

Antimitotic and Antiangiogenic Assay of Fucoidan from *Sargassum oligocystum*

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ABSTRACT

Algae are the large group of autotrophic organisms that do not have organized cell structures but have unique biological and pharmacological activities. Mitosis and angiogenesis are standard biological processes that needed in the maintenance of life. The inappropriate activity of these processes can lead to severe complications like cancer. Due to the abundance of algal species in Panay, Philippines. This study determined the antimitotic and antiangiogenic activities of fucoidan from *Sargassum oligocystum*. *Sargassum oligocystum* was extracted with hot distilled water (80°C for 4 hours) and subjected to lyophilization which yielded an average of 7.67% fucoidan from Aklan, Antique and Capiz brown algae. Fucoidan showed identical spectra when compared to the reference standard using FTIR Spectrometer at a wavelength of 650 to 4000cm⁻¹. Antimitotic assay revealed that 100mg/ml of fucoidan solution has a significant result in mitotic index and the effectiveness was comparable with Methotrexate. Likewise, 100mg and 10 mg of fucoidan in gelatin disc exhibited a reduction in the number of branches and entire branches length of the blood vessel, and the effectiveness was comparable to Retinoic acid. With distinction among the treatments, 100mg of Fucoidan is a promising therapeutic agent because it showed antimitotic and antiangiogenic activities.

Keywords: *Phycology, antimitosis, antiangiogenesis, Sargassum oligocystum, brown algae, experimental research, Philippines*

INTRODUCTION

Cancer is one of the leading causes of death and considered to be a significant public health problem in the society. Recently, there are eleven new cancer cases recorded daily by the Cancer Coalition of the Philippines (Dizon, 2017). The Philippines has the highest mortality rate regarding breast and prostate cancer among Asian countries (Torrevillas, 2017) and 26.3% of the Philippine population settled beneath the national poverty line and cannot afford the standard-of-care treatments for cancer (Philippine Statistics Authority, 2016). This new scenario must be given full attention especially in the development of

appropriate scheme for cancer control and the establishment of alternative medicine within the context of cost-effective allocation of resources.

Marine Science Institute of the University of the Philippines revealed that red alga has substantial economic benefits as the source of food and ingredient for industrial products. Several active constituents were also identified that could be used for cosmetic, biomedical and pharmaceutical research. (Algae World News, 2015). There were several international studies on pharmacological activities of brown algae, but only a few works of literature were cited in the Philippines about the biological activities of *Sargassum*. Specifically, the *S. oligocystum* literature on anticancer, antimetabolic and antiangiogenic activities have not been fully established in the country. As such, this study was conducted to determine if the isolated fucoidan from *S. oligocystum* has antimetabolic and antiangiogenic activities.

Mitosis and angiogenesis are standard biological processes that needed in the development and maintenance of life. When these mechanisms become overactive and uncontrolled, these can lead to a severe complication like the formation of a tumor which can progress to cancer. Cancer formation involves mutation of genes due to alteration of DNA structure and sequence. Each gene contains a specific function and has a particular set of instructions. Modification and alteration in the DNA can cause changes in the gene, thus, creating abnormalities in its activities, like rapid cell division that can lead to the formation of a tumor, which in turn can induce the development of new blood vessels. Treatment of cancer has evolved and scientific investigation has increased by utilizing natural resources. Several studies on antimetabolic and antiangiogenic activities of plants were associated as two mechanisms of cancer treatment. Abundance of natural resources must be highly considered to maintain the ecological balance of environment in such a way that the ecosystem is not at risk. Legendary reports of sailors and usual encounter of fishermen described the abundance of brown algae in Panay Island, Philippines because of the endless span of floating algae that makes the seashore colored brown every summer. Visayas region is composed of several islands where the main livelihood of the residents is fishing. This scenario is one of the considerations for the selection of the site for sample collection since the locals are very familiar with the existence and habitat of algal species.

Algae are the large group of simple and autotrophic organisms. They do not have well-organized cell structures like plants, but marine research paved a way to use these in cancer treatment because of their unique biological activities (Hansen et al., 2013). They are considered one of the most abundant sources of pharmacologically active compounds with various therapeutic applications. In the Philippines, most literature on seaweeds discussed the agricultural benefits, its ecology, botany and chemistry but not much about the pharmacological activities. On the other hand, foreign documentaries stated the different biological and pharmacological activities of seaweeds. Thus, this study was conducted to fill in the gap between the local and international literature on pharmacological activities of fucoidan from *S. oligocystum*.

Different species of brown algae were studied for its pharmacological activities that focused on the mechanisms of anticancer property. The antioxidant and antimetabolic activities of *Padina tetrastratica* from the west coast of India (Jose et al., 2015). The cytotoxic activities of *Gracilaria foliifera* and *Cladophoropsis* species from the Persian Gulf and Oman sea (Erfani et al., 2015). The antimetabolic activity of *Phaeocystis pouchetii* from Norway (Hansen et al., 2013). The antiangiogenic properties of *Chlorella pyrenoidosa* (Kyadari et al. 2013), *Codium fragile* (Sugawara et al., 2014) and *Amansia multifida* (De Souza et al. 2012).

The isolated fucoidan from *Sargassum* species in Pangasinan revealed a highly sulfated group with heteropolymeric fucan chain under Fourier transform infrared (FTIR) spectrometric characterization, and it was found to possess an antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Nieva et al., 2015). Fucoidan is a heteropolysaccharide which is found mostly in brown algae and echinoderms. Since, the backbone of fucoidan contains hexose units, which is a monosaccharide it favors dissolution in polar solvents like water. Thus, this study was conducted to evaluate antimitotic and antiangiogenic activities of the isolated fucoidan from *S. oligocystum* using distilled water as the extracting solvent. Consequently, this study was formulated to establish additional information on the biological activities of fucoidan from *S. oligocystum* in the Philippine setting.

Framework of the Study

Marine algae are significant in the aquatic ecosystem especially for fishes, corals, and other sea creatures. Seaweed is the most extensive form of algae used as food, a source of nutritional supplements and formulated for therapeutic purposes. Fucoidan is high molecular weight, a fucose-containing sulfated polysaccharide which is freely soluble in water. High molecular weight and water-soluble polysaccharides are considered to be bioactive against cancer cells (Lemieszek and Rzeski, 2012).

