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Original Article

CHARACTERIZATION, FTIR SPECTRA PROFILE AND PLATELET ANTI-AGGREGATION ACTIVITY OF CRUDE FUCOIDAN FROM *SARGASSUM CRASSIFOLIUM*

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ABSTRACT

Objective: This research was aimed to investigate the characterization and FTIR spectra profile in correlation with platelet anti-aggregation activity of crude fucoidan from *Sargassum crassifolium*.

Methods: Dried brown seaweed powder was extracted using 0.1 M of HCl solution after pre-macerated with 85% of ethanol, precipitated with ethanol, and lyophilized as crude fucoidan. It was analyzed for fucose, sulfate, carbohydrate content, FTIR spectra and evaluated the *in vitro* platelet anti-aggregation activity. Partial least square (PLS) analysis was conducted to identify the functional group that contributes to the platelet anti-aggregation activity.

Results: The fucose, sulfate, and carbohydrate content were 3.64-9.44%, 12.05-18.01%, and 11.45–21.41%, respectively. The results of the statistical analysis showed significant differences in fucose, sulfate, carbohydrate content, and platelet anti-aggregation activity of crude fucoidan extracted from *Sargassum crassifolium* at different harvest times. According to the result of PLS analysis using FTIR spectra data and the value of IC50, the functional group that contributed to the platelet anti-aggregation activity were OH, C=O, and S=O.

Conclusion: Platelet anti-aggregation activity of crude fucoidan was proportional to the level of sulfate not to fucose and carbohydrates levels. The functional group of crude fucoidan which were correlated with this activity could be identified.

Keywords: FTIR spectra, PLS analysis, Platelet anti-aggregation, *Sargassum* sp, Sulfated fucan

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INTRODUCTION

Sargassum sp is one of the brown seaweed. About 621 species and variant of *Sargassum* sp was described [1], with 79 species are grown in Indonesia [2]. The availability of *Sargassum* in Java is a potential source for self-sufficiency in raw materials, especially for cardiovascular diseases. Cardiovascular diseases are one of four main types of non-communicable diseases, according to the World Health Organization (WHO), in addition to chronic respiratory diseases, cancer, and diabetes [3]. One of the causes of cardiovascular disease is abnormal platelet aggregation.

Sargassum sp is a good source of sulfated polysaccharides, namely fucoidan. The exploration of *Sargassum* for cardiovascular disease has been reported in several publications. *Sargassum polycystum* extract has *in vivo* platelet anti-aggregation activity [4, 5], as well as the crude fucoidan nanoparticles of *Sargassum polycystum* [6]. Fucoidan, as the marker of *Sargassum* sp, has a very complex structure and varies from each species. Some factors that cause differences in the structure and content of fucoidan are the environment (temperature, ocean currents, depth of growth, salinity), harvest period, species, stage of growth, season, extraction process, type of solvent extraction, temperature, and time of extraction [7]. Fucoidan content has been shown to vary over the year, with the highest in autumn [8].

Sargassum crassifolium is one of the brown seaweed that grows in Java. This name is currently regarded as the synonym of *Sargassum aquifolium* (Turner) C. Agardh [1]. Fucoidan from this species had anti-gastric ulcer activity in mice [9], immunomodulatory [10], and inhibitory of Icam-1 and Vcam-1 in lipopolysaccharide-induced raw 264.7 cell [11]. Besides the various biological activity of fucoidan, most of the FTIR analyses reported only the types of its functional groups. Multivariate analysis using FTIR had given information on which the functional group in such extract correlated with biology activity are [12-14]. Many publications described this species; however, fucoidan characteristics and its FTIR profile in correlation with ADP-induced platelet aggregation inhibition activity of *Sargassum crassifolium* had not been reported before. In this study, we investigate the partial characterization and FTIR spectra profile of crude fucoidan derived from *Sargassum crassifolium* during four months and partial least square (PLS) analysis to investigate the functional group of sulfated fucan that has a strong affected on platelet anti-aggregation activity.

MATERIALS AND METHODS

Materials

The brown seaweed was obtained from Cicalobak coast, Karangwangi, Garut (S 7°33'; E 107°33'), during September-December 2018. A taxonomic determination was performed by the Marine Biology Laboratory, Biology Faculty, University of Indonesia. L-cysteine hydrochloride, L-fucose (>99%), and adenosine diphosphate (ADP) were purchased from Sigma-Aldrich (St. Louis, USA), potassium bromide (KBr FTIR grade), ethanol, and the other chemicals were pro-analysis grade from Merck (Darmstadt, Germany). Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP) were obtained from the Indonesian Red Cross.

