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The Properties of Brown Marine Algae *Sargassum turbinarioides* and *Sargassum ilicifolium* Collected From Yogyakarta, Indonesia

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ABSTRACT

Brown marine algae are the prominent source of marine natural products having bioactive metabolites. *Sargassum turbinarioides* (ST) and *Sargassum ilicifolium* (SI) were dominated in Indonesia as brown marine algae that well known as a source of fucoidan. In this study, we investigated and identified the yield of aqueous crude and purified extracts using different extraction temperatures (60°C, 70°C, 80°C, 90°C). The highest yield of *S. turbinarioides* crude extract (7.36%) was obtained at temperatures 90°C and 80°C while the highest return of *S. ilicifolium* was 3.49% at 80°C. Each of the extracts was screened using Thin Layer Chromatography (TLC), Fourier transforms infrared spectroscopy (FTIR) analysis and Nuclear Magnetic Resonance (NMR) spectroscopy. The FTIR spectrum of the *S. turbinarioides* (ST) *and S. ilicifolium (SI) extract* refer to the presence of ester sulfate groups through showing peaks at 1300 to 1200 cm^{-1} and 980 to 950 cm^{-1}. The NMR analysis proposed that the main structure of extract is L-fucopyranose and presence of different type of fucose sulfate groups. The result indicated that *S. turbinarioides* (ST) *and S ilicifolium* (SI) contained sulfate polysaccharide. This may be a potential basic for development of marine nutraceuticals.

Keywords: *S turbinarioides, S ilicifolium,* Fucoidan, FTIR spectroscopy, NMR spectroscopy

INTRODUCTION

Marine algae have been increasingly studied over the years in pharmaceutical science to discover bioactive compound to develop as medicine, nutraceutical product, cosmeceutical, functional, healthy food and to design of drug delivery system (Cunha and Grenha, 2016). Based on the exhibited photosynthetic, marine algae can be classified into three types, brown marine algae *(Phaeophyta),* green marine algae *(Chlorophyta),* and red marine algae *(Phaeophyta)* (Fleurence and Levine, 2016). Brown marine algae, largest and most complex types of algae in the world, usually have yellow-brown color. About 1800 species of brown marine algae were distributed from tropical ocean area to polar ocean area in the world (Indrawati *et al*., 2015). The Brown marine algae are the massive spread of a lot of Indonesian ocean.

Brown marine algae include the genera *Sargassum sp*., *Turbinaria sp*., and *Padina sp*., have a large number of biological compounds such as a polysaccharide (fucoidan), alginate, laminarin which account for 40-80 % of the extract (Sinurat, *et al*., 2016). Content and structured fucoidan (polysaccharide) for water-soluble fucoidan from different source depend on species (Kusaykin, *et al*., 2008), habitat and season of harvesting (Sinurat, *et al*., 2016). Numerous reports showed that fucoidan from brown marine algae

present a broad range of biological activities (Kusaykin, *et al*., 2008; Wijesinghe and Jeon, 2012) such as antithrombotic (Jolly and Iyer, 2015), antioxidant (Ponce, *et al*., 2003), immunomodulator (Raghavendran, *et al*., 2011), anticoagulant, antibacterial, anticancer, antiviral, antitumor (Senthilkumar, *et al*., 2013), gastro protector (Ahmed, *et al*., 2014).

The biological activities of fucoidan are usually reported to depend on the position and content of sulfate groups in the fucoidan backbones (Thuy, *et al*., 2015), molecular weight, the composition of monosaccharide type of sugar, glucuronic acid and fucose content (Ale, *et al*., 2011; Thuy, *et al*., 2015; Zhao, *et al*., 2008). The bioactivity of crude fucoidan was generated by the fucoidan branches and backbone structural especially of the sulfate group. It needs to elucidate the underlying factors of fucoidan activity (Ale, *et al*., 2011). Structural characteristics of fucoidan are likely dependent on the technique of extraction (Ponce, *et al*., 2003), species of marine algae, the season of harvesting, geographic location (Sinurat, *et al*., 2016), and maturity of marine algae (Zvyagintseva, *et al*., 1999). Fucoidan can be extracted and purified from brown marine algae with various multi-step processed by involving with hot water, dilute acid, dilute alkali, physical and enzymatic treatment. Many purification and fractionation steps using a large volume of solvents and long extraction times used to get purified fucoidan (Holtkamp, *et al*., 2009).

