Original Article

Anti-Inflammatory and Antimicrobial Activity of Protein Isolated from Spirulina Fusiformis

Janani Priya V*, Gomathi V*, Kumar Ebenezar K**

*PG Students, **Professor, Natural Medicine and Molecular Physiology Lab, Faculty of Allied Health Sciences, Chettinad Academy of Research & Education, Kelambakkam-603103, Chengalpattu Dt., TN. India.

Ms. V. Janani Priya has Completed her M.Sc. Medical Biotechnology at the Faculty of Allied Health Sciences, CHRI, Chettinad Academy of Research and Education, Kelambakkam. At present, she is a trainee in TamilNadu Government Laboratory at Coimbatore.

Corresponding author - K. Kumar Ebenezar (jkebi23@gmail.com)

Chettinad Health City Medical Journal 2021; 10(1): 21 - 31

DOI: https://doi.org/10.36503/chcmj10(1)-04

Abstract

Inflammation is a pathologic condition that includes a variety of diseases including infectious diseases as they can form on the surface and persist after treatment with diverse antibacterial agents. The natural products have demonstrated their effectiveness in many fields and plant based drugs were also used for human diseases. In this study, the protein from green seaweed (S. fusiformis) was extracted, purified using gel chromatography and the active fractions were identified by using UV-spectroscopy, pooled, dialyzed and quantified. Enriched protein from green seaweed was extracted and mineral components were determined using Atomic absorption spectroscopy (AAS). The presence of protein was confirmed by SDS-PAGE and structure elucidation was portrayed by FT-IR spectroscopy. The antioxidant and the teratogenicity of purified protein from S. fusiformis were characterized by the zebrafish embryo (ZFE) model and also antibacterial activity of purified protein was tested with agar well diffusion method. The protein extracted from the green seaweed shown to have the potential of being used as therapeutics with anti-inflammatory property and posses anti-microbial effect which may be taken for further studies.

Keywords: Antioxidant, anti-inflammatory, Anti-microbial activity, S. fisiformis, zebrafish.

Introduction

Inflammation is the essential response by the immune system to ensure the survival of a cell during the injury and tissue damage. It is the host response that assists the removal of a harmful stimulus and also the healing of damaged tissue.[1] The symptoms of inflammation include tenderness, swelling, pain and heat[2] and these inflammatory responses are the primary defensive mechanisms of the body which also involves the physiology. As a pathological immune response, the Macrophages get activated and the inflammation stimulates the cells to produce inflammatory mediators like the nitric oxide (NO), prostaglandins (PGs)and the inflammatory cytokines which leads to numerous signaling pathways of inflammation.[3] Inflammation is triggered by oxidative stress where the reactive oxygen species (ROS) induces the mitogens response at a physiological level and causes damage in the internal components of the cells. ROS can induce the inflammatory mediator signals through the NF-κB pathway-related genes in the cells. Lipopolysaccharide (LPS) is an endotoxin molecule and significant component of the external layer of all Gram-negative bacteria directly causes pathophysiological damage for example, metabolic changes, fever, endotoxic stun, and cause death.[4] These impacts are related to the incitement of LPS to receptors which intercede the signaling cascades and afterward lead to the stimulation of neutrophils, macrophages and the appearance of inflammatory cytokines like IL-6, IL-8, and TNF-α. The acute inflammatory response to the pathogens is caused by the interleukin 8 (IL-8, CXCL8, CXC ligand 8) and the other inflammatory cytokines in various cell types. The LPS, the major component of the cell wall of gram-negative bacteria, increases the production of pro-inflammatory cytokines, nitric oxide (NO), and prostaglandin E2 (PGE2), which are the main cytotoxic and pro-apoptotic mediators involved in the innate response in many mammals[5] Therefore, LPS is associated with a wide variety of inflammatory diseases and has been widely used to mimic the features of inflammatory diseases.