Physicochemical testing and Fourier transform infrared characterization was used to identify the presence of key functional groups that played an essential role in the antimitotic and antiangiogenic activities.

Another vital structure of fucoidan is the sulfate group, which disrupted the Gap0/Gap1 (G0/G1) phase of cell division (Atashrazm et al., 2015, Zhang et al., and 2011) and suppressed cyclins (Boo et al., 2013 and Banafa et al., 2013). Fucoidan also reduced the expression of vascular endothelial growth factor (VEGF) for angiogenesis (Xue et al., 2012) and its high sulfate content inhibited the binding of VEGF to the receptor in the cell membrane (Koyanagi, 2003).

Meristematic onion root tip model was used to evaluate the antimitotic activity because the roots grow fast and numerous. Thus many cells in different stages of mitosis could be observed. On the other hand, chorioallantoic membrane assay was used because of the high degree of vascularization and easier to use with limited ethical concern (Ribatti et al., 2010).

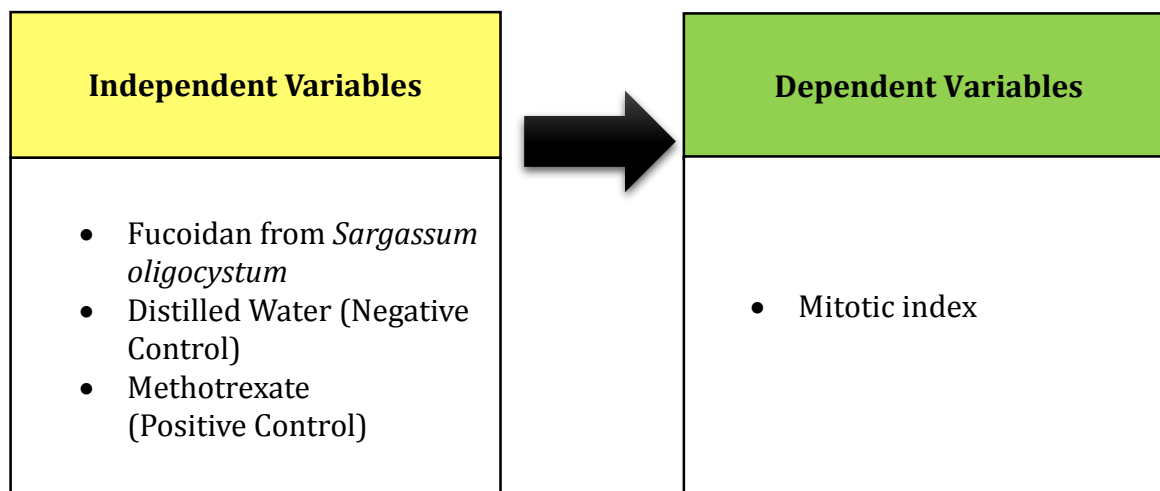


Figure 1. Independent and Dependent Variables for Antimitotic Assay

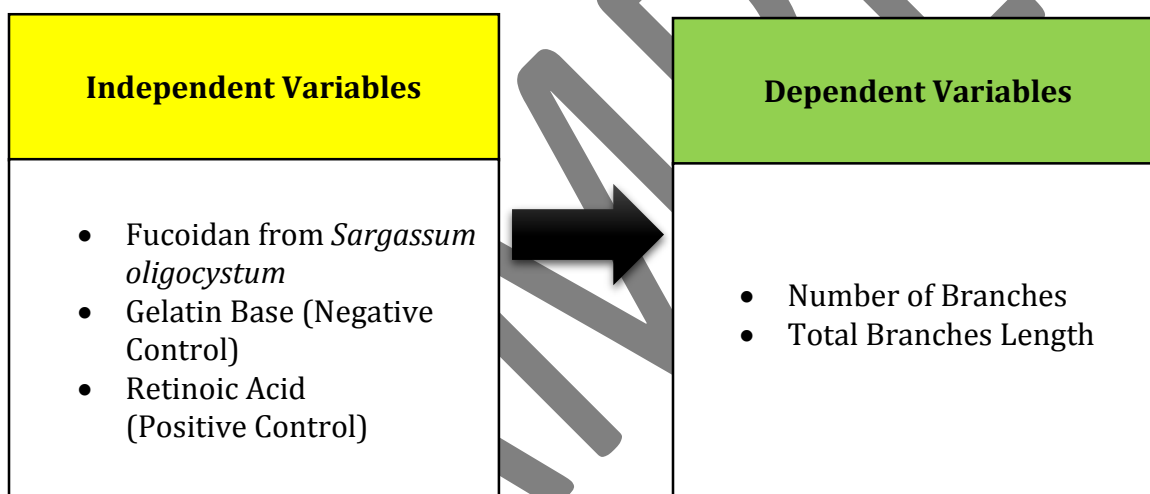


Figure 2. Independent and Dependent Variables for Antiangiogenic Assay

OBJECTIVES OF THE STUDY

The study aimed to determine the antimitotic and antiangiogenic activities of Fucoidan from *Sargassum oligocystum* collected in the Provinces of Aklan, Antique and Capiz, Philippines. Specifically, this study aimed to: (1) measure the percentage yield of Fucoidan from *Sargassum oligocystum*; (2) characterize the fucoidan through Physicochemical Testing and Fourier Transform Infrared Spectrometry; (3) evaluate the antimitotic activity of Fucoidan using the Meristematic Onion Root Tip (MORT) Model; (4) determine the antiangiogenic activity of Fucoidan using Chorioallantoic Membrane (CAM) Assay through Image J Angiogenesis Analyzer in terms of number of branches and total branches length;

and (5) determine if there is a significant difference in the antimitotic and antiangiogenic activities of fucoidan compared with the positive control.

MATERIALS AND METHODS

Collection and Preparation of Materials

Sargassum oligocystum. Fresh and mature brown algae (*Sargassum oligocystum*) were collected from the subtidal zone of Aklan, Capiz and Antique Provinces. The fresh and whole plant of *S. oligocystum* was stored in a clear, wide-mouth glass bottle for macroscopic analysis and plant authentication.

Duck Eggs. Fertilized eggs were collected from a poultry farm at Brgy. Matanga, New Buswang, Kalibo, Aklan. The number of days of eggs was strictly considered to ensure uniformity of the test media. The eggs were incubated using a digital analytical incubator at a temperature of 37.5°C and with 62.5% relative humidity (Mondol, 2006).

