ANGIOSUPPRESSIVE PROPERTY OF SULFATED POLYSACCHARIDES GEL DISC FROM SARGASSUM OLIGOCYSTUM MONTAGNE

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ABSTRACT

Algae are the group of marine photosynthetic microorganisms with diverse biological and pharmacological activities. Angiogenesis plays a vital role in sustaining a growing body but over activity of this mechanism can lead to tumor formation. This study was conducted to determine the angiosuppressive property of sulfated polysaccharides (SPs) from *Sargassum oligocystum* using chorioallantoic membrane (CAM) assay. Hot water extraction and lyophilization were used to extract SPs with a yield of 8.56%. FTIR characterization of SPs showed identical spectra with the reference standard under 650 to 4000cm^{-1}. CAM model was used to assess the effect of SPs on angiogenesis. One-way ANOVA and DMRT revealed that 100mg of SPs disc did not differ significantly when compared to retinoic acid at $p<0.01$. The result suggested that the SPs from *S. oligocystum* is a promising alternative for inhibition of angiogenesis. Thus, the use of specific cancer cell line is recommended.

KEYWORDS: *Medicine, Cancer, Angiosuppression, CAM Assay, Philippines*

INTRODUCTION

The importance of health is only realized when one has acquired a disease. It affects the daily activities of men and all aspects of life. The Philippine Statistics Authority revealed that cancer is one of the leading causes of death next to cardiovascular disease [1]. Cancer cell develops when there is an alteration in the composition and metabolism of the normal cell. The cancer cell is considered to be a new growing body which divide more rapidly than the normal cell. In order for this cancer cell to grow, angiogenesis is needed.

Angiogenesis is the formation of new blood vessels from the existing one in a branching pattern which is a normal and complex biological mechanism in the body. Blood vessels are needed to transport oxygen and nutrients in order for the normal and cancer cells to grow [2]. This process is affected by endogenous local chemical signals or circulating endothelial precursors [3,4] which are vital in some physiological processes such as wound healing, embryonic development, tumor formation and cancer metastasis [3]. Angiogenesis include protease assembly, endothelial cell transport, and proliferation, vascular tube development, cross-connection of newly formed tubes, formation of a new basement membrane, and integration of smooth muscle cells and pericytes [4]. Angiogenesis are regulated by several signaling proteins like, basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), vascular endothelial growth factor (VEGF) and tumor necrosis factor-α (TNF-α). These regulatory proteins are normally present in the body and has specific mechanism in inducing angiogenesis [5]. However, the over activity and abnormal response of these precursors affect the balance and normal physiological and pathological processes. The metabolism of endothelial cells of both normal and cancer cells can be correlated to the presence and amount of oxygen needed in order for the blood vessel to sprout or form a vascular network [6]. In cancer patients, the activity of endothelial cells is vigorously active due to the release of proteins which can activate endothelial cell growth and induces metastasis when the angiosuppressive factors' production is reduced [2,7].

The unstable economic condition of the Philippines drives pharmaceutical companies to utilize herbal plants in formulating life-saving medicines. Knowledge on plant chemistry, biochemistry and pharmacology is essential in dealing with drug discovery. In the Philippines, few studies were conducted on algae as source of pharmaceutical alternative. *S. oligocystum* is one of the species brown macroalgae or seaweed from the family Sargassaceae under class Phaeophyceae in the order Fucales. It is distributed widely in coastal countries, like Philippines. *S. oligocystum* is found mostly in the subtidal zone, which is characterized by brownish coloration of the seaside when it dried up [8]. It is a good source of many metabolites such as alginates, alginic acid, pigments, sulfated fucoidans, sterols, oils and mannitols [9].

Fucoidan is one of the most interesting metabolites of Sargassum. Fucoidan is derived from its structural composition made of fucose, which is a hexose deoxysugar. Fucose is the fundamental sub-unit of fucoidan, a

sulfated polysaccharide and can be found in N-linked glycans on plant surface [10] which is one of the components of cell wall. The water extract of *Sargassum oligocystum* revealed a notable antiproliferative and antitumor activities against K562 and Daudi human cancer cell lines. using MTT (Microculture Tetrazolium) Assay and TBE (Trypan Blue Exclusion) Test. [11, 12]. Thus, this study was formulated to determine the angiosuppressive property of the sulfated polysaccharides *Sargassum oligocystum* Montagne using chorioallantoic membrane assay. Specifically, this study aimed to: (a) measure the percentage yield of SPs, (b) characterize the SPs using physicochemical testing and fourier transform infrared spectrometric analysis, (c) determine the antiangiogenic activity of SPs using chorioallantoic membrane (CAM) assay and (d) determine the significant difference between the angiosuppressive property of SPs and retinoic acid (positive control).