Many analytical techniques can be used to investigate and detect bioactive compounds from the extract. Thin-Layer Chromatography (TLC), Fourier Transform Infrared spectroscopy (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopy analysis could be used to identify compounds. FTIR is regarded as a potential tool for determining the molecules. There are no two samples have the same infrared (IR) spectrum. Fourier Transform Infrared spectroscopy (FTIR) studies revealed different characteristic peak values of a various chemical component in the extract. The FTIR was used to detect the typical functional groups (Ashokkumar and Ramaswamy, 2014). Nuclear Magnetic Resonance (NMR) spectroscopy was used to identify molecules in an extract, quantify functional groups, and detected impurities and minor components. NMR spectroscopy analysis can provide detailed sequence distribution, a composition of structural information, molecular weight and substitution pattern (Cheng dan Neiss, 2012). This research focused on investigating the properties of *S. turbinarioides* (ST) *and S ilicifolium* (SI) collected from Gunung Kidul, Yogyakarta, Indonesia which has not been reported so far.

MATERIAL AND METHODS

Materials

S ilicifolium (SI) and *S turbinarioides* (ST) were collected from Drini Beach, Gunungkidul, Yogyakarta, Indonesia in June 2017. The reference standards of fucoidan from *Undaria pinnatifida* were purchased from Sigma-Aldrich Chemical Co. Used solvents included aquades, ethanol, acetone, dietileter, methanol, and chloroform. All solutions used for analytical and extraction process were obtained from E. Merck (Darmstadt, Germany).

Instrumentation

Absorption band of crude extract was monitored by Fourier transform infrared (FTIR) spectroscopy (Nicolet iS10 Thermo Scientific, USA), Nuclear Magnetic Resonance (NMR) spectroscopy (JEOL 400MHz), centrifuge (HeraeusChrist GmbH, Osterode, Germany), volume pipette (Pyrex), micropipette 100-1000µL (Thermo Scientific).

Sample preparation

S. ilicifolium (SI) and *S. turbinarioides* (ST) were collected in June 2017 from Drini beach, Gunungkidul, Yogyakarta, Indonesia. Samples were identified and authenticated by Taxonomy Laboratory of Biology Departement, Faculty of Biology, Universitas Gadjah Mada to determine the taxonomic position. Brown marine algae samples were cleaned of epiphytes to remove waste and necrotic parts. Samples were washed with fresh water and were dried in the sun for two days in an oven at a temperature 50°C for two days. The samples were cut into small pieces and then pulverized into fine powder in a mixer grinder in 40 mm mesh size. The dried powder sample was kept in a dried, clean, and sealed container well.

Extraction procedure

The extraction procedure was performed as described by Li, *et al*. (2006) with slight modification. Each of *S. ilicifolium* (SI) and *S. turbinarioides* (ST) (10g) were extracted with hot water (1:10g/mL) with a variety of different temperatures of extraction such as 60, 70, 80, and 90°C for one hour at room temperature. The obtained hot water extracts were filtered through

Buchner vacuum. Each filtrate was centrifuged at 6000rpm for 10min. The supernatant was filtered by nylon cloth. Ethanol 96% was added to supernatant as far as consist of precipitation and produced double layer. The precipitate was filtered through with Buchner vacuum, washed with Ethanol 96%, acetone, and diethyl ether. The yield was air dried at 40°C for 24h and then pulverized into fine powder. The yield was calculated based on equation 1.

$$\text{Yield (\%)} = \frac{\text{Weigh of the extract (g)}}{\text{Weight of the dried biomass (g)}} \text{ x100} \quad \text{.....(1)}$$

Purification of crude extract

The dried of crude extract (1g) was dissolved in water (40mL). The solution was mixed with 3M $CaCl_2$ (1.5mL). Ethanol 96% was added to the solution. The precipitate was filtered through Buchner funnel and vacuum pump, washed with acetone, and diethyl ether. The precipitate was dried at 40°C for 24h to get a purified fucoidan (Li, *et al*., 2006).

Thin-Layer Chromatography (TLC)

The crude extract was hydrolyzed with 0.01M HCl for 60min (Pielesz and Biniaś, 2010). Samples were spotted on TLC plate by micropipette standardized for five times. TLC was performed on a silica gel aluminium plate (silica gel GF 254) to the fractionated active compound of the crude extract. The chloroform: methanol (1:1v/v) was the mobile phase. The separated spots were market, and the Rf value was calculated (Jolly and Iyer, 2015). The spot was visualized after spraying H_2SO_4: methanol (1:1v/v) and heating until the formation of dark spot on TLC plate.

