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Full Length Research Article

Study on the Effects of *Dictyota ciliolata* Polysaccharides on Proliferation and Anti-Oxidation Status of *Cyprinus carpio (common carp) leukocytes*

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ABSTRACT

The brown seaweed (*Dictyota ciliolataa* Sonder ex Kutzing) has been identified as a valuable source of anionic polysaccharides with remarkable biological properties that may render them suitable for application in aquaculture. In this study, the chemical composition of *D. ciliolata* polysaccharides (DCP) was analysed using spectrophotometry and High-Performance Anion-Exchange Chromatography/ Pulsed Amperometric Detection (HPAEC-PAD). The result showed that DCP had a total carbohydrate content of 56.56 ± 1.17 %, sulphate content of 4.58 ± 0.26 %, uronic acid content of 6.54 ± 0.26 %, and protein content of 7.31 ± 0.24 %. HPAEC-PAD revealed that DCP composed of fucose (27.16 ± 4.27 mg/l), galactose (11.47 ± 1.38 mg/l), arabinose (11.88 ± 1.24 mg/l) and glucose (1.64 ± 0.07 mg/l). DCP was non-toxic to carp leukocytes at dosages ≤ 50 µg/ml but was found to induce cell proliferative activity. Cellular antioxidant assays showed that DCP did not disrupt glutathione and superoxide dismutase activities in carp leukocytes cells when compared to the control. However, a decrease in malondialdehyde levels in carp leukocytes cells indicated that DCP did not induce oxidative stress. Therefore, DCP is a promising antioxidant source that could be effectively utilised in enhancing the health status of cultured fish species.

Keywords: *Dictyota ciliolata* Polysaccharides; Chemical composition; Common carp leukocytes; Cell proliferation; Oxidative stress

INTRODUCTION

Global aquaculture is growing tremendously, providing a major sustainable source of protein for human consumption (Kubečka *et al.,* 2016). However, aquaculture husbandry practices such as confinement, changes in environmental conditions, transportation, handling, and high stocking density are stress inducers that may adversely affect the normal physiological functions of cultured fish species (Harper and Wolf, 2009; Wedemeyer, 2012; Yousefi *et al.,* 2016). Like other organisms, fish respond to the stressor by increasing the production of reactive oxygen species (ROS) and this may consequently disrupt intracellular redox homeostasis (Lushchak, 2011). The inability of the organism to combat these reactive intermediates via the antioxidant defence system may lead to oxidative stress (OS). Several disease conditions have been linked to OS since it could cause damage to cellular macromolecules such as nucleic acid, lipids, and protein and ultimately cell death (Martínez-Álvarez *et al.*, 2005). Specifically, the degradation of lipids by oxidants is a significant biomarker of OS, where the polyunsaturated fatty acids (PUFAs) present in cellular membrane bilayer are disposed to free radical attack, thereby initiating a chain reaction leading to membrane structure modification (Hematyar *et al.,* 2019).

Considering the significance of OS in fish farming, particularly in contemporary rearing systems which are highly intensive and stressful, research attention is focused on the improvement of cultured fish species' antioxidant defence system. The application of chemically synthesized antioxidants (butylated hydroxyl toluene, butylated hydroxyl anisole) in fish farming to ameliorate antioxidant defence status is restricted due to their negative impacts on the environment and public health (Williams *et al.,* 1999). Therefore, alternate solutions including the use of naturally derived bioproducts that could inhibit oxidative damage are presently of research interest.

The ocean is a distinct aquatic ecosystem endowed with a vast number of bioresources including algae ranging from microscopic diatoms to macroalgae reaching about 30m in length (Yasuhara-Bell and Lu, 2010). Although being underexploited, studies have recognised algae as an abundant resource of carotenoids, polysaccharides, fatty acids, acetogenins, amino acids, peptides, vitamins, and phenolic compounds (La Barre *et al*., 2010; Moghadamtousi *et al*., 2015). Specifically, polysaccharides from marine macroalgae (seaweeds) have emerged as prospective bioactive macromolecules that are effective and considerably non-toxic substances (Alves *et al.,* 2013; Rodrigues *et al.,* 2013) with potent antioxidant activity (Batista-Gonzalez *et al.,* 2012). However, the extent of biological properties of seaweed polysaccharides varies greatly since their composition and structure are heterogeneous (Rodrigues *et al*., 2017). Currently, seaweed-derived polysaccharides including alginate, fucoidan and laminaran extracted from various brown seaweeds have been found to possess potent biological activities that may render them suitable as antioxidants in cultured fish species (Mohan *et al*., 2019). For example, fucoidan from the brown seaweed *Saccharina japonica* improved superoxide dismutase and catalase activities and decreased malondialdehyde levels in yellow catfish (Yang *et al.,* 2014). However, to the best of our knowledge, the effects of *Dictyota ciliolata* polysaccharides extract on common carp (*Cyprinus carpio*) oxidative stress responses are yet to be reported

in literature. Hence, the present study examined the chemical constituents of *D. ciliolata* polysaccharides (DCP) and their impacts on cell proliferation and the anti-oxidation status of cultured carp leukocytes.