Several classes of drugs available commercially, for example nonsteroidal anti-inflammatory drugs (NSAIDs) including aspirin, ibuprofen, and naproxen are a widely administered which inhibit the prostaglandins, apart from that glucocorticoids and immunomodulatory drugs for anti-inflammatory and

analgesic purposes. Though these engineered drugs have been widely used for treatment, the side effects, however, are often severe and range from gastric ulcers to kidney damage and death remains essential on long term use. The search for anti-inflammatory agents is challenging due to the complexity of the inflammatory process and its role in host defense. However, research studies on the progress in understanding the mechanisms of inflammation has made the identification of new targets possible and open a range of search for new compounds with potential therapeutic effects on acute or chronic inflammatory diseases. Several drug development programs are underway and focuses on the search for bioactive compounds obtained from natural resources. Many drugs used for the treatment of several diseases have been developed and focused on natural products which have been demonstrated to posses' minimal side effects even at higher doses. The studies in terrestrial organisms have been extended to the marine environment, a resource with an enormous potential for drug discovery.

Marine biological system contains essential and optional metabolites gives various assets to human wellbeing and nourishment when contrasted with the earth bound substances.[7] One such source, microalgae are considered as the sustainable source of protein, where the protein level similar to the other protein sources like the meat, egg, soybean and milk. The use of protein algae production has many important values in terms of productivity and nutritional value.[8] Spirulina fusiformis is a microscopic and filamentous spiral-shaped cyanobacterium which is blue-green algae belong to the family Oscillatoriaceae and mainly used as a source of protein and vitamins.[9] It contain high amount of proteins (60–70%) and vitamins (Eg. vitamin B12 and provitamin A (beta-carotene), minerals, and essential fatty acids.[10] S. fusiformis has recommended as a potential food supplement in preventing the Protein Energy Malnutrition (PEM) and the Protein Energy Wasting (PEW). The S. fusiformis has several benefits where it reduces the weight and it has been used as a medicine for many diseases like the attention deficit-hyperactivity disorder, diabetes, hay fever, nervous weakness, depression, premenstrual syndrome and other health issues.[11] The use of microalgae in traditional medicine has been known for the treatment of precancerous development in the mouth, enhancing the immune system, accumulative of energy and metabolism, improving memory, reducing cholesterol thereby preventing heart disease, therapeutic for wounds and improving digestion. In the 21st century, Spirulina has been be considered as the one of the most used healing and prophylactic component of nutrition due to its nutrient profile, therapeutic effects and also the lack of toxicity (Fig.1).[12]

Figure 1: Biological benefits of S. fusiformiss

The natural antioxidants are regularly shown to be a key complement as a preventive medicine that there is an inverse link between the dietary consumption of antioxidant-rich foods and prevalence of human illness. Spirulina has received increased attention by researchers, mainly for its protein content in addition to the pigments that they posses, and also with pharmaceutical and cosmetic industries. In addition to the treatment of certain diseases, this microalga has potential an alternative for production of functional foods and nutraceuticals. Many naturally occurring substances are anti-oxidant and have the potential of reducing inflammation thereby reduce illness and also protect cells. The scientific literature related to protein fractionation from S. fusiformis is still limited, thus further studies on its functional and other properties are needed. Hence, the present study is proposed to extract and fractionate the protein and further analyze the in vitro anti-oxidant and anti-inflammatory activities of S. fusiformis.

Materials and Methods

Collection of sample

The S. fusiformis (wet weight 2 kg) was purchased from the SAB Research Centre, Pudukkottai main road, Thanjavur, Tamil Nadu.