FTIR spectroscopy analysis

The qualitative investigation of the purified fucoidan was done by FTIR spectrophotometer *(Nicolet iS10 Thermo Scientific, USA)*. The spectra were recorded between 4000 and 400cm^{-1} wavenumber, and the trembling was marked as a graphic representation. FTIR spectrophotometer equipped with a deuterated triglycine sulfate (DTGS) detector and a KBr/germanium as the beam splitter, interfaced to a computer having Horizon MB software and Windows operating system, was used for FTIR acquisition for spectra. A little of *S. turbinarioides* (ST) *and S. ilicifolium* (SI) *extract* was positioned in contact with attenuated total reflectance (ATR). The ATR was carefully cleaned by tissue with acetone twice before filling in with next sample. These spectra were recorded as absorbance values at each data point.

NMR spectroscopy analysis

The dried of crude extract (20 mg) was dissolved in 1.0mL Deuterium Oxide (D_2O). ^1H nuclear magnetic resonance was acquired on a JEOL 400MHz at 65°C. The measurement of ^1H NMR in ppm relative to internal reference Deuterium Oxide (D_2O) at 4.7ppm (Li *et al*., 2006; Sinurat *et al*., 2016b).

RESULTS AND DISCUSSION

Extraction of brown marine algae

S. ilicifolium (SI) and *S. turbinarioides* (ST) were extracted with hot water (1:10) at 60, 70, 80, and 90°C for one hour to get water soluble in polysaccharide (fucoidan). Centrifugation at 6000 rpm was done to separate supernatant and filtrate. Ethanol 96% was added to the supernatant to make a precipitate of the polysaccharide. Ethanol 96%, acetone, and diethyl ether were added to rinse the impurities that soluble in the extract. The yield of crude extract of various extraction temperature from *Sturbinarioides* (ST) and *S ilicifolium* (SI) (Table I). In general, the value of the yield of the crude extract is higher in case of *S. turbinarioides.* The highest yield of *S. turbinarioides* crude extract was 7.36%, obtained at temperature 90 and 80°C. The highest yield of *S. ilicifolium* crude extract was 3.49%, derived from temperature 80°C.

Table I. The yield of crude extract of various extraction temperature from *S. turbinarioides* (ST) *and S. ilicifolium* (SI)

Temperature (°C)	Yield of crude extract (%)	
	(ST)	(SI)
90	7.36	3.38
80	7.36	3.49
70	7.26	3.18
60	6.00	2.89

The temperature had an essential role in yield of extraction. Fucoidan content of the extract decreased with increasing temperature, while sulfate content decreased as the temperature increased. The polysaccharide molecules are relatively stable at 70-80°C. Time extraction and high temperature caused depolymerization of polysaccharide into decreased solubility and free sugars (Sugiono, *et al*., 2014). Fucoidan usually

exists in dry brown marine algae about 5-10%, depending on the harvesting period, thallus part that is being used, and the species of algae (Cunha and Grenha, 2016). The result of extraction can be concluded that different brown marine algae species and different condition of extraction giving the different yield of extract.

Purification of crude extract

The dried crude extract was dissolved in distilled water. The solution was mixed with $CaCl_2$ for the purification of crude extract and elimination of algin (Li, *et al*., 2006). Ethanol 96% was added to the solution to make polysaccharide precipitation. Acetone and diethyl ether was washed to remove the impurities on the extract. The precipitate was dried at 40°C for 24h to get a purified fucoidan. The highest yield powder of purified extract *S. turbinarioides* (ST) 84.61% at temperature 90°C. The highest yield powder of purified extract *S. ilicifolium* (SI) 75.67% at temperature 80°C.

Thin-Layer Chromatography (TLC)

Thin-Layer chromatography (TLC) is used to separate compound on the crude extract of *S. turbinarioides* (ST) and *S. ilicifolium* (SI) by the distribution between two phases (solid-liquid). The advantages of TLC are the ability of it to separate the compounds quickly as well as it is an inexpensive tool. Like-dissolves-like can be applied to separate molecule in the extract. The polar stationery can be attracted polar molecule more strongly. The polar solvent and nonpolar solvent were combined to use for mobile phase because the polysaccharide is the polar molecule.

The bands obtained showed the Rf value of crude extract of *S. ilicifolium* (SI) and crude extract of *S. turbinarioides* (ST) similar to the standard Rf value of fucoidan commercial (0.68). The spot was visualized by UV lamp and spray with H_2SO_4: methanol (1:1 v/v) to heating until formed dark spot on TLC plate. The dark spot is identified that monosaccharide on the extract. If the color of the spot and Rf value were the same, the extract and the commercial standard fucoidan have the same biological metabolites (Wang, *et al*., 2015).