MATERIALS AND METHODS

Collection of Sample

The brown seaweed *D. ciliolata* was collected from the Lagos coast (Latitude 6° 39'N Longitude 3° 40'E), Nigeria, in March 2020. The sample was conveyed to the Department of Marine Sciences laboratory in an ice-cold box, thoroughly cleaned with freshwater to detach epiphytes and remove salt. Afterward, the cleaned seaweed was sun-dried for 72 h and pulverised. The seaweed species was identified by Dr. O.W. Elegbeleye (a phycologist) and a typical specimen was kept at the research laboratory of the Department of Marine Sciences for reference purposes.

Extraction of Aqueous Polysaccharides from *D. ciliolata*

The *D. ciliolata* powder (10 g) was pretreated with 200 ml of 75% ethanol at 40 ℃ for 1 h (twice) to get rid of pigments and other impurities. The residue was recovered by filtration and weighed, following extraction using deionized water (1:40 w/v; 80 ℃; 2 h) (twice). The mixture was centrifuged at 4,400 rpm for 10 mins, followed by decantation and concentration of the supernatant under reduced pressure at 55 ℃ using a Rotary vacuum evaporator (Rotavapor R-114). The concentrate was precipitated with absolute ethanol at 4 ℃ overnight. The precipitate was retrieved by centrifugation at 4,000 rpm for 10 mins and then freeze-dried to obtain *D. ciliolata* polysaccharides (DCP).

Chemical Analysis of DCP

Chemical Colorimetric and Turbidimetric Assays

Total carbohydrate content of DCP was determined according to the phenol-sulfuric acid method previously described by Dubois *et al.,* (1956). D-glucose was used as standard. Total sulphate content of DCP was evaluated using Barium Chloride-gelatin turbidimetric method according to Dodgson and Price (1962) and potassium sulphate was used as standard.

Protein content was measured using the Bradford assay (Bradford, 1976). Total protein content of DCP was estimated from a bovine serum albumin (BSA) standard curve. The uronic acid content of DCP was determined following the sulfamate/m-hydroxydiphenyl method (Tullia *et al.,* 1991) using Galacturonic acid as the standard.

Determination of Monosaccharides Unit in DCP

Firstly, 4 mg of DCP was subjected to hydrolysis for 2 h at 121 ℃ in a sealed Pyrex test tube containing 2 ml of 2 M TFA. The diluted solution was (1:10 v/v ultrapure water; Thermo Scientific) analysed by HPAEC-PAD on a Dionex ICS-3000 Ion Chromatography System (Dionex Corporation, CA, USA) equipped with a Dionex AS-AP Auto Sampler, Dionex ICS 3000 Detector Chromatography and Dionex ICS 3000 Dual Pump System. A CarboPac PA20 analytical column (3x150 mm 6.0 µm particle size) was used for separation. 10 mM NaOH with an adapted flow rate of 0.3 ml/min and an injection volume of 20 µL was used as the mobile phase. Six standard sugars (arabinose, fucose, mannose, galactose, glucose and xylose) were serially diluted with ultrapure water and monosaccharides composition of DCP was quantified from each standard sugar calibration curve. Chromeleon® version 6.8 was used in the data processing.

Fourier Transform Infrared (FT-IR) Spectroscopy

DCP spectrum was recorded with FT-IR spectroscopy (Nicolet 380, Thermo Fisher Scientific) at the frequency range of 4000 – 400 cm^{-1}. Measurement was taken thrice for consistency.