Apparatus and Instruments. The apparatus and instruments used in the study were Mettler balance, graduated cylinder, electric grinder, dissecting/stereomicroscope with a built-in camera, a biological microscope with a built-in camera. Also, digital analytical egg incubator with humidity meter, Fourier transform spectrometer and high-resolution camera were used. A 2-cm diameter ring, stirring rod, test tubes, Erlenmeyer flask, beakers, reagent bottles, surgical blade, forceps, sterile transpore, candling light, digital microcaliper and polyethylene bags were used as supporting devices.

Test Drugs. Solutions of brown algae (*Sargassum oligocystum*) extract (1mg/ml, 10mg/ml and 100mg/ml for antimitotic assay; 1mg, 10mg and 100mg per disc for antiangiogenic assay). Methotrexate and Retinoic Acid.

Chemicals and solutions. The chemicals used in the study were: water, ethyl alcohol, glacial acetic acid, Molisch's Reagent, Lugol's solution, barium chloride solution, concentrated sulfuric acid, dilute hydrochloric acid, KBr pellets, KBr powder, gelatin USP, and aceto-carmin.

Test Media. The test medium employed in the study were the meristematic onion root tip for antimitotic assay and fertilized chicken eggs for antiangiogenic assay. (Mondol, 2006).

Groupings. The grouping of test media and their treatment was accomplished through Completely Randomized Design (CRD). Distilled water for antimitotic and USP gelatin disc for antiangiogenic studies as the negative controls, Methotrexate solution (Positive control for antimitotic assay) and Retinoic Acid solution (Positive control for antiangiogenic test). There were two sets of assay namely: antimitotic and antiangiogenic assays. Each group in each assay was composed of three trials with ten determinations each trial.

Extraction and Physicochemical Identification

The brown algae were washed thoroughly with fresh water to remove dirt, dust and extraneous matter. The brown algae were air-dried for seven days and milled using an electric grinder to increase the surface area of contact with the solvent. Four kilograms of brown algae were macerated with 8000 mL distilled water and subjected to electrothermostatic water bath heating at 80°C for 4 hours (Sugiono, Widjanarko, & Soehono, 2014; Ayesha, Sultana, Ara, & Ehteshamul-Haque, 2010; Guevara, 2004). The extractive was filtered using fluted filter paper. The filtrate (extractive) was placed in several

containers with a maximum volume of 100 mL extract in each container, then covered with aluminum foil and subjected to freeze-drying (lyophilization) using Eyela Lyophilizer.

The physical and chemical properties of the isolated fucoidan were compared to the standard fucoidan (pharmaceutical/food grade).

Physical Tests. This includes odor, color, taste, appearance, solubility, and pH of the lyophilized extract.

Chemical Tests. This includes tests for carbohydrate, sulfate, and polysaccharide. Carbohydrate is the major macromolecule where fucoidan is classified based on its basic unit (fucose). Sulfate and polysaccharides are two chemical sub-groups present in the chemical composition of fucoidan.

Carbohydrate Test. Molisch Test is the general test for carbohydrates. One (1) ml of Molisch's reagent (α -naphthol dissolved in ethanol) was added to fucoidan solution (0.5g in 1ml water). The solution was mixed gently and 0.5 ml concentrated sulfuric acid was added slowly down the sides of the sloping test tube without mixing to form a layer. A positive reaction is indicated by the appearance of a purple ring at the interface between the acid and test layers (Sadasivam, & Balasubramanian, 1987).

Sulfate Test. Barium Chloride Test is the common test used to detect the presence sulfate. One (1) ml of fucoidan solution (0.5g in 1ml water) was acidified with few drops of dilute hydrochloric acid. Few drops of barium chloride were added to the acidified solution. The formation of a white precipitate is an indication of a positive reaction (Housecroft & Sharpe, 2012).

Polysaccharide Test. Lugol's Test (Iodine Test) is the general test for polysaccharides. One (1) ml of iodine solution was added drop by drop to fucoidan solution (0.5g in 1ml water). The formation of a blue-black or dark purple solution is an indication of a positive reaction (Ball, Hill, & Scott, 2012).

The characterization of fucoidan was accomplished through the use of Fourier Transform Infrared Spectroscopy to determine the quality of fucoidan in comparison to the reference standard fucoidan.

Instrumental Methods (Characterization). Functional groups of fucoidan were analyzed by Agilent Technologies Fourier Transform Infra-Red (FTIR) spectrometer using 32 sample and background scans with 8-labeled resolution, Happ-Genzel Apodization at 650-4000 cm^{-1} frequency range. (Sugiono et al. 2014). The quality of each fucoidan sample including the reference standard was compared against ten samples stored in the Attenuated Total Reflection Library of the FTIR Spectrometer (Agilent Technologies).

Antimitotic Assay

The fresh onion bulbs weighing 40-50 grams were obtained from Kalibo Fruit and Vegetable Stand (Shivasharanappa & Londonkar, 2014). The dry outer layers of onion bulbs were peeled off and subjected to a series of containers with tap water (Fiskesj , 1993). The bulbs were allowed to grow in dark area for 48 hours at ambient temperature until the roots measured 2-3 cm, the tap water was changed at 24-hour interval until the desired root measurement was achieved.

The bulbs were divided into eleven (11) groups, each group contained three (3) trials, and each trial contains ten (10) bulbs. Bulbs were treated according to treatment assignment below:

Treatment A: Methotrexate	:	0.10mg/ml
Treatment B: Aklan Fucoidan	:	100mg/ml
Treatment C: Antique Fucoidan	:	100mg/ml
Treatment D: Capiz Fucoidan	:	100mg/ml
Treatment E: Aklan Fucoidan	:	10mg/ml
Treatment F: Antique Fucoidan	:	10mg/ml
Treatment G: Capiz Fucoidan	:	10mg/ml
Treatment H: Aklan Fucoidan	:	1mg/ml
Treatment I: Antique Fucoidan	:	1mg/ml
Treatment J: Capiz Fucoidan	:	1mg/ml
Treatment K: Distilled Water	:	

The bulbs were incubated at 22±2°C for 72 hours away from direct sunlight. The treatment solutions were changed every 24-hour interval (Shrivastava, Sijoria, Dey, Pandey, Jadhav, & Wanjari 2015). The root tips with 2-3 mm length were collected and placed in a watch glass. The roots were transferred into the vial containing fixative of acetic acid and ethanol solution (1:3) and soaked for 24 hours. Each root was placed on a glass slide, and one drop of 0.1N HCl was added, followed by 2-3 drops of 0.5% acetocarmine red stain (Saboo, Khadabadi, & Tapadiya, 2012). The slide was warmed gently over the alcohol lamp. The excess dye was blotted with filter paper. The more stained tip portion of the root was cut using a surgical blade, and a drop of water was added. The root was mounted using cover slip and was squashed by gently tapping the cover slip with the blunt end of the needle (Amrita, 2015).