Extraction of sulfated fucan

The brown seaweed was cleaned with running water, air-dried, and milled into powder. The extraction of fucoidan was conducted according to the procedure described by Fletcher *et al.* [8] with modification. The seaweed powder was macerated with 85% ethanol (1:10) for 6 h at room temperature. The residue was then washed with acetone and dried. About 200 g of residue was extracted by kinetic maceration with 2000 ml of 0.1 M HCl at 80 °C for 4 h. The extraction results were then filtered. The filtrate was adjusted to a pH of 7 using 1 M $Ca(OH)_2$, then added with 1% $CaCl_2$ (1: 1.25) and store at 4 °C overnight. The solution was centrifuged at 3000 rpm for 15 min. The supernatant was collected and 96% v/v ethanol was added until the final concentration was 40% v/v. The mixture was allowed for 4 h at 4 °C to precipitate laminarin. After centrifugation, the filtrate was collected and added with 96% v/v

ethanol until the final concentration becomes 70% v/v. The precipitate was collected and lyophilized as crude fucoidan (CF).

Determination of total carbohydrates

Total carbohydrate content was carried out based on DuBois method. About 5 mg of CF dissolved in 5 ml of water, then 0.5 ml of 0.3% phenol and 5 ml of concentrated sulfuric acid were added, mixed, and allowed to cool (soaked in ice) for 30 min. The solution absorbance was measured at 490 nm using Shimadzu UV 1800 spectrophotometer. Carbohydrate content was calculated using the calibration curve of glucose with concentrations of 40, 60, 80, 100, and 120 µg/ml. The calibration curve was generated by plotting the glucose concentration versus absorbance [15].

Determination of L-fucose content

About 10 mg of CF was diluted with water until the concentration was 500 µg/ml. About 1.0 ml of test solution was poured into the test tube. The reaction tube containing the solution was cooled for 2-3 min, 4.5 ml of 85% H_2SO_4 was added, homogenized using a vortex. The reaction tube was covered, put in a boiling water temperature (±100 °C) for 10 min, then cooled under running water, and 1.0 ml of 0.1% L-cysteine hydrochloride was added homogenized using a vortex. Allow standing in a dark room for 2 h. The greenish-yellow color was formed [16]. The absorbance of this solution was measured using Shimadzu UV 1800 spectrophotometer (Shimadzu, Kyoto, Japan). Fucose content was calculated using the calibration curve of L-fucose with a concentration of 20, 40, 60, 80, 100, 120 µg/ml. The calibration curve was generated by plotting between the L-fucose concentrations versus absorbance in the wavelength of 400 nm [17].

Determination of sulfate content

The determination of sulfate content was carried out by Dogson and Price method using 0.5% barium chloride in 0.5% gelatin which was left for 2-3 h before use. About 2 mg of CF was dissolved in 2 ml of water, added with 2 ml of 4% TCA and 2 ml of barium chloride-gelatin. The absorbance of this solution was measured in the wavelength of 420 nm. Sulfate content was calculated using a standard curve of sodium sulfate solution with concentrations of 100, 150, 200, 250, 300 µg/ml [18]. These assays were conducted using Shimadzu UV 1800 spectrophotometer (Shimadzu, Kyoto, Japan).

Measurement of FTIR spectra

FTIR analysis was done using FTIR spectrophotometer Shimadzu IR Spirit-T with DLATGS (deuterated L-alanine doped triglycine sulfate) detector (Shimadzu, Kyoto, Japan), equipped with IR solution software. About 2 mg of dried extract was mixed and homogenized with about 200 mg of KBr, pressed with a pressure of 80 KN for 5 min. The spectra of the sample were recorded in the middle IR region (4000-400 cm^{-1}) in absorbance mode, 32 scans/min, and a resolution of 4 cm^{-1}. Spectra data were saved in ASCII, then converted to MS excel format. Each sample was recorded in triplicate. The obtained spectrum was analyzed by chemometric for identification and detecting any functional groups that might contribute to platelet anti-aggregation activity. All absorbance spectra were then submitted to smoothing, normalization, and baseline correction. Standard normal variation (SNV) was applied before PLS analysis [19].