METHODOLOGY

Research Design. Completely randomized design and experimental methods was used in angiosuppressive assay of SPs on chorioallantoic membrane of duck egg. Post-test only control group design was used in each treatment with three trials and ten measurements each trial.

Sample Collection. Fresh and mature *S. oligocystum* were collected from the subtidal zone of Tangalan, Aklan. The whole plant was stored in a clear, wide-mouth glass bottle for macroscopic analysis for specific algal authentication by the Aquaculture Department of Southeast Asian Fisheries and Development Center, Region VI.

*Duck Egg Collection***.** Five-day old fertilized duck eggs were collected from Brgy. Matanga Poultry Farm of New Buswang, Kalibo, Aklan. The eggs were then further incubated at the laboratory using a digital analytical incubator at 37.5°C with 62.5% relative humidity [13].

Plant Extraction. S. oligocystum was washed thoroughly with distilled water to remove dirt and extraneous matter. The algae were air-dried for seven days and then dry-milled using an electric grinder. Four kilograms of brown algae were soaked in 8000 mL distilled water and subjected to electrothermostatic water-bath heating for 4 hours at 80^{o}C [14, 15, 16]. The algal residues were separated using suction filtration and the aqueous filtrate was lyophilized to obtain a powder extract.

Characterization. The physicochemical characteristics and FTIR spectra of SPs were compared to the standard-pharmaceutical grade fucoidan. Physical tests include color, taste, odor, appearance, pH and solubility of SPs. Chemical Tests include tests for carbohydrate, sulfate, and polysaccharide. Protein test was performed to ensure that the extract was purely made of sulfate group and polysaccharide chain since amino acid group/s is/are major contaminant/s. The characterization of SPs was performed through the use of Fourier Transform Infrared Spectroscopy to determine the quality of SPs in comparison to the reference standard fucoidan at 650-4000cm^{-1} frequency range. [14]. The quality of SPs including the reference standard was compared against ten samples stored in the Attenuated Total Reflection Library of the FTIR Spectrometer (Agilent Technologies).

Angiosuppressive Assay. The 5-day old fertilized eggs were incubated for another 5 days at 37.5°C with 62.5% RH using a digital analytical incubator. The windowing of eggs was madeusing a candling device at the blunt end of egg for air sac identification and prominent blood vessel marking especially the Y-shaped blood vessel. A small window was made perpendicular to the previously identified blood vessel in the center of the egg by the use of a surgical blade. A mild suction using tuberculin syringe was applied to the hole to remove the excess liquid albumin. The CAM was separated from the shell where the identified Y-shaped blood vessel was secured. A 0.5 cm diameter [17] and 0.1 cm thick gelatin-based disc was placed on the Y-shaped vascular area. The USP gelatin discs were formulated and infused with the following treatments: 10mg/disc of retinoic acid (Treatment A/positive control), 100mg/disc of SPs (Treatment B), 10mg/disc of SPs (Treatment C), 1mg/disc of SPs (Treatment D) and gelatin disc-base (Treatment E/negative control). The gelatin disc served as the matrix for delivery of SPs into the CAM. After placing the discs on the vascular area, the window was sealed with sterile transpore, and the eggs were returned to the incubator and incubated for the next 3 days. The eggs were totally opened and transferred to Petri dishes. A 1-inch diameter ring was placed around the disc where it was planted to limit the area of analysis [13]. A digital camera was used to capture the image on the CAM [18] at a distance of 6-inch focus. The measurement of angiogenesis was made by measuring the number of branches using Image J Angiogenesis Analyzer [19].

Statistical Treatment of Data. For descriptive statistics, mean was used to describe the results of SPs, positive and negative controls on angiogenesis. Analysis of Variance was used for assessing the significant difference in the

number of branches and total branch length at $p<.01$ level of significance. On the other hand, the Duncan Multiple Range Test was used to further evaluate the differences among three SPs gelatin disc concentrations when compared to retinoic acid (positive control).