The squashed roots were examined under the bright field microscopy using OMAX LED Compound Microscope. Documentation and visualization were accomplished through the use of OMAX 18 Megapixel Microscope Camera with Toupview Imaging Software running under Windows Platform. The dividing cells were noted by locating the metaphase, anaphase, and telophase using the printed table for mitotic study. The counting of nondividing and dividing cells was validated by the three research assistants against the counting made by the researcher.

The mitotic index was calculated through the use of the following formula:

$$\text{Mitotic Index} = \frac{\text{Number of Dividing Cells}}{\text{Total Number of Cells}} \times 100$$

Antiangiogenic Assay

Anas platyrhynchos (duck) eggs were obtained from the Farm Poultry in Matanga, New Buswang, Kalibo, Aklan. The eggs were incubated at a temperature of 37.5°C and with 62.5% relative humidity for ten days using HHD Digital Analytical Incubator. The eggs were candled using a candling device at the blunt end of each egg to identify the air sac and prominent blood vessels (mainly Y shape). The chorioallantoic membrane (CAM) was separated from the shell by making a shallow burr hole perpendicular to the previously identified blood vessel in the center of the egg. A mild suction was applied to the blunt end

burr hole to displace the air sac and drop the CAM away from the shell. With the use of a surgical blade, a small window was made on the shell above the identified blood vessel (Y shape). A 0.5 cm diameter (Tan and Tantiado, 2012) and 0.1 cm thick gelatin-based disc with corresponding treatment below were placed or planted on the Y-shaped vascular area.

Treatment A: Retinoic Acid	:	10mg/disc
Treatment B: Aklan Fucoidan	:	100mg/disc
Treatment C: Antique Fucoidan	:	100mg/disc
Treatment D: Capiz Fucoidan	:	100mg/disc
Treatment E: Aklan Fucoidan	:	10mg/disc
Treatment F: Antique Fucoidan	:	10mg/disc
Treatment G: Capiz Fucoidan	:	10mg/disc
Treatment H: Aklan Fucoidan	:	1mg/disc
Treatment I: Antique Fucoidan	:	1mg/disc
Treatment J: Capiz Fucoidan	:	1mg/disc
Treatment K: USP Gelatin Base	:	

The window was sealed with sterile transpore, and the eggs were returned to the incubator. On day 13, the eggs were harvested, opened and transferred to Petri dishes. A ring with 1-inch in diameter was placed on the location where gelatin disc was planted on the Y-shaped area to limit the field of analysis (Mondol, 2006). A digital camera was used to capture the vascularized area on the CAM (Deryugina & Quigley, 2009). The quantitation of angiogenesis was made by measuring the number of branches and total branches length through the use of Image J Software on Angiogenesis Analyzer running under JAVA Platform (Carpentier, Martinelli, Courty, & Cascone, 2012).

Statistical Treatment of Data

The data gathered were subjected to appropriate descriptive and inferential statistics. For descriptive statistics, mean was used to describe the average results of fucoidan, positive and negative control. Analysis of Variance measured the significant difference among the different concentrations of fucoidan in antimitotic and antiangiogenic assays. On the other hand, the Duncan Multiple Range Test was used to evaluate the differences between fucoidan and the positive controls (Methotrexate for antimitosis and Retinoic Acid for antiangiogenesis).

RESULTS AND DISCUSSION

Percentage Yield Determination of Fucoidan

The significance of percentage yield is to ascertain the amount of fucoidan obtained from the specific weight of brown algae. Extraction temperature and solvent contact time are critical parameters that are useful in optimizing extraction of fucoidan. The cell walls of brown algae, *Sargassum* becomes more porous at a temperature ranging 70 – 80°C (Sugiono et al., 2014). Hence, extraction of fucoidan was carried out at a temperature of 60°C for 4 hours to avoid degradation of the active constituent.

After lyophilization, brown algae samples from Aklan, Antique and Capiz yielded 8.56% by weight, 7.87% by weight and 6.58% by weight, respectively. The difference in the yield of fucoidan depends on the geographical and climatic condition of their habitat.

Characterization of Fucoidan

Fucoidan was characterized using Fourier Transform Infrared (FTIR) Spectrometer by Agilent Technologies under default method set at a wavelength ranging 4000 cm^{-1} to 650 cm^{-1} . Pharmaceutical or food grade fucoidan was used as the reference standard in comparing fucoidans obtained from *Sargassum* of Aklan, Antique, and Capiz. In figure 1, fucoidan showed several bands in the regions from 700 cm^{-1} to 3400 cm^{-1} . The broad peak between 3300 and 3400 cm^{-1} suggested the presence of OH group in the monosaccharide monomer, at 2900 cm^{-1} for the aliphatic C-H, a peak at 1700 cm^{-1} for C=O stretching vibration for acetate groups (Zayed et al., 2016). The bands in the region 700 to 1000 shows C-O-C bending vibrations in glycosidic linkage (Fernando et al., 2017). The bands between 1600 cm^{-1} and 1000 cm^{-1} corresponded to the glycosidic linkage stretch vibration of C-O-C and C-O-H. Also, the signals close to 1600 cm^{-1} and 1500 cm^{-1} are due to the asymmetric and symmetric stretch vibration of C-O-O of uronic acid (Mao et al., 2005; Singthong, Cui, Ningsanond, & Goff, 2004). The weak band at 1000 cm^{-1} indicate the presence of S=O stretching vibration of sulfate group (Sinurat et al. 2015). On the other hand, there was no stretch observed at wavenumber 3400 - 3500 cm^{-1} which is the characteristic band of the amino group. It denotes that protein is absent in the three samples of fucoidan. Protein is considered in this determination because it is the principal contaminant of fucoidan.