Platelet anti-aggregation assay

Platelet anti-aggregation activity was conducted using a modification of the Light Transmission Aggregometry method. The turbidity of the sample was measured using a spectrophotometer in a wavelength of 600 nm instead of aggregometry [20, 21]. About 1 ml of PPP and 4 ml of 0.9% NaCl were poured into the glass tube, 1 ml PRP and 4 ml of 0.9% NaCl were into the second tube, the other tubes were filled with 1 ml PRP, 3 ml NaCl 0.9%, and 1 ml of clopidogrel, 1 ml of CF (each of 100, 200, 500, 800 and 1000 µg/ml). All of the tubes were incubated at 37 °C for 5 min, then added 20 µl of 5 µM ADP, re-incubated for 30 min at 37 °C. Tube 1 was used as a blank, tube 2 as a negative control. Clopidogrel was used as a positive control. The absorption of these mixtures was measured using Shimadzu UV 1800 spectrophotometer at a wavelength of 600 nm, then decreased absorption compared to the negative control, and calculated the percentage of antiplatelet inhibition activity [22]. The aggregation inhibition activity is expressed as % inhibition calculated by using the following equation:

$$\%\text{inhibition} = \frac{\text{Abs PRP} - \text{Abs(PRP+sample)}}{\text{Abs PRP}} \times 100\% \text{ [23]}$$

The IC50 value were obtained from the plot between concentrations versus % inhibition.

Data analysis

Determination of fucose, sulfate, carbohydrate content and evaluation of activity had been performed in triplicate. The average values were reported along with the standard deviation. One-way analysis of variance (ANOVA) with (p-value<0.05) was conducted using SPSS software 23.0 (IBM, Armonk). Partial least square analysis to process FTIR spectra data in correlation with IC50 of inhibition activity was conducted using the Unscrambler X ver. 10.4. (CAMO, Norwegia).

RESULTS AND DISCUSSION

Extraction of CF was performed using maceration with heating for four hours. CF extracted from seaweed during four months has the same light brown color.

Fucose, sulfate, and carbohydrate content

The yields of extraction and analysis content results are summarized in table 1. Determination of fucose, sulfate, carbohydrate content and evaluation of platelet anti-aggregation activity had been performed in triplicate and expressed as the average value±SD.

Table 1: The yields of extraction and analysis content results

	Yields (%)	Fucose (%)*	Sulfate (%)*	Carbohydrate (%)*
September	1.08±0.11[b]	5.98±0.03[b]	15.98±0.04[c]	17.24±0.30[b]
October	1.12±0.09[c]	9.44±0.13[c]	12.05±0.45[a]	21.41±0.37[d]
November	1.05±0.98[b]	5.80±0.01[b]	13.70±0.13[b]	19.74±0.18[c]
December	0.42±0.05[a]	3.64±0.03[a]	18.01±0.20[d]	11.45±0.18[a]

Information: data was given in mean±SD, n=3. The value in the same column, followed with different superscript letters, indicates a significant difference at the level of p<0.05

The brown seaweed used in this study was taken during September-December 2018. Determination of fucose, sulfate, carbohydrate content and evaluation of activity had been performed in triplicate and expressed as the average value±SD. Based on the results, the highest yield was obtained in October (1.12%) and the lowest yield in December (0.42%) (table 1). Differences in harvest time and season caused this variation. In December, the sea waves in the territory of Indonesia are higher and greater than during the transition in September-October-November. This is very closely related to the occurrence of seasonal winds that affect changes in sea waves. Changes in sea waves affect the absorption of nutrients and the formation of fucoidan compounds.

The good-growth brown seaweed generally occurs in months where rainfall and temperatures are low. This period had minimum wind speed conditions. Conversely, unproductive seasons generally occur

when rainfall is high with strong winds so it affects the environmental conditions of the waters and the conditions of sea waves. The productive season occurs in June-November, while in November-March, the seaweed is unproductive [24]. The difference in harvest time will affect the brown seaweed constituent between low rainfall and high rainfall.