Cell Proliferation Assay

The cell line model, carp leukocyte culture [CLC (ECACC 95070628)] was established from a healthy two-year-old female *C. carpio* peripheral blood mononuclear cells (PBMC) which possess distinctive characteristics of macrophages (Weyts *et al.,* 1997). The cell line as a suitable biological surrogate for the preliminary screening of new compounds as drug candidates for aquaculture was proven by earlier studies (Vidal *et al*., 2009; Kareem *et al*., 2018). For this experiment, 200 µl of carp leukocytes suspended in a CO_2-independent medium (Gibco, UK) containing 10% FBS, 1% L-Glutamine and 1% penicillin-streptomycin were seeded out in a 96-well plate (SARSTEDT, Germany) at a density of 100,000 cells per well. Adhered cells with about 70% confluence were treated with DCP (0, 5, 25, 50, 100, 200, 400 µg/ml) and incubated at 27 ℃ (VWR Incu-Line®, IL-10). After a 24 or 48 h incubation period, 10 µl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added into respective wells and then incubated for additional 4 h at 27°C. Following incubation, 100 µl of DMSO (dimethyl sulfoxide) was added to the wells and kept in the dark for 2 h at room temperature. The OD (optical density) was measured at 570 nm using a Tecan Infinite F50 microplate reader. The absorbance value was interpreted into a cell proliferation ratio (%) = $\frac{AV_t}{A_{Vc}}\ x\ 100\ \%$, where AV_t and AV_c are the absorbance values of the treated and control groups, respectively. The experiment was independently replicated thrice. The concentrations of DCP that did not reduce carp leukocytes proliferation rate < 95 % were selected for further experiments.

Measurement of Antioxidant Activity and Malondialdehyde Level

Carp leukocytes suspended in serum-rich CO_2-independent medium were seeded at a density of 500,000 cells per well in a 6-well plate. Varied concentrations of DCP (0, 12.5, 25, 50 µg/ml) were added to adherent carp leukocytes and incubated at 27 ℃. After 24 h, the culture medium was aspirated and cells were washed using ice-cold Dubelcco's phosphate-buffered saline (DPBS) and then suspended in 100 µl chilled cell lysis buffer (Cell Signaling Technology, USA). The cell lysates were collected after centrifugation (4 ℃,

14 000 rpm, 10 mins) and analysed for superoxide dismutase (SOD) activity, glutathione (GSH) and malondialdehyde (MDA) level using respective commercial colorimetric assay kits (Abcam, USA: ab65354 Invitrogen®ThermoFisher Scientific: EIAGSHC; R&D systems, USA: KGE013). The optical density (OD) for each assay was read on a microplate reader (Infinite F50, Tecan) at the manufacturer's recommended wavelength. All the assays were independently replicated thrice.

Statistical Analysis

Chemical constituents of DCP were calculated by using linear regressions obtained from each assay standard calibration curve. Preceding data analysis, SOD, GSH and MDA concentrations were normalised to percentages relative to the control (DCP-free) for each experiment. Data are represented as mean ± standard deviation (SD) for independent triplicates (n=3) and tested for significance level of difference ($p<0.05$) with post hoc Dunnett's multiple comparison test (GraphPad Prism v.8).

RESULTS

Chemical Composition

The yield and total contents of sulphate, carbohydrate, protein and uronic acid of DCP are presented in Table 1. The results obtained show that the polysaccharides made up 17.97 ± 2.84 % of *D. ciliolata* algal dry weight. DCP had a total carbohydrate content of 56.56 ± 1.17 %, sulphate content of 2.58 ± 0.26 %, uronic acid content of 6.54 ± 0.26 % and protein content of 7.31 ± 0.24 %. As summarised in Table 1, four monosaccharide units including fucose, arabinose and galactose were identified and quantified in DCP. Fucose occurs at a high proportion of 27.16 ± 4.27 mg/l followed by galactose (11.47 ± 1.38 mg/l), arabinose (11.88 ± 1.24 mg/l) and glucose (1.64 ± 0.07 mg/l).

Table 1: Yield, chemical and monosaccharide composition of *D. ciliolata* polysaccharides

PARAMETERS	CONTENT
DCP Yield (%)	17.97 ± 2.84
Chemical Composition (%)	
Total carbohydrate	56.56 ± 1.17
Sulfate	2.58 ± 0.26
Uronic acid	6.54 ± 0.26
Protein	7.31 ± 0.24
Monosaccharide Content (mg/L)	
Fucose	27.16 ± 4.27
Galactose	11.47 ± 1.38
Arabinose	11.88 ± 1.24
Glucose	1.64 ± 0.07
Xylose	ND
Mannose	ND

ND = not detected

The infrared spectrum of DCP, ranging from 4000 – 400 cm^{-1}, is presented in Figure 1. DCP showed characteristic peaks of seaweed polysaccharides: the broad band at 3296 cm^{-1} originated from O-H groups stretching vibrations; the signal at 2930 cm^{-1} was as a result of C-H stretching vibrations. There were peaks around 1640 and 1400 cm^{-1} regions indicating asymmetric and symmetric stretching vibrations of C-O and C=O of carboxylate groups respectively. The peak centred at 1028 cm^{-1} is attributed to the occurrence of alduronic acid while the absorption band at 927.8 cm^{-1} is due to C-O stretching vibrations in 3,6-anhydrogalactose.