RESULTS AND DISCUSSION

Percentage Yield

Table 1 shows that 8.56%w/w of SPs were extracted from 4000 g of dried *S. oligocystum*. Extraction temperature and solvent contact time are critical parameters essential in optimizing SPs extraction. The cell wall of *S. oligocystum* becomes more porous at a temperature ranging 70 – 80ºC when carried within 3-4 hours extraction time [14]. This condition favors the extraction and solubility of SPs in water. The amount of SPs from brown algae varies according to habitat, freshness of the sample, maturity, period of collection and species [20].

Table 1: Percentage yield of sulfated polysaccharides

Weight *S. oligocystum*	Weight of SPs Extract	Percentage Yield
4000 grams	342.40 grams	8.56%w/w

Physicochemical Properties of SPs

Table 2 reveals that the standard fucoidan and SPs have the same properties in terms of odor, taste and form except the color. The difference in the color is due the presence of darker spots of *S. oligocystum* [21] than kelp, a variety of brown algae which is the source of standard fucoidan. Standardization and commercialization of fucoidan involves the removal of dark pigments during the process of extraction to give an aesthetic appeal. The solubility of both standard fucoidan and SPs were the same in different solvents. Fucoidan and SPs are composed of L-fucose, sulfate, mannose, galactose, glucose, and xylose units [14]. Structurally, it is made up of several hydroxyl groups making it freely soluble in water and soluble in dilute acidic and basic solvents due to hydrogen bonding. Fucose units are insoluble in semi- and nonpolar solvents due to minimal hydrogen bonding and low dielectric constant property of ethanol, acetone and hexane. The pH of both samples are slightly alkaline and this serves as basis of fucoidan and SPs' stability and optimization of biological activities. Fucoidan and SPs contain polyhydroxyaldehydes and these are prone to undergo the process of oxidation [22] which will yield polyhydroxyacid. Thus, a decrease in the pH value of SPs is an indication of decomposition.

Table 2: Comparison of physical properties of standard fucoidan and sulfated polysaccharides

Property	Standard Fucoidan	Sulfated Polysaccharide
Color	Light yellow	Brown
Odor	Fishy	Fishy
Taste	Salty	Salty
Form	Powder	Powder
Solubility in:		
Distilled water	Freely soluble; 1:10	Freely soluble; 1:10
5%NaOH	Soluble; 1:15	Soluble; 1:15
5%$NaHCO_3$	Soluble; 1:20	Soluble; 1:20
5%HCl	Soluble; 1:15	Soluble; 1:15
Ethanol	Insoluble	Insoluble
Acetone	Insoluble	Insoluble
Hexane	Insoluble	Insoluble
pH	7.78 slightly alkaline	7.83 slightly alkaline

Table 3 shows that the standard fucoidan and SPs do not contain proteins and amino acids, it suggests that the SPs are chiefly carbohydrate in nature with the presence of sulfate and absence of traces of proteins and amino acids. Detection of proteins was conducted to ensure the quality of the extracted SPs because proteins are major

contaminants of fucoidan or sulfated polysaccharides extracted from macroalgae [22]. Thus, the biological activity of SPs is affected by the presence of other compounds and functional moieties other than carbohydrates.

Table 3: Comparison of chemical properties of standard fucoidan and sulfated polysaccharides

Tests	Positive Results	Fucoidan	SPs
Molisch *Carbohydrate*	Purple ring at the junction	+	+
$BaCl_2$ *Sulfate*	White precipitate	+	+
Lugol *Polysaccharide*	Blue-black solution	+	+
Biuret *Peptide bond*	Blue-violet solution	-	-
Xanthoproteic *Protein*	Yellow precipitate	-	-
Ninhydrin *Amino acid*	Intense Blue solution	-	-

Fourier Transform Infrared Spectrum of SPs

Fig. 1 shows that the FTIR spectrum of SPs is identical to the standard fucoidan and Agilent pectin library sample. The specific functional groups that were characterized are summarized in Table 4.