Fucoidan was the sulfated polysaccharide found in *Sargassum* sp. L-fucose is the major component of fucoidan. L-fucose content can be determined based on the greenish-yellow of fucose-cysteine complex formation [16]. Based on the calibration curve using L-fucose as the reference standard, the L-fucose content during four months could be seen in table 1. The highest content was crude fucoidan which was harvested in October, which was 9.44%, and the lowest was in December. Based on statistical analysis, the fucoidan content was significantly different from all months. There were differences in the fucose level based on different harvest times. Variation of fucose content was also met in fucoidan from *Fucus serratus*, *Fucus vesiculosus*, *Ascophyllum nodosum*, and *Sargassum horridum* [8].

Sulfate content was one of the polysaccharide characteristics of *Sargassum* sp. The range of sulfate content was 12.05–18.01%. Based on the statistical analysis, there were significant differences between the samples. The range of carbohydrate content during four months were 11.45–21.41% and there were significant differences during four months. Carbohydrate total as sugar also had monthly variation in the range of 17.7–29.8%, while the ratio of sulfate-total sugar were in the range of 0.91–1.12 [25].

Platelet anti-aggregation activity

The potency of crude fucoidan as an aggregation inhibitor was expressed as IC_{50} value as seen in table 2. Platelet anti-aggregation activity of the brown seaweed fucoidan was determined in some concentrations series, which were added with Platelet Rich Plasma (PRP). Clopidogrel was used as a positive control, while the negative control was PRP with the addition of 0.9% NaCl. Percent inhibition was calculated from the decreasing plasma uptake of ADP-induced negative controls. The potency of sulfated polysaccharide as aggregation inhibitor was expressed as IC_{50} value (table 2). The Platelet anti-aggregation activity parameter in this method was IC_{50} value. IC_{50} value was obtained from the linear regression equation of the concentration versus percentage of inhibition plot. The smaller the IC_{50} value the higher the activity.

Clopidogrel is a platelet anti-aggregation drug that was obtained from the selectively inhibiting adenosine diphosphate (ADP) binding on the platelet ADP receptor, thereby inhibiting ADP-mediated activation of the glycoprotein complex GPIIb/IIIa and causing inhibition of platelet aggregation. This drug has long been used in antithrombotic treatment [21]. Based on the value of IC_{50} there were significant differences, except September and October.

Table 2: Platelet anti-aggregation activity of crude fucoidan and clopidogrel

Sample	IC_{50} (mg/l)*
clopidogrel	465.39±1.65[a]
September	478.50±7.94 [ab]
October	490.33±13.65[b]
November	643.67±108.08[d]
December	577.33±12.86[c]

*data was given in mean±SD, n=3. The value in the same column, followed by different superscript letters, indicates a significant difference at the level of $p<0.05$. Fucose, sulfate, total carbohydrate content, and % aggregation inhibition of 1000 µg/ml CF generated the profile as seen in fig. 1.

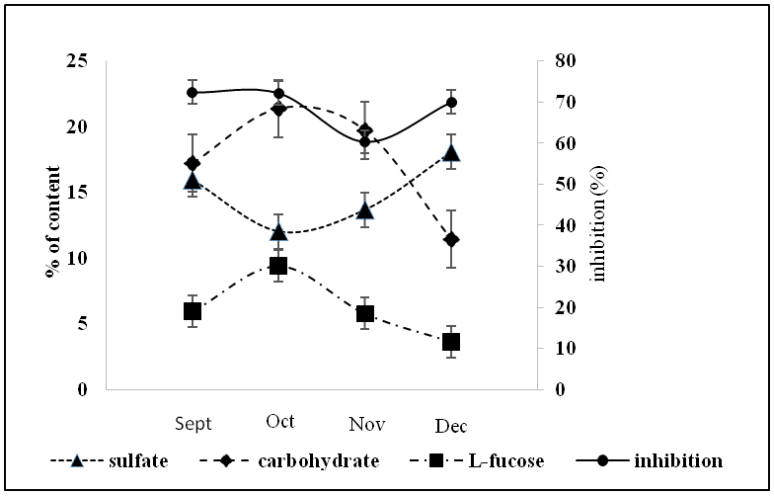

Fig. 1: Fucose, sulfate, total carbohydrate content, and % platelet aggregation inhibition during four months

The L-fucose content profile was the same as the carbohydrate (fig. 1). The greater the carbohydrate content, the greater the L-fucose content. Based on fig. 1, the platelet anti-aggregation activity was not affected by fucose content. Besides L-fucose as a major component of fucoidan, there were minor amounts of other monosaccharides, mainly galactose and mannose [26, 27]. Monosaccharide composition is not a factor that influences fucoidan activity [28]. Some publications mentioned that sulfate influenced the activity. The anticoagulant, cytotoxic, and antitumor activities of fucoidan from *Sargassum aquifolium* increased with the degree of sulfation [29]. High sulfate content (21%) is required to inhibit the α-amylase activity of fucoidan [28], and decreasing sulfate decreases the anticancer activity of fucoidan [30]. According to fig. 1, the activity from the trend data of September, November, and December showed the higher the sulfate content, the higher the inhibition percentage.