Fucoidan standard (pharmaceutical/food grade) purchased from Xian Sonwu Biotech Co., Ltd, Xian; China has a quality of 89.19%, Aklan fucoidan (92.40%), Antique fucoidan (88.64%) and Capiz fucoidan (84.32%).

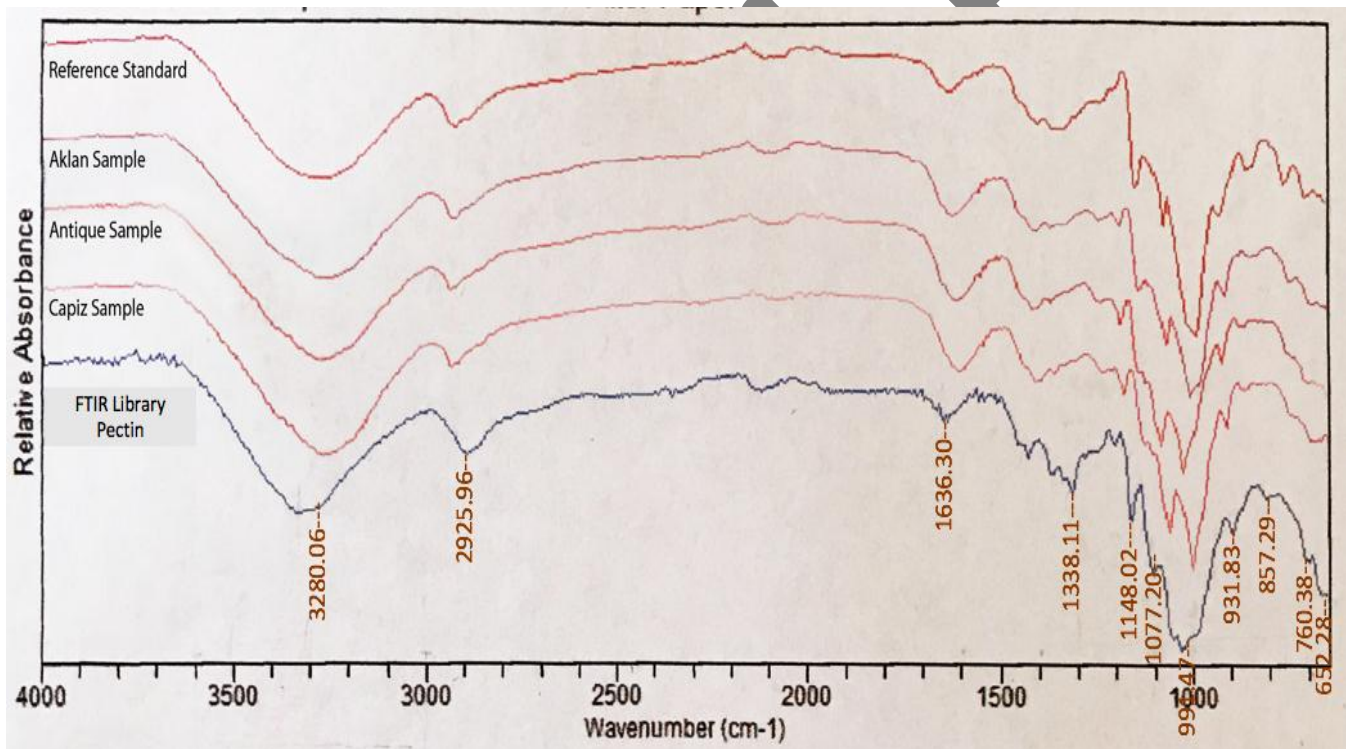
In addition to spectral characterization, physical and chemical tests were performed to ascertain the identity of the sample fucoidans (Aklan, Antique and Capiz). The lyophilized fucoidan obtained was brown in color, fishy in odor, salty in taste, powder in form and with pH of 7.83 (Aklan), 7.70 (Antique) and 7.67 (Capiz). Fucoidan is freely soluble in distilled water (0.1g in 1ml), and soluble in 5% NaOH (0.1g in 15ml), 5% NaHCO_3 (0.1g in 20ml) and 5% HCl (0.1g in 15ml). On the other hand, it is insoluble in ethanol, acetone, and hexane. Both the reference standard and fucoidan samples have the same physical properties except for the color which is light yellow and a pH of 7.78 in the case of the reference standard.

Fucoidan is a component of the cell wall of brown algae, composed of L-fucose, sulfate, mannose, galactose, glucose, and xylose (Sugiono et al., 2014). Monosaccharides are significant components of fucoidan and based on the structure of sugars. It contains more -OH, thus, hydrogen bonding is favored making it freely soluble in water. For semi-polar and non-polar solvents, fucoidan has difficulty in dissolution due to limited hydrogen bonding, and these solvents have low dielectric constant.

Chemical tests were also performed to compare the chemical characteristics of the sample fucoidan with that of the reference standard. The formation of the purple ring at the interface between the acid and test layers after the addition of Molisch's reagent indicated the presence of carbohydrate. The production of white precipitate upon addition of Barium

chloride test solution suggested the presence of sulfate group. The presence of polysaccharides was confirmed by the use of Lugol's solution through the production of blue-black solution. These three chemical identification test results were found to be identical to the reference standard.

The fibrillar part of the cell wall and intercellular spaces of brown algae contain fucoidan. Primarily, it contains sulfated polysaccharide of same or different monosaccharide units. The above tests conducted confirmed the presence of primary components, as carbohydrate in nature, with polysaccharide and sulfate regardless of the specificity of each constituent (Skriptsova, 2015).



Evaluation of Antimitotic Activity of Fucoidan

Meristematic onion root tip model was used in the assessment of the antimitotic activity of three fucoidan samples (Aklan, Antique and Capiz). In this study, metaphase, anaphase, and telophase were the stages of mitosis considered as dividing cells. Prophase was not included in the counting of dividing cells to avoid misinterpretation as the interphase.