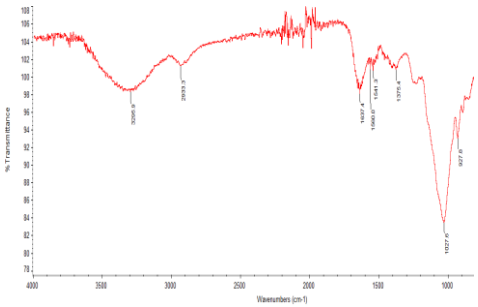

Figure 1: FT-IR spectrum of *D. ciliolata* polysaccharides

Effect of DCP on CLC Cell Proliferation Rate

As shown in Figure 2A, compared with the control (untreated cells), DCP at the lowest concentration (5 µg/ml) resulted in a significant increase ($p < 0.01$) of carp leukocytes proliferation up to 25 % increase in viable cells while at higher concentrations of DCP (200 – 400 µg/ml), the viability of CLC cells significantly decreases ($p < 0.05$) after a 24 h incubation period. As presented in Figure 2B, after 48 h treatment with DCP, the stimulating effects on the proliferation of carp leukocytes reduced with a significant decrease ($p < 0.05$) at dosages of 100 – 400 µg/ml when compared with the control.

Antioxidant Activity and MDA Level

The intracellular antioxidant status including glutathione (GSH) and superoxide dismutase (SOD) are presented in Figure 3A and 3B respectively. Carp leukocytes exposed to DCP at dosages of 12.5 – 50 µg/ml did not cause significant effects on GSH and SOD. Although a trend to decrease SOD activity was observed in DCP-treated cells. As shown in Figure 4, the malondialdehyde (MDA) level was significantly reduced in CLC cells treated with 25 µg/ml, however, dosages of 12.5 and 50 µg/ml were not significantly different from the DCP-free group (control).

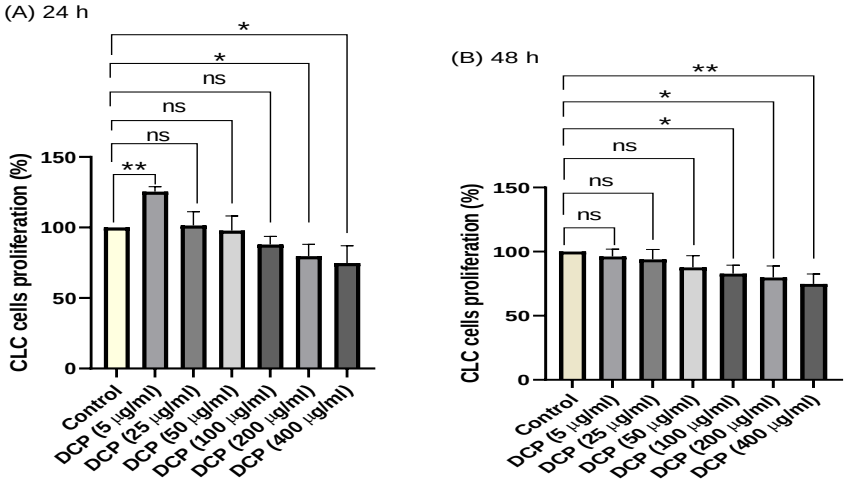

Figure 2: Effect of *D. ciliolata* polysaccharides on carp leukocyte culture (CLC) proliferation rate after 24 h (A) and 48 h (B) incubation period. Significant levels of difference between control and different concentrations of DCP using Dunnett's Multiple Comparison test were indicated as; * $p < 0.05$, ** $p < 0.01$ and not significant as 'ns'