FTIR spectra

The representative FTIR absorption spectra derived from CF during four months were seen in fig. 2. All of the functional groups of CF could be summarized as mentioned in table 3.

As mentioned in table 3, the absorbance at 3000-3500 cm^{-1} indicated a stretching vibration of the OH group in all samples. The absorption of the OH group is in the range of wavenumbers 3600-3200 cm^{-1} [31]. The absorption band at wavenumbers 1610–1651 cm^{-1} is the C=O vibration of uronic acid [32]. The presence of uronic acid indicates the presence of alginic acid, which is a constituent of alginate polymers. It showed that the extract still contained alginate even though in the extraction process, it had been removed with calcium chloride. In brown seaweed, alginate is the most dominant polysaccharide [33]. Uronic acid also is met as a contaminant in

purified fucoidan [33, 34]. The peak at 1412.3-1424 cm^{-1} indicates the C-H vibration of the polysaccharide consisting of D-glucose, D-mannose, D-xylose, and galacturonic acid [32]. The absorption band in the range of 1470-1400 cm^{-1} indicates the vibration of CH_2 belongs to galactose and mannose [32]. The presence of absorption at this wavenumber indicates the presence of CH_2 vibrations from polysaccharides. The absorption band of galactose and mannose was also found in the FTIR spectra of *Sargassum cinereum* extract [35]. The strong peak between 910.34-1126.35 cm^{-1} is a characteristic of fucose and the presence of absorption at that wave number indicates a stretching vibration of S=O bound to the axial C-4 position [36]. These results are in agreement with reports for groups wavenumbers for the hetero-oxy compound that absorbance range of 1200–1100 cm^{-1} belongs to organic sulfates [31].

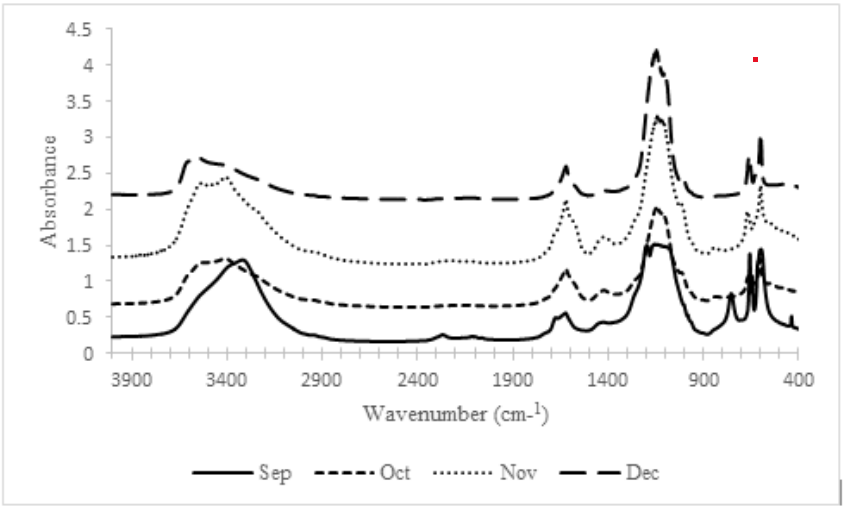

Fig. 2: The representative overlay FTIR absorption spectra of crude fucoidan

Table 3: Functional groups of crude fucoidan

No	Wavenumber (cm^{-1})	Functional group	Vibration mode
1	3300-3500	OH	Stretching
2	1610–1651	C=O	Vibration of uronic acid
3	1412-1424	CH_2	stretching
4	1100-1200	S=O	stretching
5	720	S-O-S	stretching

Pre-processing step was done before PLS analysis. This step aims to reduce variation data that didn't affect the analytical information. The effect of the absorbance of functional `toward platelet anti-aggregation activity of CF could be evaluated by PLS analysis. PLS analysis aims to build the relationship between FTIR profiling and platelet anti-aggregation activity. PLS model needs a predictor variable and response variable. The IC50 value obtained in platelet anti-aggregation activity was used as response variable (y), and absorbance data from 4000-400 $cm^{-1}$of CF were used as a predictor variable (x). PLS analysis generated a regression coefficient plot. It was used to simplify the relationship between all predictors and responses.