FTIR spectroscopy analysis

The crude extract and purified extract obtained at various extraction temperatures were analyzed by FTIR to determine the specific absorption bands present in the recovered product. Fucoidan commercial was used as a standard. FTIR spectra showed that all the evaluated samples exhibited an absorption band that is typical of fucoidan. FTIR spectroscopy has widely used to evaluate the biological metabolites. FTIR spectroscopy is a sensitive, fast and non-destructive technique. Furthermore, the instrument is easy to use, and it does not consume reagents and solvents (Berthomieu and Hienerwadel, 2009; Rohman, *et al*., 2011).

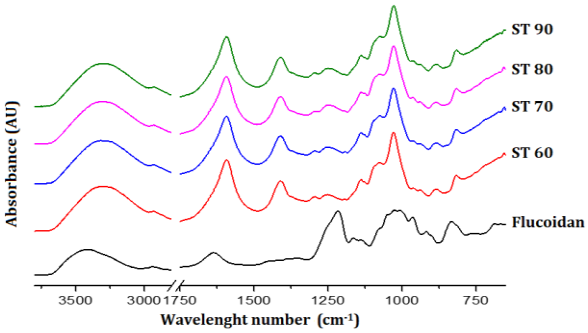

Figure 1. FTIR spectra of crude extract from *S. turbinarioides* (ST) from different extraction temperature and a commercial fucoidan from *U. pinnatifida*

Figure 1 exhibit FTIR spectra of *S. turbinarioides* (ST) crude extract obtained at extraction temperatures 90, 80. 70 and 60°C, and the standard fucoidan obtained from *U. pinnatifida* at frequency region 4000–400cm^{-1}. The crude extracts of various temperature extraction from *S. turbinarioides* (ST) showed that each sample has eight peaks in (ST) the area of wavelength number from 3316cm^{-1} to 815cm^{-1}. Fucoidan commercial has a wavelength number from 3416cm^{-1} to 833cm^{-1}. Taking in to account the spectra, it can be seen that ST 90, 80, 70, and 60°C has the same peak with fucoidan standard. The absorption band at 1216-1255cm^{-1} (S=O stretching) confirmed the presence of sulfate in the covered polysaccharides. The FTIR spectroscopy showed that the fucoidan bands were formed in the region of wave number 1500–1000 (Jolly dan Iyer, 2015). The sharp band at 840cm^{-1} and the shoulder at 820cm^{-1} (C-S-O) suggest a complex pattern of substitution at the C-4 position with other substitution at C-2 or C-3 at the equatorial position. The wavelength number 3415cm^{-1} presence of a hydroxyl group (Lim, *et al*., 2002). Fucoidan is the complex group of polysaccharide which composed of L-fucose and sulfate ester groups (Pérez, *et al*., 2016).

Table II. FTIR spectral peak values and functional groups obtained of *S. turbinarioides* (ST) and *S. ilicifolium* (SI)

Extracts prepared	Peak values (cm^{-1})	Functional groups
Sargassum turbinarioides (ST)	3315	OH group
	1593	CH bending
	1240	S=O stretching
	1255	S=O stretching
	1137	C-O stretching
	1027	carbohydrate
	939	carbohydrate
	963	carbohydrate
	816	C-S-O
Sargassum ilicifolium (SI)	3404	OH group
	1323	CH bending
	1250	S=O stretching
	1230	S=O stretching
	1137	C-O stretching
	1027	carbohydrate
	961	carbohydrate
	937	carbohydrate
	816	C-S-O

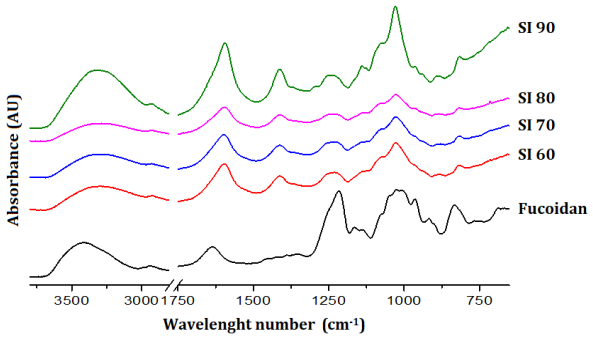

Figure 2. FTIR spectra of crude extract from *S. ilicifolium* (SI) from different extraction temperature and a commercial fucoidan from *U. pinnatifida*