Figure 4 showed that 100mg/mL concentration of each fucoidan samples has the lowest mitotic index Aklan (M=3.86, SD=1.09), Antique (M=3.99, SD=1.05), Capiz (M=4.28, SD=0.80), followed by 10mg/mL Aklan (M=13.75, SD=1.91), Antique (M=15.23, SD=1.84), Capiz (M=16.66, SD=1.38) and 1mg/mL Aklan (M=27.86, SD=2.72), Antique (M=29.56, SD=2.64), Capiz (M=30.23, SD=2.73).

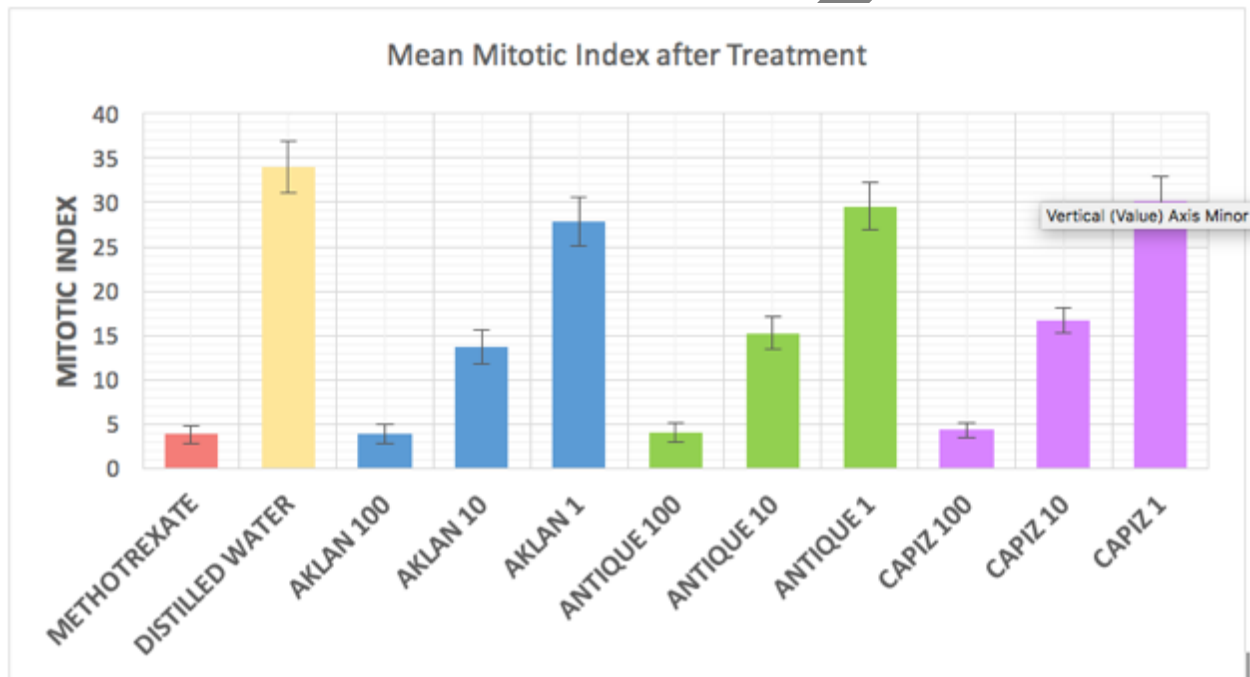


Figure 4. Mean Mitotic Indices After Treatment with Methotrexate (+control), Fucoidan (Aklan, Antique, and Capiz) and Distilled Water (-control)

The relationship between mitotic index and concentration of fucoidan was assessed by the Pearson product-moment correlation coefficient. There was a negative and strong relationship between mitotic index and concentration of fucoidan, Aklan ($r=-0.856$), Antique ($r=-0.872$) and Capiz ($r=-0.892$). This means that the mitotic index is concentration-dependent, the higher the concentration of fucoidan, the lower the mitotic index.

One-way analysis of variance in table 1 was used to compare the mitotic index of three fucoidan samples with methotrexate. There was a significant difference in the mitotic index of three fucoidan samples when compared to methotrexate at $p<.01$ level of significance, Aklan [$F(22884.59, 632.20) = 1312.19, p=0.000$], Antique [$F(23752.73, 609.510) = 1412.67, p=0.000$] and Capiz [$F(23881.68, 566.558) = 1528.01, p=0.000$].

Table 1. Analysis of Variance of Mitotic Indices after Treatment with Fucoidan

(Aklan, Antique and Capiz)

		Sum of Squares	df	Mean Square	F	Sig.
Aklan	Between Groups	22884.598	4	5721.150	1312.190	.000
	Within Groups	632.200	145	4.360		
	Total	23516.798	149			
Antique	Between Groups	23752.735	4	5938.184	1412.671	.000
	Within Groups	609.510	145	4.204		
	Total	24362.244	149			
Capiz	Between Groups	23881.681	4	5970.420	1528.018	.000
	Within Groups	566.558	145	3.907		
	Total	24448.239	149			

p < 0.01

Post hoc comparisons using Duncan multiple range tests indicated that the mean score for the mitotic index of three fucoidan samples with the concentration of 10mg/mL and 1mg/mL were significantly different than the mean mitotic index of methotrexate. However, the three fucoidan samples with the concentration of 100mg/mL did not differ significantly from the mean mitotic index of methotrexate. These results suggest that 100mg/mL fucoidan concentration is comparable with methotrexate. Specifically, the mitotic index of Aklan fucoidan with a concentration of 100mg/mL (M=3.86, SD=1.09) is most comparable with methotrexate (M=3.78, SD=0.94). Aklan fucoidan has the highest quality regarding the presence of functional groups that closely resemble with the FTIR library standard. Also, the carbohydrate content, functional substituents and sulfate groups of fucoidan contribute to the inhibition of cell division.

Fucoidan affects the Gap phase of cell division thereby modifying the chromosomal activity in preparation for the S phase (Atashrazm et al., 2015 and Zhang et al., 2011). When this process is altered, DNA replication is not evident. If not all cells were affected in the Gap phase, another mechanism could also be inhibited, the suppression of cyclins which can lead to deactivation of cyclin-dependent kinases, thus transcription is down-regulated (Boo et al., 2013 and Banafa et al., 2013). Fucoidan can stimulate apoptosis by activating the caspase-independent pathway. This mechanism is vital in fast-dividing or overactive cells

(Philchenkov et al., 2007). Tubulin organization in the metaphase stage is also affected by fucoidan, consequently, the phases of anaphase and telophase, fucoidan can block nuclei migration and fusion respectively.