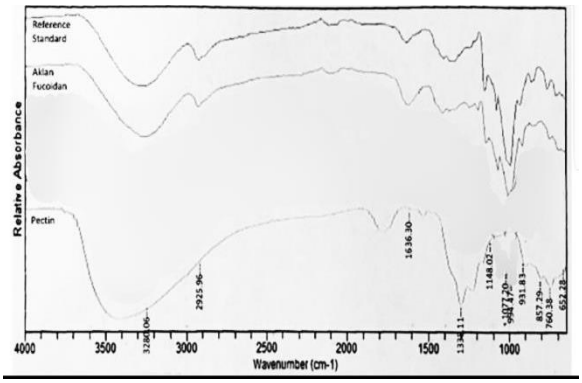


Figure 1: Fourier transform infrared spectra of standard fuoidan (top), SPs (middle) and Agilent pectin library sample (below).

FTIR spectrum of SPs shows that there is no vibration stretch observed at wavenumber 3400-3500cm^{-1} which is the specific band of the amino group. This means that protein is absent in the extracted SPs. The presence of OH group in the monosaccharide monomer is observed at 3300-3400cm^{-1}, an aliphatic C–H at 2900cm^{-1}, a C=O stretch for acetate at 1700cm^{-1} [24]. The C-O-C bending vibrations in glycosidic linkage is observed at the region 700-1000cm^{-1} [25]. The glycosidic linkage stretch C–O–C and C–O–H are distinct between 1600 $cm^{-1}$and 1000 cm^{-1}. The signals near to 1600 $cm^{-1}$and 1500 $cm^{-1}$are produced by the asymmetric and symmetric stretch vibration of C-O-O of uronic acid [26,27]. The presence of S=O stretching of sulfate group is noted as a weak band at 1000cm^{-1} [28]. Therefore, the distinct band stretch of polysaccharide at 1000–3400cm^{-1} and sulfate at 1000 cm^{-1} is an indication that it is a sulfated polysaccharide. These are the essential functional moieties that can affect cell division and blood vessel formation [29].

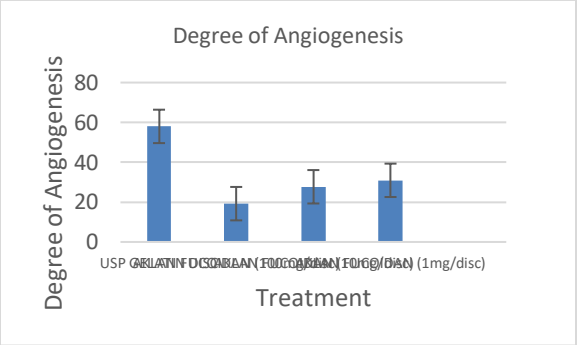
Table 4: Summary of functional groups characterized by FTIR spectrometer

Wavenumber cm^{-1}	Functional Groups
3300 – 3400	-OH group of monosaccharide
2900	Aliphatic C—H
1700	C=O for CH_3COO^-
1500 – 1600	Asymmetric and symmetric C-O-O stretch of uronic acid
1000 – 1600	C-H-O and C-O-C
1000	S=O stretch of sulfate group
700 – 1000	C-O-C vibration of glycosidic linkage

Angiosuppressive Assay

Table 5 reveals the degree of angiogenesis which was evaluated through the use of chorioallantoic membrane (CAM) model. It shows that the treatment with 100mg/disc (M=16.05, SD=2.6) has the lowest percent angiogenesis or vascularization, followed by 10mg/disc (M=67.17, SD=2.8) and 1mg/disc (M=86.66, SD=2.15) when compared to the positive control, retinoic acid (M=16.57, SD=3.86).

Table 5: Evaluation of the degree of angiogenesis of SPs



In-ovo CAM assay is widely used [30,31] in the analysis of proangiogenic and antiangiogenic agents [32] as shown in Figure 2. This assay deals on vascularization process which occurs during fetal development including both physiological and pathological blood vessels formed by normal or overactive angiogenesis [33]. The CAM developed from day 4 to day 14 (6 cm^2 up to 65 cm^2) which acts as a respiratory organ with a rapidly developing vascular network [34]. The number of branches was quantified by Image J angiogenesis analyzer and expressed in pixel unit.