Processing of S. fusiformis

The collected S. fusiformis sample was totally washed once in tap water and shadow dried for about a week. Then the dried sample was finely powdered with mechanical grinder. Approximately 10 g of the algal sample was dissolved in the following solvent such as ethanol, methanol, chloroform, petroleum ether, and aqueous solution. Then it was

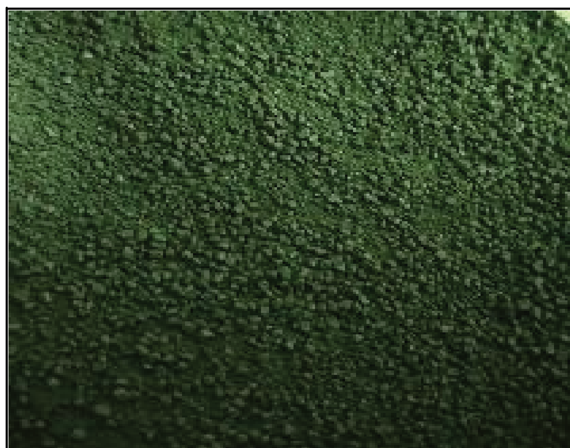

Figure 2: Powder of Spirulina fusiformis

subjected to non-stop agitation for 3 consecutive days at 180–220 rpm,[13] filtered gently in filter paper (no 42) and deposited in a sealed container for further use (Fig. 2).

Protein extraction

The dried S. fusiformis was extracted by ultrasound extraction method. About 5.0 g sample was defatted by hexane where the lipid compounds will be removed then the sample was mixed with distilled water and kept in magnetic stirrer for 60 min at room temperature at pH 8.0 and the pH was altered by using 0.1M HCl. Then the sample was treated with utrasound device sonicator for 60 min. It was then kept in the water bath shaker for 60 min at 37oC and then centrifuged at 4000 rpm for 30 min at 4oC. The pH of the supernatant was adjusted to 3.010. Then the sample was centrifuged at 4000 rpm for 30 min at 4oC, supernatant was discarded and the precipitate was stored at 4oC for further analysis.[14]

Fractionation of protein

The precipitate was dissolved in milliQ water and taken for protein purification using silica gel chromatography (70 x 1.5 cm). Fractions of protein were collected using PBS (pH 7.2) with different concentrations 0.01 M, 0.05 M, and 0.1 M. The elution rate was 0.3 ml/min and the active fractions were identified using UV-spectroscopy, pooled dialyzed against the same buffer, freeze dried and stored at -20oC for further study.[15]

Total protein estimation

The total protein was estimated by using the method of Bradford[16] (1976) using BSA as standard. Total protein estimation was done for partial purified (dialysed) and crude samples. A graph was plotted based on the absorbance values obtained in spectrometer at 595 nm and the concentrations of protein in the samples were calculated.

SDS-PAGE

The presence of protein in the purified extract was confirmed using SDS-PAGE following the method of Karthik et al. (2016).[17] The proximate weight range of the crude, fractionated and purified protein was estimated using 12% polyacrylamide gels and the bands were stained using coomassie brilliant blue R250 and visualized using a Merck Gel documentation system.

Minerals contents estimation by using Atomic Adsorption Spectroscopy

About 2.0 g of purified protein of S. fusiformis was taken in a glass container. 10 ml of perchloric acid was added and left without disturbance for 5 min. Then, 10 ml of conc. HNO3 was added to it, incubated for 5 min and then 10 ml of conc. HCl was added. The mixture was allowed to evaporate and the final residue was dissolved in 10 ml of conc. HCl. The filtrate was subjected to analysis in atomic absorption spectrophotometer. The same producedure was adopted from the raw material of S. fusiformis. The minerals analyzed results were expressed as ppm and percentage.[18]

Antimicrobial activity

The minimum inhibitory concentration (MIC) was tested on purified protein from S. fusiformis for antimicrobial activity. For this, LB broth was prepared for 100 ml, transferred into glass test tubes, inoculated the bacterial culture (E. coli, Acinobacter, Pseudomonas, Staphylococcus and Klebsiella) and incubated overnight at 37oC for growth of organisms. To the MH agar plates, wells were made and purified protein (1µg/ml) was added to each well ranging from 20 µl to 60 µl, then the plates were incubated at 37 ° C for 24 hr. Antibacterial activity was evidenced by the presence of clear inhibition zone. The diameter of this zone was measured zone of inhibition (mm) of each well.[19]