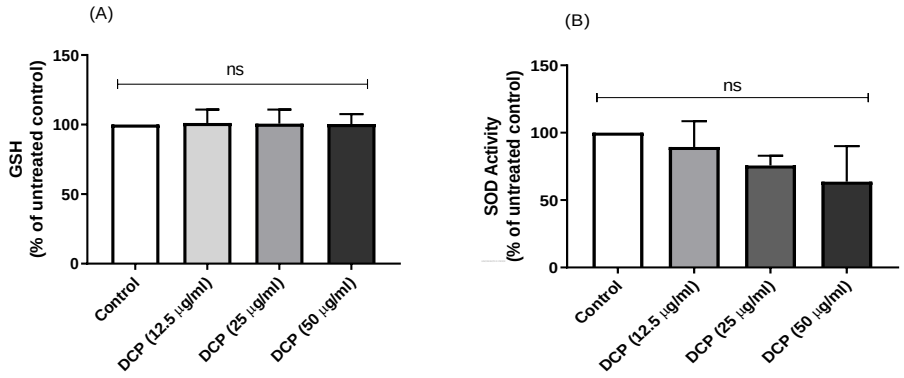

Figure 3: Intracellular antioxidant activity (A) glutathione and (B) superoxide dismutase measured in carp leukocytes treated with *D. ciliolata* polysaccharides. Dunnett's Multiple Comparison test indicated not significant (ns) difference between control and different concentrations of DCP.

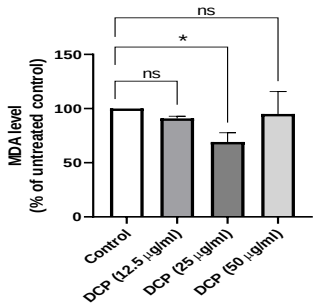

Figure 4: Effects of *D. ciliolata* polysaccharides on malondialdehyde level in carp leukocytes. Dunnett's Multiple Comparison test indicates significant difference (* $p < 0.05$) from untreated control; (ns = not significant).

DISCUSSION

Various techniques have been applied to extract carbohydrate polymers from seaweeds, however hot water extraction (HWE) method serves as an effective method since polysaccharides tend to have a higher solubility in water (Khalil *et al.,* 2018). The extraction method together with the environmental and physiological status of the marine macroalgae species could greatly influence the yield and also chemical constituents of the algal polysaccharides (das NevesAmorim *et al.,* 2011; Souza *et al.,* 2012). The extraction yield of polysaccharides from *D. ciliolata* using HWE was higher than that of the brown seaweeds *Dictyopteris membranacea* (3.89 %), *Turbinaria conoides* (7.89 %) and *Laminaria japonica* (10.25 %) (Abou-Zeid*et al.,* 2014; Sun *et al.,* 2018; Venkatesan *et al.,* 2019). Chemical composition analysis indicated that the DCP chain comprises neutral and acidic sugars together with minute quantities of sulfate esters and proteins. Furthermore, multiple sugar units were identified including fucose as the predominant monosaccharide, arabinose, galactose, and glucose, signifying that DCP is a heteropolysaccharide. Similar monosaccharides were detected in *L. japonica* polysaccharides with a high proportion of fucose also suggesting its dominance in brown seaweed polysaccharides (Sun *et al.,* 2018). The FT-IR spectrum exhibited similar characteristic absorption bands assigned to brown seaweed saccharides moiety (Rupérez *et al.,* 2002; Peng *et al.,* 2012; Cui *et al.,* 2016). FT-IR peaks further confirm the colorimetric

measurements that DCP contains sugars with uronic acids and galactan residues.

Immune cells play remarkable roles in the physiological function of organisms such as development, homeostasis maintenance and protection against pathogens by orchestrating appropriate functional responses (Hodgkinson *et al.,* 2015). Therefore, the evaluation of the viability level and/or proliferation rates of cells are widely used as an indicator of immune cell health when screening for new compounds as drug candidates in biomedical research (Oh *et al.,* 2018). In this study, lower concentrations (≤ 100 µg/ml) of DCP showed no cytotoxicity over 24 h exposure period but instead, carp leukocytes proliferation rate increased at the lowest concentration. However, the proliferative activity after 48 h decreased at higher concentrations (100 – 400 µg/ml) of DCP. These findings aligned with the study of Qian *et al.,* (2021), who reported an increased proliferation rate of RAW264.7 macrophages after incubation for 24 h with lower concentrations (< 50 µg/ml) polysaccharides from brown seaweed *Sargassum fusiforme* and reduced cell proliferation rate after 48 h incubation period. Other studies have also reported the promoting and/or low toxicity effects of seaweed polysaccharides on immunocompetent cells (Tabarsa *et al.,* 2018; Kidgell *et al.,* 2020; Wang *et al.,* 2021).