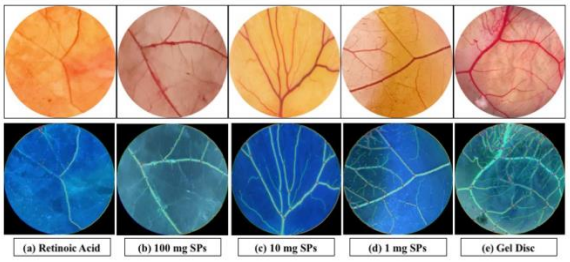


Figure 2: Normal and phase-contrast images of CAM

Data in Table 6 reveals that there was a significant angiosuppressive effect on the number of vascular branches at the p<.01 level for the five treatments, namely, retinoic acid, gelatin disc, 100mg, 10mg and 1mg SPs gel disc [F(4, 10) = 11.14, p = 0.00105]. Pearson product-moment correlation coefficient (r=-0.982) suggests that the the degree of angiogenesis and concentration of SPs is strongly indirectly proportional.

Table 6: Analysis of variance

	SS	df	MS	F	Sig
Between groups	3256.58	4	814.1	11.14	.00105
Within groups	730.84	10	73.08		
Total	3987.42	14			

Data in table 7 shows that there was no significant difference in the angiosuppressive effect between retinoic acid and the three treatments of SPs. Thus, the inhibitory effect of SPs was comparable with the positive control with the distinction of 100mg/disc which has the lowest number of branches compared to 10mg and 1mg.

Sulfated polysaccharide structure plays a vital role in the inhibition vascular growth. O-acetylated and heterogeneous sulfate groups attach to polysaccharide was found to have an anticancer activity [35]. The high molecular weight, water solubility of SPs [29] and complex polysaccharide chain contribute to its pharmacological activity against suppression of tumor. The anticancer and antiangiogenic effects of fucoidan is due to its fucose units linked with alpha-l-(1→3) and (1→4)-chained fucopyranosy and beta-d-(1→6) bonds in the backbone of polysaccharide [36,37,38]. The anticancer mechanism of fucoidan is contributed by the blockade of the VEGF receptor [39,40] and inhibition of FBGF [41] thereby, blocking signal transduction needed for angiogenesis and tubulogenesis. Fucoidan can also induce the action of natural killer cell [36,42,43] and can activate apoptosis [36].

Table 7: Duncan multiple range test

Treatments	N	Subset for alpha = 0.01	
		1	2
Retinoic acid	3	16.57	
100 mg SPs	3	19.23	
10 mg SPs	3	27.70	
1 mg SPs	3	30.93	
Gel Disc	3		58.0000
Sig.		.084	1.000

CONCLUSION

Based on the tests performed the researcher concluded that the sulfated polysaccharide (SP) gel discs exhibited an angiosuppressive effect on chorioallantoic membrane of duck egg. Specifically, 100 mg SPs disc was more closely comparable with retinoic acid. Thus, sulfated polysaccharides from *S. oligocystum* is a potential alternative antiangiogenic agent.

REFERENCES

Lisa Grace Bersales. 2018. Deaths in the Philippines, 2016. Ref. No. 2018-045. Retrieved on June 07, 2018 from https://psa.gov.ph/content/deaths-philippines-2016.

Shaker Mousa and Mehdi Rajabi. 2017. The Role of Angiogenesis in Cancer Treatment. (June 2017). Retrieved October 2, 2018 from http://www.mdpi.com/2227-9059/5/2/34/html.

Anon. (PDF) Angiogenesis Inhibitors for Cancer Therapy. Retrieved October 2, 2018 from https://www.researchgate.net/publication/324029467_Angiogenesis_Inhibitors_for_Cancer_Therapy

Alisa E. Koch and Oliver Distler. 2007.(2007). Retrieved October 2, 2018 from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2072889/

Jaywant Jadhav, Aruna Kanase and Anuya Mane. 2011. Antiangiogenic properties of Boerhaavia diffusa extracts in

chick Chorioallantoic Membrane (CAM) International Journal of Drug Development & Research. 3(4). 307-317.

Christopher Butt, Sooyeol Lim, Celia Greenwood, and Proton Rahman. 2007. VEGF, FGF1, FGF2 and EGF gene polymorphisms and psoriatic arthritis. (January 2007). Retrieved October 2, 2018 from https://bmcmusculoskeletdisord.biomedcentral.com/articles/10.1186/1471-2474-8-1

Helena Pavlakovic, Werner Havers, and Lothar Schweigerer. Multiple angiogenesis stimulators in a single malignancy: Implications for anti-angiogenic tumour therapy. Retrieved October 2, 2018 from https://link.springer.com/article/10.1023/A:1016045012466

Justin Healey. 2009. *Marine conservation*, Thirroul, N.S.W.: Spinney Press.