Determination of Antiangiogenic Activity through Number of Branches and Total Branches Length

Figure 5 showed the average number of branches of the blood vessel in the CAM after treatment with 100mg/disc, 10mg/disc and 1mg/disc fucoidan from 3 provinces. The values in the graph are expressed regarding pixel as suggested by the Image J Angiogenesis Analysis Software. The graph below revealed that Retinoic acid (M=16.57, SD=3.86) has the least number of branches, followed by 100mg/disc Aklan (M=19.23, SD=2.6) Antique (M=21.13, SD=2.12) and Capiz (M=27.13, SD=3.93), then 10mg/disc Aklan (M=27.70, SD=2.8) and Capiz (M=30.1, SD=8.32). Next is 1mg/disc of Aklan (M=30.93, SD=2.15), 10mg/disc Antique (M=35.60, SD=1.56), 1mg/disc Antique (M=38.07, SD=4.71) and Capiz (M=41.20, SD=9.34) and the most number of branches is in USP gelatin disc (M=58.00, SD=18.2). 100mg/disc fucoidan Aklan has the closest value to retinoic acid.

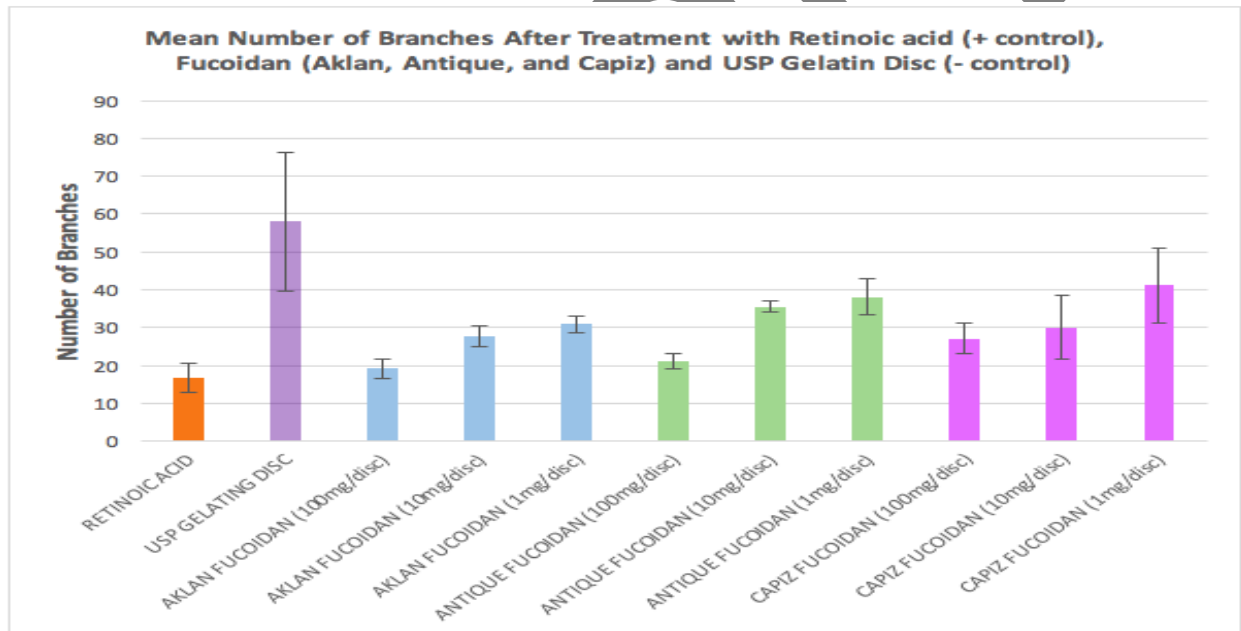


Figure 5. Mean Number of Branches After Treatment with Retinoic acid (+ control), Fucoidan (Aklan, Antique, and Capiz) and USP Gelatin Disc (- control)

The relationship between the number of blood vessel branches and concentration of fucoidan was assessed by Pearson product moment correlation coefficient. There was a negative and strong relationship between the number of branches and concentration of fucoidan, Aklan ($r=-0.982$), Antique ($r=-0.999$) and Capiz ($r=-0.722$). This means that the number of blood vessel branches is concentration-dependent, the higher the concentration of fucoidan, the lesser the number of branches.

Figure 6 revealed the average number of branches of the blood vessel in the CAM after treatment with 100mg/disc, 10mg/disc and 1mg/disc fucoidan from 3 provinces. The values in the graph are expressed regarding pixel as suggested by the Image J Angiogenesis Analysis Software. The graph below showed that 100mg/disc Aklan (M=2153.30, SD=256.36) has the least branches length, followed by Retinoic acid (M=2192.40, SD=177.15), 100mg/disc Antique (M=2734.80, SD=256.36), 10mg/disc Antique (M=2735.57, SD=54.61), 100mg/disc Capiz (M=2879.23, SD=699.87), 10mg/disc Aklan (M=3307.57, SD=41.3), 10mg/disc Antique (M=3299.27, SD=312.94), 1mg/disc Aklan (M=3894.73, SD=31.14), 1mg/disc Antique (M=4049.60, SD=84.95), 1mg/disc Capiz (M=4411.43, SD=117.83) and USP gelatin disc (M=5713.93, SD=943.55). Among the treatments used, Aklan (100mg/disc) has the lowest value in terms of total branches length and it is even lower than the positive control, the retinoic acid.