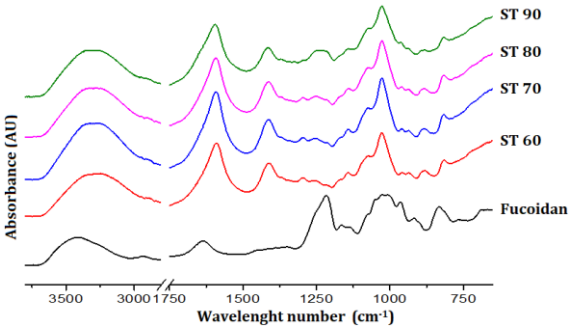

Figure 3. FTIR spectra of purified extract from *S. turbinarioides* (ST) from different extraction temperature and a commercial fucoidan from *U. pinnatifida*

The FTIR spectra (Figure 2) clearly show that all evaluated samples of crude extract of *S. ilicifolius* (SI) exhibited absorption bands. The fucoidan standard has eight peaks the same as the crude extract of *S. ilicifolius* (SI) temperature 70 and 60°C. That was different from the crude extract of *S. ilicifolius* (SI) temperature 80 only have seven peaks and temperature 80°C only have six peaks. The absorption band at 1240-1255cm^{-1} of all samples was drawn that S=O stretching confirmed the presence of sulfate in all the crude extract. The band at 815-840 confirmed that suggest a complex pattern of substitution of C-S-O. The wavelength number around 3383 to 3420cm^{-1} and near 2925cm^{-1} due to vibration of O-H and C-H. FTIR spectral peak values and functional groups obtained of the crude extract of *S. turbinarioides* (ST) and crude extract of *S. ilicifolium* (SI) (Table II).

FTIR Analysis of purified extract from *S. turbinarioides* (ST) (Figure 3). The spectrum of FTIR analysis showed characteristic absorbance bands. All of the absorption bands from purified extract and fucoidan commercial has eight peaks.

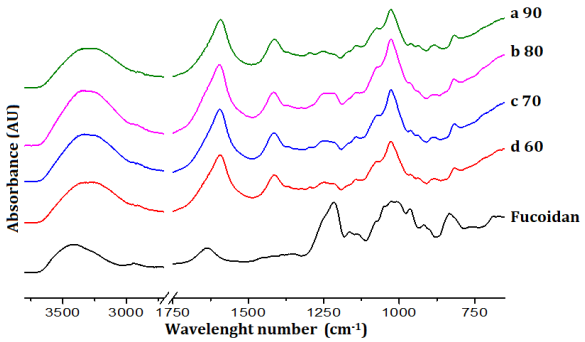

Figure 4. FTIR spectra of purified extract from *S. ilicifolium* (SI) from different extraction temperature and a commercial fucoidan from *U. pinnatifida*

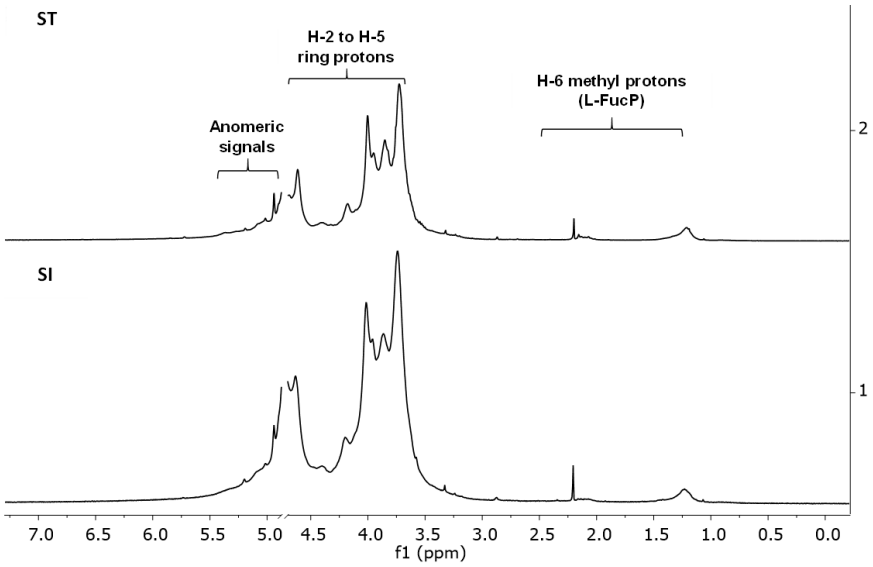

Figure 5. The stacked plot of 1H NMR spectra of crude extract from *S. ilicifolium* (SI) and crude extract from *S. turbinarioides* (ST)

The peaks at wavelength number near 1250cm^{-1} are due to the presence of asymmetric S=O stretching vibration and symmetric C-O vibration associated with the CO-SO_3 group (Manoj, *et al*., 2013). The wave near 820 cm^{-1} was present in fucoidan standard is due to more sulfation at equatorial at the position in C3 and C2 (Patankar, *et al*., 1993).