In vitro anti-oxidant activity

DPPH (1, 1, diphenyl-2, picrylhydrazyl) scavenging activity

The DPPH radical scavenging assay has been done in a 96 well microtiter plate as this is much more penetrating, fast and environmentally-friendly. 50 µl of 100 µg/ml methanolic DPPH solutions was added to 200 µl of different concentration of purified protein sample (12.5-100 µg/ml) and mixed in a vortex. Ascorbic acid existed as a standard. The solution has incubated in dark at room temperature. The absorbance of the purple solution has recorded with microplate reader at 517 nm. A standard curve of ascorbic acid (5-50 µg/ml), has used as a positive control. The percentage of scavenging

on the purified protein from S. fusiformis was calculated by using the formula

DPPH scavenging activity (%) =
(1-λ517-S/λ517-C) x 100

The λ517-S was the absorbance of remaining DPPH in the presence of scavenger.[20]

H_2O_2 radical scavenging activity

The H_2O_2 scavenging activity of purified protein activity was determined by the method of Karthik et al. (2016).[17] About 0.3 ml of purified protein was reacting with 600 µl H_2O_2 solution (40 Mm) and kept for 10 min at room temperature. The absorbance has measured at 239 nm in UV spectroscopy. The percentage of H_2O_2 scavenging activity of a purified protein can be calculated by using the equation

H_2O_2 scavenging (%) =
[(A control -A control) / A control] x 100

Control used was H_2O_2 and the standard is the ascorbic acid.

Total antioxidant activity

Total antioxidant capacity of the purified protein from S. fusiformis was determined. About 0.3 ml of the sample was made to react with the 3.0 ml of total antioxidant capacity (TAC) reagent (0.6 M H2SO4, 28 mM Sodium phosphate, 4 Mm ammonium molybdate). The mixture was incubated at 950C for 90 min in a water bath. The absorbance of the reaction was measured at 695 nm in UV spectrophotometer. The TCA was calculated with reference to the standard ascorbic acid, stated by mg Ascorbic acid equivalents/g.[21]

Phosphomolybdenum activity

The Phosphomolybdenum activity was estimated the purified protein by the green phosphomolybdenum complex formation. The reagent solution contains 0.588 ml of H_2SO_4, 0.036 g sodium phosphate, and 0.049 g ammonium molybdate which was made up to 10ml with distilled water. The sample with different concentrations (0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml) were mixed with 1 ml of reagent, then it has been kept in water bath at 90°C for 90 minutes. Then the absorbances can be measured in UV-spectrophotometer at 695 nm with ascorbic acid as standard. The phosphomolybdenum reduction potential (PRP) of purified protein can be reported in percentage as previously reported.[22]

Ferric reducing/antioxidant power (FRAP) assay

The FRAP reagent was prepared by adding 300 mM acetate buffer (pH-3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution and 20 mM FeCl3.6H2O in a 10:1:1 ratio and heated in a water bath at 370C. 3.0 ml of this working standard was mixed with 100 µg of purified protein, 300 µl of distilled water and allowed to stand for 4 mins. Then the concentration was measured at 593 nm using spectrophotometer. 200µl of the working solution as blank was added to the microtiter plate. The result has been expressed as the concentration of the antioxidant that has the ability to reduce the ferric ion.[23]

In vivo anti-inflammatory activity

Wild type zebrafish was handled as described by animal welfare regulations and maintained according to standard protocols (zfin.org). Adult zebrafish were maintained at 25 ± 0.5°C with 14:10 h photoperiod (light: dark) as bought and assimilated for 10 days before the experiment. Commercially available brine shrimp was fed twice a day. Healthy matured female and male fish were selected in the ratio of 2:1 and kept in the breeding tank undisturbed on the day before the spawning. Eggs were collected by natural spawning and the embryos were separated within 1h of light exposure and rinsed in fresh embryo medium (EM). The fertilized and normal embryos obtained at approximately 2 h post-fertilization (hpf) were selected for the test and inspected under microscope.[24]

