02	5.029	0.0094*
Error	10	123.32	12.33		
C. Total	19	681.507			
Lack of fit	5	97.39	19.47	3.76	0.863
Pure error	5	25.92	5.18		
Total error	10	123.32			

**Significant at 5% (Fischer's least significance difference test)*

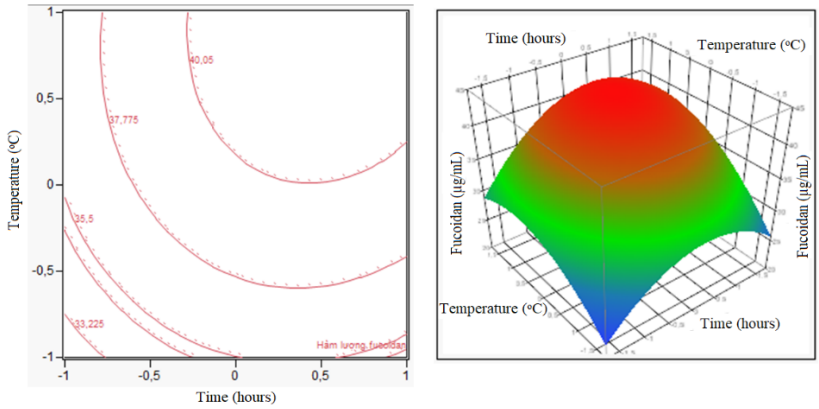

Fig. 4: The response surface and contour plots for the effects of temperature and time extraction on fucoidan content

The non-significant value of lack of fit (F = 3.76) showed that the model is fitted with a good prediction (R^2 = 0.819) (Table 3). The non-significant value of lack of fit (F = 3.28) showed that the model is fitted with good prediction (R^2 = 0.819) (Table 3). Various response 3D surface graphs were generated for fucoidan content and shown in Figure 4. The interaction effect of solvent concentration (X1), extraction temperature (X2) and time (X3) showed significant ($p < 0.01$) positive effect on fucoidan content (Eq. 1). The quadratic effect of variables (X_1^2, X_2^2, X_3^2) significantly affected fucoidan extraction. The software of JMP 10 was used to determine the optimal conditions of fucoidan extraction: X_1 = 0.77 (1.39 M), X_2 = 0.51 (85.1°C), X_3 = 0.64 (3.64 hours). At the optimal extraction conditions, fucoidan content was 47.9 µg/mL.

Three experiments were carried at optimal conditions. Experimental results showed the highest fucoidan content (45.5 µg/mL) when the extraction process was at optimal conditions, this result was consistent with the predictive model (47.9 µg/mL).

3.5 Fucoidan spectra

3.5.1 FT-IR fucoidan spectra

The region around 800–850 cm^{-1} was used to infer the position of the sulfate groups in sulfated polysaccharides. The FT-IR spectroscopy showed typical absorption bands of sulfated polysaccharides (Ribeiro *et al.,* 1994). The FT-IR spectrum of crude fucoidan from *Kappaphycus alvarezii* (Fig. 5) contained broad peak of 800-1732 cm^{-1} (sulfate at axial or equatorial positions), 1028-1637cm^{-1} (C-O-C and C-O-H stretching) (Shanthi *et al*., 2014), 1210-1250

cm^{-1} (S=O stretching) and 840-848 cm^{-1} (C-O-S stretching), which is a characteristic band for deoxysugars as fucose. In addition, crude fucoidan from *Kappaphycus alvarezii* has the characteristic wide band at 1218 cm^{-1} (S=O stretching), the peak of 846 cm^{-1} (C-O-S stretching at C4 of α-L-fucopyranose). The infrared spectrum data only indicates that the sulfate group is in the axial (C4) or equatorial (C2 or C3) of the pyranose ring of fucose or galactose (Bilan *et al*., 2004; Rodriguez-Jasso *et al.*, 2011; Yuan *et al.*, 2015).