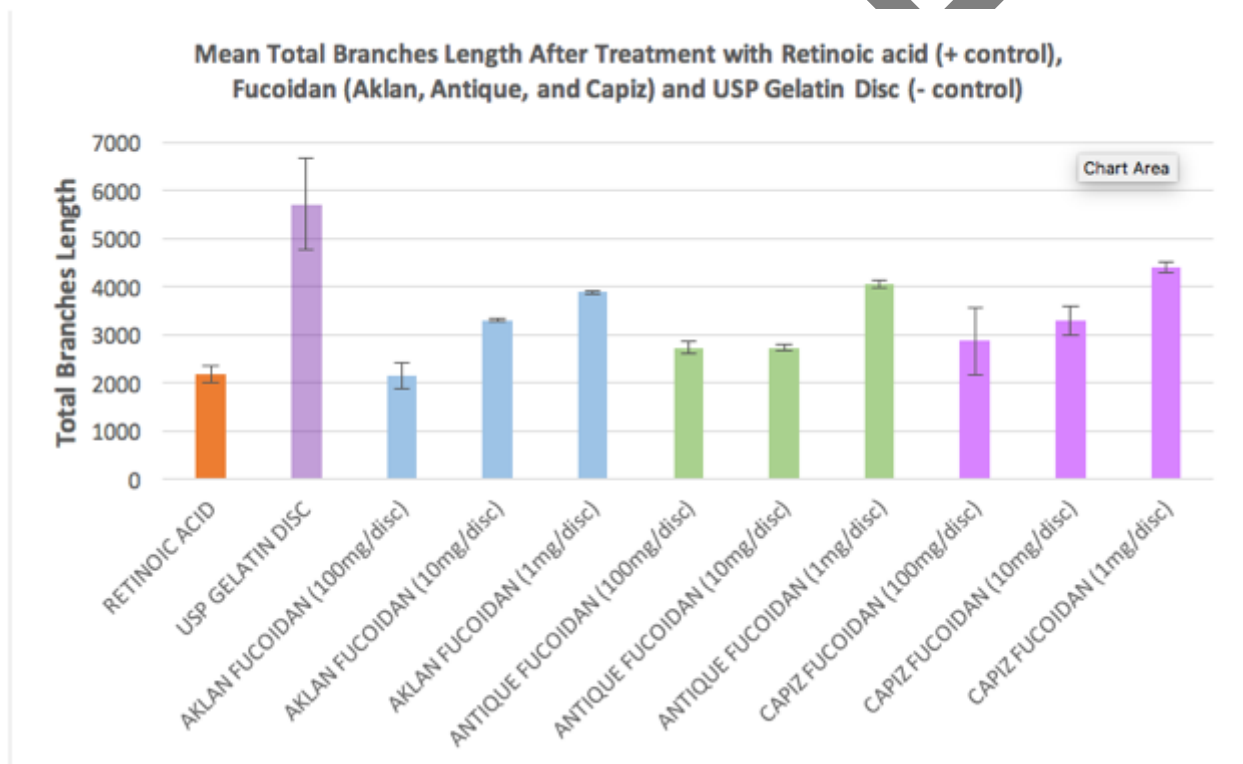


Figure 6. Mean Total Branches Length After Treatment with Retinoic acid (+ control), Fucoidan (Aklan, Antique, and Capiz) and USP Gelatin Disc (- control)

Pearson product-moment correlation coefficient was used to assess the relationship between total branches length and concentration of fucoidan. There was a negative and strong relationship between entire branches length and concentration of fucoidan especially from Aklan ($r=-0.967$), followed by Capiz ($r=-0.767$) and Antique ($r=-0.569$). This means that the entire branches length is concentration-dependent, the higher the concentration of fucoidan, the shorter the branches length.

Table 2 showed the analysis of variance of the number of branches and total branches length of blood vessels after treatment with fucoidan. The p-value is lower than 0.01. Thus, the null hypothesis is rejected. Therefore, there is a significant difference in the number of branches (F=7.267, p<0.01) and of the entire branches length (F=25.328, p<0.01).

Table 2. Analysis of Variance of Number of Branches and Total Branches Length of Blood Vessels After Treatment with Fucoidan

		Sum of Squares	df	Mean Square	F	Sig.
No. of branches	Between Groups	2742.277	4	685.569	7.267	.005
	Within Groups	943.340	10	94.334		
	Total	3685.617	14			
Total Branches length	Between Groups	1.912E7	4	4780548.426	25.328	.000
	Within Groups	1887460.640	10	188746.064		
	Total	2.101E7	14			

p < 0.01

Duncan Multiple Range Test was used to further evaluate the significance level for the differences in the number of branches and total branches length after treatment with 100mg/disc, 10mg/disc and 1mg/disc fucoidan from Aklan, Antique and Capiz. Regarding the number of branches, 100mg, 10mg and 1mg fucoidan discs (Aklan, Antique and Capiz) were comparable with the positive control.

Aklan has the most comparable antiangiogenic property regarding the number of branches and total branches length to retinoic acid. About FTIR characterization, Aklan has the highest quality and matching percentage when compared to the library standard. The functional groups of fucoidan present in Aklan fucoidan are nearly identical to the library standard.

Heterogenous sulfate (Usol'tseva et al., 2017) and O-acetylated sulfate groups (Synytsya et al., 2010) attach to the polysaccharides possess anticancer activity. Likewise, the high molecular weight property and water solubility of fucoidan contribute to its biological activity against suppression of tumor (Lemieszek and Rzeski, 2012). The sulfate and acetyl groups present in the 1,3-linked fucose residues (Menshova et al., 2016) and the beta (1→3) and (1→6) linkages in the backbone of polysaccharide (Lemieszek and Rzeski, 2012) were found to contribute in the anticancer activity of fucoidan. These structural orientations were associated with its biological activity. The anticancer mechanism of fucoidan can be attributed to the blockade in the receptor of vasculoendothelial growth

factor or VEGF (Xue et al., 2012) and inhibit fibroblast growth factor or FBGF (Sugawara et al., 2014) thus, suppressing signal transduction needed for angiogenesis and tubulogenesis. Fucoidan can also stimulate the action of the natural killer cell and activation of apoptosis (Ale et al., 2011, Teruya et al., 2009 and Maruyama et al., 2003).

Chromosomes play an important role in cell division. Consequently, mitosis is also needed for growing structures like blood vessel formation. When these processes remain uncontrolled, cancer cell formation becomes evident. The antiangiogenic property of fucoidan is related to its antimitotic activity. Both mechanisms are significant to each other. If there is overactivity of mitosis, then the branching of blood vessels is also increased. When a vast network of blood vessels formed, then there would be more channels to carry the nutrients needed for the maintenance of growing new cells like cancer cells. If one of these mechanisms is suppressed, then the other process would be inhibited too.

CONCLUSIONS

Based on the tests performed and results obtained the researcher concluded that 100 mg of Fucoidan possessed antimitotic and antiangiogenic properties. Thus, fucoidan from *S. oligocystum* is a potential alternative agent.

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