Twelve FTIR spectra and twelve IC_{50} data were further analyzed by PLS using Unscrambler X ver. 10.4 (CAMO, Norwegia). PLS analysis results show some functional groups that had negative regression values. These are OH in the 3315 cm^{-1} regions, C=O in the wavenumber of 1650 cm^{-1}, and S=O that gave the absorbance in 1140 cm^{-1} (fig. 3).

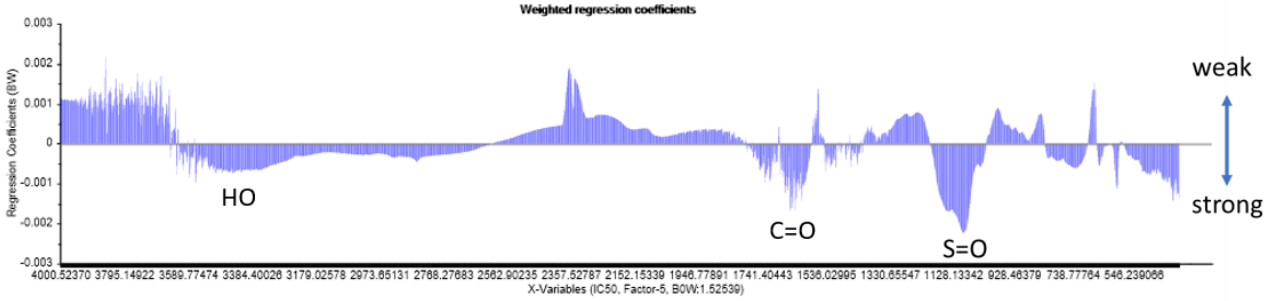

Fig. 3: Partial least squares (PLS) plot weighted regression coefficients of platelet anti-aggregation activities of CF using FTIR spectra

PLS, is often used to correlate plant's biological activities with spectroscopic data (absorbance value). Line loading plot (weighted regression coefficients) in the PLS model was used to evaluate the functional groups [13]. A regression coefficient plot was used to summarize the correlation of all predictors and responses at PLS. X variable that had high regression coefficient are such variable that has an important role in the regression model. Using PLS analysis, functional groups that had a role in α-glucosidase inhibition expressed as IC_{50} value had negative regression coefficient value [37].

There are reference plots and prediction plots. Both of them correspond to a linear relation between prediction value with measure value. In the quality analysis using PLS, there are R^2 (linearity) and RMSE (Root Mean Square Error). R^2 value in accordance with linearity between the predictor variable and response variable. RMSE value shows value variation using the PLS model toward observation value [38]. In this research, the PLS model of correlation between functional groups toward platelet anti-aggregation generates the R^2 value of 0.985 and RMSE of 0.01. The OH functional group that contributed the activity may be OH belongs to non-carbohydrate compounds. Fucoidan had not been further purified, so maybe there were such impurities in companion with crude fucoidan. The purified fucoidan of *Sargassum siliquosum* still had polyphenol as impurity [34]. The C=O functional group belongs to alginate. Alginate had *in silico* activity in purinoceptor P_2Y_{12} [39]. P_2Y_{12} receptor is the most clinical drug target for inhibition of platelet aggregation [40, 41]. The third functional group that had strong contribution to platelet inhibition was S=O of sulfate. This is in agreement with sulfate content results.

CONCLUSION

Platelet anti-aggregation activity was not directly proportional to the levels of fucose and carbohydrates. Sulfate content gave a contribution to platelet anti-aggregation activity. The functional group which was associated with the platelet anti-aggregation activity are OH, C=O, and S=O.

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AUTHORS CONTRIBUTIONS

All the authors contributed to the design and implementation of the research, the analysis of the results, and the writing of the manuscript.

CONFLICT OF INTERESTS

The authors report no conflicts of interest.

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