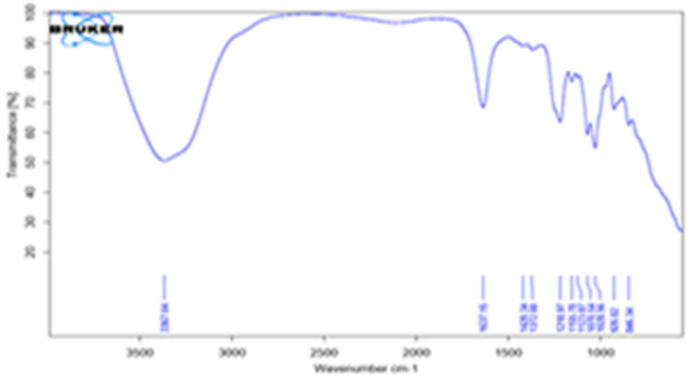

Fig. 5: FT-IR fucoidan spectra

The FT-IR was typical of fucoidans in the range of 1240-1255cm^{-1} (Marais M.F and Joseleau J.P, 2001; Rodriguez-Jasso *et al*., 2011). Rodriguez-Jasso (2013) also suggested that the typical IR absorption spectrum of fucoidan from 1240 to 1255cm^{-1} is characterized by S = O bond sulfate in polysaccharides (Rodríguez-Jasso *et al*., 2013). The absorption band at 1220-1230 cm^{-1} for S = O, 840 cm^{-1} for C4 axial, 820 cm^{-1} for C-O-S at equatorial C2 and C3 of sulfate (Yuan *et al.*, 2015). Zayed *et al*., 2016 reported the FT-IR spectrum of fucoidan_M is defined and explained: a peak at 1218 cm^{-1} (S = O stretching), 1005 cm^{-1} (C-O ether), 835 cm^{-1} (C-O-S stretching) of sulfate. Fucoidan infrared spectra at peaks: 3200-3550 cm^{-1} (strong and pointed) for O-H, 1020-1080 cm^{-1} (mean and sharp) for C-O glycoside, 1240-1260 cm^{-1} (mean) for S = O of sulfate. In addition, the two peaks at 820 and 840 cm^{-1} were determined for O-S ester sulfate, where the equatorial sulfate and axial ester are characterized at the peak of 820 cm^{-1} and 840 cm^{-1}, respectively. These peaks are characterized by fucoidan (Thi *et al.,* 2012).

3.5.2 ^{13}C-NMR spectroscopy

The ^{13}C-NMR spectra of fraction 2 are showed in Figure 6.

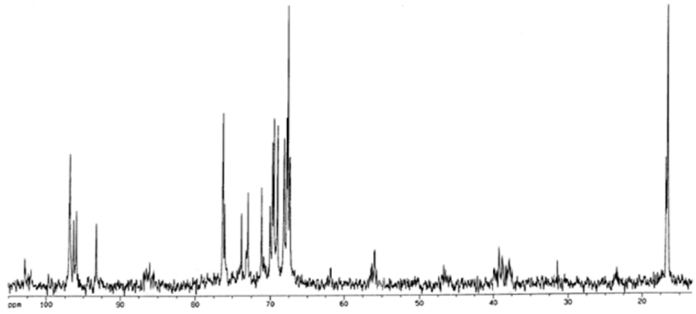

Fig. 6: ^{13}C-NMR spectra of fucoidan from *Kappaphycus alvarezii*

Like many other algal fucoidans, sulfated polysaccharides from *Kappaphycus alvarezii* had very complex ^{13}C-NMR spectrum, which was difficult to interpret completely (Figure 6). The ^{13}CNMR spectrum of fucoidans exhibited several major signals, most of them showing a certain degree of multiplicity. The multiplicity of signals with slightly different chemical shifts suggested either diversity in the positions of glycosidic bonds, or/and multiplicity of the patterns of sulfation in the fucose residues. ^{13}C-NMR spectrum of fucoidan contained several intense signals in the anomeric (97-102 ppm) and high-field (15-18 ppm) regions, which are typical of a-L-fucopyranose residues, the signal anomeric 67-86 ppm is C2-C5 carbon of the pyranoid ring (Bilan

et al., 2002). In addition, the ^{13}C-NMR spectrum also shows the presence of β-D-Galactose through the signal of the non-bonding C6 and C1 of the β-D-Galactose, corresponding to signal in the anomeric 61-62 ppm and 103-104 ppm (Vishchuk *et al.,* 2011). Thus, the ^{13}C-NMR spectrum depicted that the fucoidan samples had the structural characteristics belonged to the sulfated carrageenan group.

3.6 Monosaccharide determination

Monosaccharide composition in fucoidan such as fucose, mannose, galactose, xylose, and glucose was determined by HPLC. The content of D-xylose in fucoidan, sulfate and uronic acid was obtained (Table 4).

Table 4: Composition of monosaccharide in fucoidan

Fucoidan fractions	SO_4^{2-}	Acid uronic	Monosaccharide composition				
	(%)		%				
			Fucose	Mannose	Galactose	Xylose	Glucose
F-F1	15.67	10.5	18.34	11.17	17.87	8.4	6.5

Table 4 showed that fucose in fucoidan from *Kappaphycus alvarezii* occupies a higher content (18.34%) than other monosaccharides. Galactose content was 17.87%, which was equivalent to fucose. In addition, there were also other sugars with a lower content as mannose (11.17%), xylose (8.4%), and glucose (6.5%). Sulfate content and its position on the sugar were the most important factors, influencing the biological activity of fucoidan. The content of sulfate and uronic acid in fucoidan from *Kappaphycus alvarezii* was 15.67% and 10.5%, respectively. These results were also consistent with previous publications on the diversity of the chemical composition of fucoidan. Fucose had a significant content in fucoidan (35.8-55.85%), galactose was equal to fucose, mannose (2.5-19.2%), xylose (1.3-11.5%) and glucose (0-20.6%). This showed that monosaccharide content in fucoidan from *Kappaphycus alvarezii* is a remarkable difference with fucoidan from other seaweeds. It was lower fucose and higher uric acid content. This inferred the diversity of the chemical composition of fucoidan in different species.

4 CONCLUSIONS

For fucoidan extraction from *K. alvarezii,* HCl is a suitable solvent, the ratio of material/solvent 1/40 (w/v), the temperature of 80°C in 3 hours. The optimal condition for fucoidan extraction is 1.39 M HCl, temperature 85.1°C, 3.64 hours (CCRD experiment design), fucoidan content of 47.9 µg/mL. FT-IR, ^{13}C-NMR spectrum and monosaccharides referred the sulfated ester absorption from *Kappaphycus alvarezii* are characterized for fucoidan.

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