Induction of Inflammation

Inflammation was induced in zebrafish larvae using a stock solution of 0.5 mg/ml LPS (E. coli - O55:B5 L2880; Sigma) by static immersion for 48 h. The 3 days post fertilization (dpf) larvae (2/well in 250 µl) were introduced into "U" bottom micro titer plates (Greiner Cellstar, USA) with embryo medium. The experiment was carried out for 5 days with 6 groups (n=10/group) divided as untreated control, LPS induced, LPS induced and treatment with different concentrations of purified protein from S. fusiformis samples, positive control with Betamethasone at a final concentration at 100 mg in 1% DMSO. The larvae were pre-treated with the purified protein in serial dilutions. The zebra fish larvae were constantly monitored for intestinal inflammation and treatment effects in a microscope (Nikon Eclipse Ni-U, USA).[25]

Group 1	Normal control (normal diet and water)
Group 2	Induced LPS (0.5 mg/ml)
Group 3	LPS (0.5 mg/ml) + 200 mg of purified protein
Group 4	LPS (0.5 mg/ml) + 100 mg positive control (Betamethasone)

Table 1: Experimental groups in zebrafish larva

Embryo toxicity

The early life stages of zebrafish embryo were monitored with a stereomicroscope and the mortality and developmental malformations in exposure to LPS observed at 24, 48, 72, and 96 and 120 hpf. To detect toxicity using zebrafish embryos, diluted test concentrations were prepared to avoid complete inhibition due to high toxicity of slurry. The selected normal embryos (at approximately 3 hpf) were transferred into 24 well plates, such as one embryo per well. Each well contained 1.0ml control or exposure waste water. Two replicates for the controls and exposure groups were used. For each control and exposure group, the early embryonic development was examined under the microscope and mortality was recorded at an interval of 24 h. After 72 h exposure experiments, mortality, heart beat rate and malformations of embryos in each group were documented.[26]

In vivo antioxidant assay

The antioxidant enzymes levels of SOD, Catalase, GPx and GSH in zebrafish larve tissues were determined by using commercial kit (Sigma).

Histology

On termination of the study, the zebrafish larvae were anaesthetized using tricaine (120 µg/ml) solution and the larva are processed for histopathology. The larvae are fixed in 10% neutral buffered formalin and were subjected dry heat at 70°C. The dehydrated larvae are fixed with molten paraffin wax. The zebra fish larvae fixed in paraffin were embedded transverse axes in paraffin blocks. The paraffin blocks were trimmed and sections of 3 µm thickness were taken using a microtome (brand, make). The sections were kept at 70°C for 10 minutes, deparaffinized in multiple washes of xylene, rehydrated using absolute alcohol and 70% alcohol. The rehydrated sections were stained using hematoxylin and exosin, alcian blue (1%) and Sudan black (0.7%). The stained segments has washed in running distilled H_2O, rehydrated using alcohols, cleared using xylene and fixed by DPX mountant and visualized under the dissection microscope (Nikon Eclipse Ni-U, USA) for cytological changes associated with induction of inflammation.

Statistical Analysis

Each test is performed from 10 zebrafish larvae and all results are expressed as the means plus-minus their corresponding standard deviation (SD). Statistical analysis was performed using SPSS software (SPSS, IBM, version 21.0).

Results and discussion

Extraction of purified protein from S. fusiformis

The extraction was done by Ultrasound extraction method where the proteins get extracted (Fig.3) from 2 kg of S. fusiformis obtained. The purified protein from S. fusiformis may be alternative marine based natural product which has taken for further analysis.

Figure 3: Extraction of protein from S. fusiformis

Column Chromatography

Column chromatography was used to fractionate the protein from the S. fusiformis and the fraction of the column has been measured in UV- Spectrophotometer with 280-700 nm against the protein concentration (Fig.4) and the 280 nm showed the protein concentration. The purified protein should absorb maximum UV spectrum at 280 nm, because of the presence of aromatic amino acids15.