Ching-Lee Wong, Sook-Yee Gan, and Siew-Moi Phang. 2004. Morphological and molecular characterisation and differentiation of Sargassum baccularia and S. polycystum (Phaeophyta). *Journal of Applied Phycology* 16, 6 (2004), 439–445.

D.J. Becker and J.B. Lowe. 2003. Fucose: biosynthesis and biological function in mammals. (July 2003). Retrieved October 2, 2018 from https://www.ncbi.nlm.nih.gov/pubmed/12651883

K. Zandi et al.2010. Anticancer activity of Sargassum oligocystum water extract against human cancer cell lines. (August 2010). Retrieved October 3, 2018 from https://www.ncbi.nlm.nih.gov/pubmed/20707286

Yoshinobu Aisa et al. 2004. Fucoidan induces apoptosis of human HS-Sultan cells accompanied by activation of caspase-3 and down-regulation of ERK Pathways. (December 2004). Retrieved October 3, 2018 from http://onlinelibrary.wiley.com/doi/10.1002/ajh.20182/full

Mojid Mondol 2006. Antiangiogenic Study of Two Nonsteriodal Antiinflammatory Compounds Using Chick Chorioallantoic Membrane Assay, Journal of Medical Sciences. 6: 609-614.

Adi Soehono, S. Sugiono and Bambang Widjanarko. 2014. Extraction Optimization by Response Surface Methodology and Characterization of Fucoidan from Brown Seaweed Sargassum polycystum. Int'l Journal of ChemTech Research. 6(1). 195-205.

Ara Jehan, Ayesha Khan, And Syed and Sultana Viqar. 2010. In Vitro cytotoxicity of seaweeds from Karachi Coast on Brine Shrimp. Pakistan Journal of Borany. 42(5): 3555-3560.

Beatrice Guevarra. 2004. A Guidebook to Plant Screening: Phytochemical and Biological, Manila, Philippines: Research Center for the Natural Sciences, University of Santo Tomas.

Virgie Tan and Rey Tantiado. 2012. Evaluation of the Angiosuppresive Activity of Tinospora rumphii Boerl. Stem Extract Using the Chorioallantoic Membrane Assay in Anas platyrhynchos Embryos. International Journal of Bio-Science and Bio-Technology. 4(2): 93-102

Elena Deryugina and James Quigley. 2009. Chick embryo CAM model systems to study and visualize human tumor cell metastasis. Methods Enzymology Journal. 444:21-41.

G. Carpentier, I. Cascone, J. Courty and M. Martinelli. 2012. Angiogenesis Analyzer for ImageJ. 4th ImageJ User and Developer Conference proceedings. Mondorf-les-Bains, Luxembourg. ISBN: 2-919941-18-6: 198-201.

Martin Beaulieu, Laurie-Eve Rioux and Sylvie Turgeon. 2007. Characterization of polysaccharides extracted from brown seaweeds. Carbohydrate Polymers, 69, 530-537.

Nicholas Guanzon, Anicia Hurtado, Ma. Rovilla Luhan. 2006. Seaweeds of Panay. 2nd ed. Southeast Asian Fisheries Development Center. Aquaculture Department. Tigbauan, Iloilo Philippines.

Scott Mackenzie. 2013. Oxidation of Quench Oil. Conference: Heat Treat 2013 American Society for Metals. Retrieved on July 14, 2018 from https://www.researchgate.net/publication/267529127_Oxidation_of_Quench_Oil.

K.OU H.AY AK AW A1 and. 2009. KOU HAYAKAWA. (April 2009). Retrieved October 2, 2018 from http://ar.iiarjournals.org/content/29/4/1211.abstract

Ahmed Zayed et al.2016.(April 2016). Retrieved October 2, 2018 from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4849083/

Fernando et al. FTIR characterization and antioxidant activity of water soluble crude polysaccharides of Sri Lankan marine algae. Retrieved October 2, 2018 from https://www.e-algae.org/journal/view.php?number=2825.

Joana Marques, Eduardo Vilanova, Paulo A.S. Mourão, and Xavier Fernàndez-Busquets. 2016.(2016). Retrieved October 2, 2018 from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4829872/.