ROS participates in the regulation of several intracellular and extracellular biochemical processes such as cell signalling and homeostasis, cellular growth, progression and differentiation and apoptosis (Turrens, 2003; Sena and Chandel, 2012). However, excessive ROS production initiates oxidative stress responses that cause structural modification of cellular components including lipids (Valko *et al.,* 2007). The principal defence mechanism against oxidative stress in fish is attributed to the activities of endogenous antioxidants including SOD and GSH (Wang *et al.,* 2018; Zhang *et al.,* 2019). For this study, DCP did not disrupt the intracellular activities of GSH and SOD in carp leukocytes, thereby maintaining redox homeostasis but could significantly reduce MDA level which is a biomarker for the oxidative degradation of lipids within cellular membranes. The non-reduction in GSH level suggests that DCP may not have induced the accumulation of hydrogen peroxide radicals in carp leukocytes. The decline in MDA levels is directly linked to the reduction of intracellular ROS and an indication of impeded oxidative stress. Jayawardena *et al.,* (2020) reported that zebra fish treated with polysaccharides from the brown seaweed *Padina boryana* had decreased ROS concentrations and MDA levels. In addition, Zhang *et al.,* (2013) observed that administered polysaccharides purified from the brown seaweed *Sargussum graminifolium* inhibited MDA formation and improved antioxidant defense status in Wistar rats. Some studies have suggested the antioxidant potential of brown seaweed polysaccharides is greatly associated with their monosaccharide composition, most importantly the presence of fucose and galactose, and functional groups (Lo *et al.,* 2011; Jayawardena *et al.* (2020). These chemical properties have been reported to synergistically induce the scavenging of free radicals.

CONCLUSION

These results suggested that DCP possesses potent effects in promoting carp leukocytes proliferation and reduction of intracellular oxidative. Furthermore, the observed biological activities of DCP are attributed to the occurrence of sulfate esters groups with high amounts of fucose and galactose as revealed by preliminary chemical composition analysis. Therefore, DCP is a promising antioxidant source that could be effectively utilised in improving the well-being of common carp.

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REFERENCES

Abou-Zeid, A. H., Aboutabl, E. A., Sleem, A. A. and El-Rafie, H. M. (2014). Water soluble polysaccharides extracted from *Pterocladia capillacea* and *Dictyopteris membranacea* and their biological activities. *Carbohydrate Polymers*, **113**:62-66.

Alves, A., Sousa, R. A. and Reis, R. L. (2013). *In vitro* cytotoxicity assessment of ulvan, a polysaccharide extracted from green algae. *Phytotherapy Research*, **27**(8):1143-1148.

Batista-Gonzalez, A. E., Silva, A. M., Vidal-Novoa, A. L., Pinto, j. R., Mancini, D. A. and Mancini-Filho, J. O. (2012). Analysis of *in vitro* and *in vivo* antioxidant properties of hydrophilic fractions from the seaweed *Halimedamonile* L. *Journal of Food Biochemistry*, **36**(2):189-197.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**(1-2):248-254.

Cui, C., Lu, J., Sun-Waterhouse, D., Mu, L., Sun, W., Zhao, M., and Zhao, H. (2016). Polysaccharides from *Laminaria japonica*: structural characteristics and antioxidant activity. *LWT - Food Science Technology,* **73**(Supplement C): 602–608.

Dodgson, K. S. and Price, R. G. (1962). A note on the determination of the ester sulphate content of sulphated polysaccharides. *Biochemical Journal*, **84**(1):106-110.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. T. and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, **28**(3):350-356.

Harper, C. and Wolf, J. C. (2009). Morphologic effects of the stress response in fish. *ILAR Journal*, **50**(4):387-396.

Hematyar, N., Rustad, T., Sampels, S. and KastrupDalsgaard, T. (2019). Relationship between lipid and protein oxidation in fish. *Aquaculture Research*, **50:**(5):1393-1403.

Hodgkinson, J. W., Grayfer, L. and Belosevic, M. (2015). Biology of bony fish macrophages. *Biology*, **4**(4):881-906.

Jayawardena, T. U., Wang, L., Sanjeewa, K. A., Kang, S. I., Lee, J. S. and Jeon, Y. J. (2020). Antioxidant potential of sulfated polysaccharides from *Padina boryana*; Protective effect against oxidative stress in *in vitro* and *in vivo* zebra fish model. *Marine Drugs*, **18**(4):212.

Kareem, N., Yates, E., Skidmore, M. and Hoole, D. (2018). *In vitro* investigations on the effects of semi-synthetic, sulphated carbohydrates on the immune status of cultured common carp (*Cyprinus carpio*) leucocytes. *Fish & Shellfish Immunology*, **74**:213-222.