Absorption bands near 850cm^{-1} were present in the crude extract of *S. turbinarioides* (ST) indicating that there is more sulfatation position at C4 (axial) (Pielesz and Biniaś, 2010). All species and fucoidan standard showed wavelength number around 3383 to 3420cm^{-1} due to vibration of O-H and C-H. Bands at 3400cm^{-1} refer to OH stretching. The bands near 1620cm^{-1} were due to COO^- groups vibration (Pielesz and Biniaś, 2010).

The FTIR characterization of the purified extract of *S. ilicifolius* (SI) (Figure 4). All the samples of a purified extract of *S. ilicifolius* (SI) have eight peaks at temperature 80°C (Pielesz and Biniaś, 2010). The FTIR bands in the region of 900-1200cm^{-1} are characteristic of carbohydrates (Pielesz and Biniaś, 2010). Comparing the spectra of Fucoidan standard and *S. turbinarioides* (ST) showed that the sharp peak in both compound at 820cm^{-1} was related to the sulfate at the axial C-4 position, while only sulfate fucoidan showed at 833cm^{-1} corresponds to sulfate that is substituted at C-6. The information on the molecular structure and chemical bond were obtained from FTIR spectra. Mainly, the intense and wide band that was formed in 3200–3550cm^{-1} corresponded to the stretching vibration of the OH bond (-OH

stretching) and associated with the presence of hydrogen bond. The wavelength number 1635cm^{-1} is assigned to stretching vibration of -C=O bond. Furthermore, the wavelength number 963cm^{-1} and 833cm^{-1} are attributed to the bending vibration of =CH and =CH_2 bond (Chranioti, *et al*., 2016). Absorption at wavelength number 1259cm^{-1} corresponds to S=O bonds. It was supported of a peak at 817cm^{-1} has compared to sulfate at a central a position assumed that sulfate group at the position at C-2 of fucose to sulfate fucose the same as fucoidan commercial at wavelength 833cm^{-1} (Zvyagintseva, *et al*., 1999). The ester sulfate of FTIR spectrum is revealed from peak 1300 to 1200cm^{-1}. The ester sulfate at an equatorial was observed wavelength number from about 980 to 950cm^{-1} (Halling, *et al*., 2015).

Nuclear Magnetic Resonance (NMR) spectroscopy analysis

The ^{1}H NMR spectrum of *S. ilicifolium* (SI) and *S. turbinarioides* (ST) (Figure 5). The ^{1}H NMR spectrum analysis was used to determine the anomeric configuration structure. The ^{1}H NMR analysis contained chemical shift from 5.0 to 5.8 ppm attributed to α-linked-L-fucose and β-sugars (H1β) of anomeric protons (H1α). The spectrum resonance characteristics of fucoidan with signals from ring protons (H-2 to H-5) at 3.5 to 4.5ppm. It was confirmed the presence of different types of fucose sulfate groups with changes in monosaccharide patterns and glycosidic linkage positions. The chemical shift of ^{1}H NMR analysis between 1.1 to 2.1 ppm confirmed H-6 methylated proton signals determined L-fucopyranose (L-FucP) (Alwarsamy *et al*., 2016; Lim *et al*., 2016).

CONCLUSION

The present study was demonstrated that the fucoidan could be successfully extracted from *S. ilicifolium* (SI) and *S. turbinarioides* (ST) by water extraction. The highest yield of crude extract from *S. turbinarioides* (ST) was 7.36%, (90 and 80°C) and crude extract from *S. ilicifolium* (SI) was 3.49%, (80°C). The crude extract or purified extract were contained phytochemical metabolites especially ester sulfate that revealed of FTIR spectrum from peak 1300 to 1200cm^{-1}. Fucoidan from brown seaweed is complex polysaccharide with complicated structures. The ^{1}H NMR spectrum analysis was used to determine the anomeric configuration structure. The ^{1}H NMR has determined L-fucopyranose as the primary structure of extract and presence of different type of fucose sulfate groups.

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