Steve W. Cuib, H. Douglas Goffc, Suwayd Ningsanonda and Jittra Singthonga. 2004. Structural characterization, degree of esterification and some geling properties of Krueo Ma Noy (*Cissampelos pareira*) pectin. Carbohydr Polym; 58:391-400.

Rosmawaty Peranginangin, Endang Saepudin and Ellya Sinurat. 2015. Purification and Characterization of Fucoidan from the Brown Seaweed *Sargassum binderi sonder*. Squalene Bulletin of Marine and Fisheries Postharvest and Biotech. 10 (2) 2015, 79-87.

Marta Lemieszek and Wojciech Rzeski. 2012.(2012). Retrieved October 2, 2018 from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3687424/

D. Ribatti. Chick embryo chorioallantoic membrane as a useful tool to study angiogenesis. Retrieved October 2, 2018 from https://www.ncbi.nlm.nih.gov/pubmed/19081537.

A. Vargas, M. Zeisser-Labouèbe, N. Lange, R. Gurny, and F. Delie. 2007. The chick embryo and its chorioallantoic membrane (CAM) for the in vivo evaluation of drug delivery systems. (September 2007). Retrieved October 2, 2018 from https://www.ncbi.nlm.nih.gov/pubmed/17870202.

F.P. Cantatore, E. Crivellato, B. Nico, and D. Ribatti. 2005. Osteocalcin is angiogenic in vivo. (July 2005). Retrieved October 2, 2018 from https://www.ncbi.nlm.nih.gov/pubmed/15979904.

I.S. Moreira, P.A. Fernandes, and M.J. Ramos. 2007. Vascular endothelial growth factor (VEGF) inhibition—a critical review. (March 2007). Retrieved October 2, 2018 from https://www.ncbi.nlm.nih.gov/pubmed/17348829.

Patrycja Nowak-Sliwinska-Tatiana Segura-M. Iruela-Arispe . 2014. The chicken chorioallantoic membrane model in biology, medicine and bioengineering. Angiogenesis 17, 779–804.

S. Badrinathan, T.M. Shiju, A.Suneeva Sharon Christa, R. Arya, and V. Pragasam. 2012.(2012). Retrieved October 3, 2018 from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3687925/

Marcel Tutor Ale, Jørn D. Mikkelsen, and Anne S. Meyer. 2011.(2011). Retrieved October 3, 2018 from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3210621/

M.I. Bilan et al. 2010. Further studies on the composition and structure of a fucoidan preparation from the brown alga Saccharina latissima. (September 2010). Retrieved October 3, 2018 from https://www.ncbi.nlm.nih.gov/pubmed/20701899

U. Adhikari, C.G. Mateu, K. Chattopadhyay, C.A. Pujol, E.B. Damonte, and B. Ray. 2006. Structure and antiviral

activity of sulfated fucans from Stoechospermum marginatum. (November 2006). Retrieved October 3, 2018 from https://www.ncbi.nlm.nih.gov/pubmed/17067880

M. Orlandini and S. Oliviero. 2001. In fibroblasts Vegf-D expression is induced by cell-cell contact mediated by cadherin-11. (March 2001). Retrieved October 3, 2018 from https://www.ncbi.nlm.nih.gov/pubmed/11108717

S. Koyanagi, N. Tanigawa, H. Nakagawa, S. Soeda, and H. Shimeno. 2003. Oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities. (January 2003). Retrieved October 3, 2018 from https://www.ncbi.nlm.nih.gov/pubmed/12504793

Maria Alessandra Gammone, Graziano Riccioni, and Nicolantonio D'Orazio. 2015.(October 2015). Retrieved October 3, 2018 from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4626686/

Z. Zhang, K. Teruya, H. Eto, and S. Shirahata. Fucoidan extract induces apoptosis in MCF-7 cells via a mechanism involving the ROS-dependent JNK activation and mitochondria-mediated pathways. Retrieved October 3, 2018 from https://www.ncbi.nlm.nih.gov/pubmed/22096572

H. Maruyama, H. Tamauchi, M. Hashimoto, and T. Nakano. Antitumor activity and immune response of Mekabu fucoidan extracted from Sporophyll of Undaria pinnatifida. Retrieved October 3, 2018 from https://www.ncbi.nlm.nih.gov/pubmed/12929574