Khalil, H. P. S., Lai, T. K., Tye, Y. Y., Rizal, S., Chong, E. W. N., Yap, S. W. and Paridah, M. T. (2018). A review of extractions of seaweed hydrocolloids: Properties and applications. *Express Polymer Letters*, **12**(4):296-317

Kidgell, J. T., Glasson, C. R. K., Magnusson, M., Vamvounis, G., Sims, I. M., Carnachan, S. M. and Taki, A. C. (2020). The molecular weight of ulvan affects the *in vitro* inflammatory response of a murine macrophage. *International Journal of Biological Macromolecules*, **150:**839–848.

Kubečka, J., Boukal, D. S., Čech, M., Hickley, P., Kitchell, J. F., Ricard, D. and Welcomme, R. (2016). Ecology and ecological quality of fish in lakes and reservoirs. *Fisheries Research*, **173**:1-3.

La Barre, S., Potin, P., Leblanc, C. and Delage, L. (2010). The halogenated metabolism of brown algae Phaeophyta: its biological importance and its environmental significance. *Marine Drugs*, **8**(4):988-1010.

Lo, T.-T., Chang, C. A., Chiu, K.-H., Tsay, P.-K. and Jen, J.-F. (2011). Correlation evaluation of antioxidant properties on the monosaccharide components and glycosyl linkages of polysaccharide with different measuring methods. *Carbohydrate Polymers*, **86**(1):320–327.

Lushchak, V. I. (2011). Environmentally induced oxidative stress in aquatic animals. *Aquatic Toxicology*, **101**(1):13-30.

Martínez-Álvarez, R. M., Morales, A. E. and Sanz, A. (2005). Antioxidant defenses in fish: biotic and abiotic factors. *Reviews in Fish Biology and fisheries,***15**:75-88.

Moghadamtousi, S. Z., Nikzad, S., Abdul Kadir, H., Abubakar, S. and Zandi, K. (2015). Potential antiviral agents from marine fungi: An overview. *Marine Drugs*, **13:**(7):4520-4538.

Mohan, K., Ravichandran, S., Muralisankar, T., Uthayakumar V., Chandirasekar R., Seedevi P., Abirami, R. G. and Rajan, D. K. (2019). Application of marine-derived polysaccharides as immunostimulants in aquaculture: A review of current knowledge and further perspectives. *Fish & Shellfish Immunology*, **86**:1177-1193.

Oh, J. M., Kalimuthu, S., Gangadaran, P., Baek, S. H., Zhu, L., Lee, H. W. and Ahn, B. C. (2018). Reverting iodine avidity of radioactive-iodine refractory thyroid cancer with a new tyrosine kinase inhibitor (K905-0266) excavated by high-throughput NIS (sodium iodide symporter) enhancer screening platform using dual reporter gene system. *Oncotarget*, **9**(6):7075.

Peng, Z., Liu, M., Fang, Z. and Zhang, Q. (2012). *In vitro* antioxidant effects and cytotoxicity of polysaccharides extracted from *Laminaria japonica*. *International Journal of Biological Macromolecules*, **50**(5):1254-1259.

Qian, W. W., Yang, S. Q., Hu, S. M., Wang, X. L., Zhu, Y. and Zhou, T. (2021). Enzymatic degradation, antioxidant and immunoregulatory activities of polysaccharides from brown algae *Sargassum fusiforme*. *Journal of Food Measurement and Characterization*, **15**:1960-1972.

das NevesAmorim, R. C., Rodrigues, J. A. G., Holanda, M. L., de Souza Mourão, P. A. and Benevides, N. M. B. (2011). Anticoagulant properties of a crude sulfated polysaccharide from the red marine alga *Halymenia floresia* (Clemente) C. Agardh. *Acta Scientiarum Biological Sciences*, **33:**(3):255-261.

Rodrigues, J. A. G., Vanderlei, E. D. S. O., de Araújo, I. W. F., Quindere, A. L. G., Coura, C. O. and Benevides, N. M. B. (2013). In vivo toxicological evaluation of crude sulfated polysaccharide from the green seaweed *Caulerpacupressoides* var. *lycopodium* in Swiss mice. *Acta Scientiarum Technology*, **35**(4):603-610.

Rupérez, P., Ahrazem, O. and Leal, J. A. (2002). Potential antioxidant capacity of sulfated polysaccharides from the edible marine brown seaweed *Fucusvesiculosus*. *Journal of Agricultural and Food Chemistry*, **50**(4):840-845.

Sena, L. A. and Chandel, N. S. (2012). Physiological roles of mitochondrial reactive oxygen species. *Molecular Cell*, **48**:(2):158-167.

Souza, B. W. S., Cerqueira, M. A., Bourbon, A. I., Pinheiro, A. C., Martins, J. T., Teixeira, J. A. and Vicente, A. A. (2012). Chemical characterization and antioxidant activity of sulfated polysaccharide from the red seaweed *Gracilaria birdiae Food Hydrocolloids,* **27**(2):287- 292.

Sun, Y., Hou, S., Song, S., Zhang, B., Ai, C., Chen, X. and Liu, N. (2018). Impact of acidic, water and alkaline extraction on structural features, antioxidant activities of *Laminaria japonica* polysaccharides. *International Journal of Biological Macromolecules*, **112**:985-995.

Tabarsa, M., You, S., Dabaghian, E. H. and Surayot, U. (2018). Water-soluble polysaccharides from *Ulva intestinalis*: Molecular properties, structural elucidation and immunomodulatory activities. *Journal of Food and Drug Analysis*, **26**(2):599-608.

Tullia. M.C., Filisetti-Cozzi, N. and Carpita, C. (1991). Measurement of uronic acids without interference from neutral sugars. *Analytical Biochemistry*, **197**:57-162

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *The Journal of Physiology*, **552**(2):335-344.

Valko M., Leibfritz D., Moncol J., Cronin M.T., Mazur M. and Telser J.J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cell Biology,* **39**(1):44–84.

Venkatesan, M., Arumugam, V., Pugalendi, R., Ramachandran, K., Sengodan, K., Vijayan, S. R. and Pugazhendhi, A. (2019). Antioxidant, anticoagulant and mosquitocidal properties of water soluble polysaccharides (WSPs) from Indian seaweeds. *Process Biochemistry*, **84**:196-204.

Vidal, M. C., Williams, G. and Hoole, D. (2009). Characterisation of a carp cell line for analysis of apoptosis. *Developmental and Comparative Immunology,* **33**(7):801–805.

Wang, C. Y., Li, Z. B., Sun, Y. Z., Chen, Q., Li, W. J., Huang, Y. C. and Lu, J. (2018). Effects of Chinese herbal medicines mixture on growth performance digestive enzyme activity immune response of juvenile Japanese seabass, *Lateolabrax japonicus*. *Aquaculture Nutrition*. **24**(2):683-693.

Wang, F., Kong, L. M., Xie, Y. Y., Wang, C., Wang, X. L., Wang, Y. B., and Zhou, T. (2021). Purification, structural characterization, and biological activities of degraded polysaccharides from *Porphyrayezoensis*. *Journal of Food Biochemistry*, **45**(4): e13661.

Wedemeyer, G. (2012). Physiology of fish in intensive culture systems. New York, NY*: Springer Science and Business Media*. p 243.

Weyts F.A., Rombout, J.H., Flik, G. and Verburg-Van Kemenade B.M. (1997). A common carp (*Cyprinuscarpio* L.) leukocyte cell line shares morphological and functional characteristics with macrophages, *Fish and Shellfish Immunology*, **7**:123–133.

Williams, G. M., Iatropoulos, M. J., and Whysner, J. (1999). Safety assessment of butylated hydroxyanisole and butylated hydroxytoluene as antioxidant food additives. *Food and Chemical Toxicology*, **37:**(9-10):1027-1038.

Yang, Q., Yang, R., Li, M., Zhou, Q., Liang, X. and Elmada, Z. C. (2014). Effects of dietary fucoidan on the blood constituents, anti-oxidation and innate immunity of juvenile yellow catfish (*Pelteobagrus fulvidraco*). *Fish and Shellfish Immunology*, **41**(2):264-270.

Yasuhara-Bell, J. and Lu, Y. (2010). Marine compounds and their antiviral activities. *Antiviral Research*, **86**(3):231-240.

Yousefi, M., Paktinat, M., Mahmoudi, N., Pérez-Jiménez, A. and Hoseini, S. M. (2016). Serum biochemical and non-specific immune responses of rainbow trout (Oncorhynchus mykiss) to dietary nucleotide and chronic stress. *Fish Physiology and Biochemistry*, **42**: 1417-1425.

Zhang, B., Li, C., Wang, X., Zhou, H., Mai, K. and He, G. (2019). The effects of dietary Eucommia ulmoides Oliver on growth, feed utilization, antioxidant activity and immune responses of turbot (*Scophthalmus maximus* L.). *Aquaculture Nutrition*, **25**(2):367-376.

Zhang, C. Y., Wu, W. H. and Lan, M. B. (2013). The protection of polysaccharide from the brown seaweed *Sargassum graminifolium* against ethylene glycol-induced mitochondrial damage. *Marine Drugs*, **11**(3):870-880.