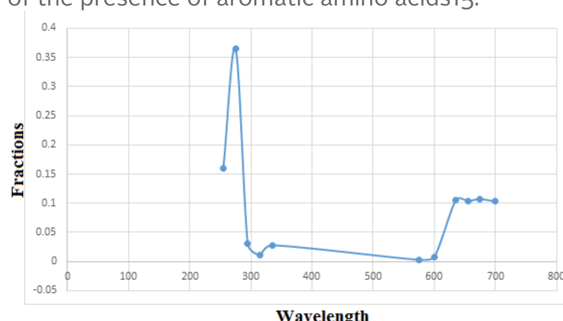

Figure 4: Column fractions of purified proteins

Protein Concentration

The Bradford's method was used to determine the protein concentration in S. fusiformis. The raw form of S. fusiformis has the concentration of 0.1174% (Fig.5) where the purified protein of S. fusiformis has the concentration of 0.219% (Fig.6). The purified protein concentrations was low when

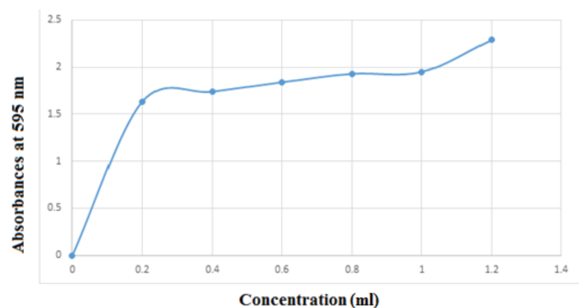

Figure 5: Concentration of crude protein from S. fusiformis

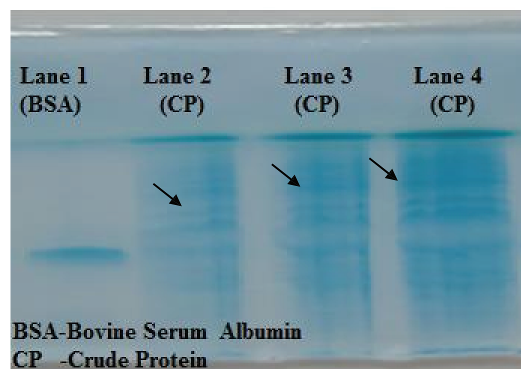

Figure 7: SDS-PAGE Crude Protein

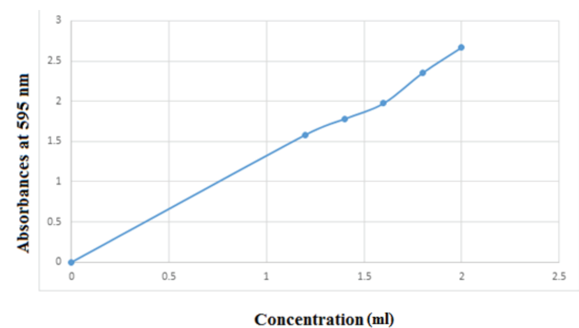

Figure 6: Concentration of purified protein from S. fusiformis

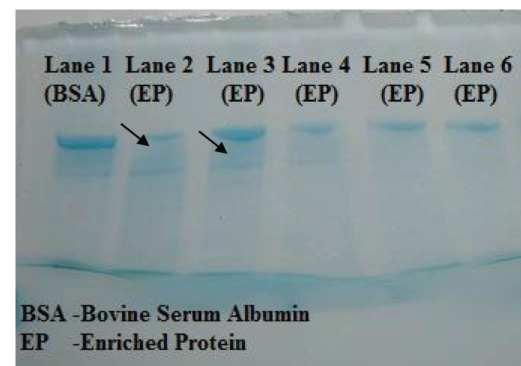

Figure 8: SDS-PAGE Purified Protein

compared to the result of Benelhadj et al.10, the difference of the yield was due to analytical method of purification.

SDS-PAGE of crude and purified protein

The figure 8 shows the SDS-PAGE pattern of a crude protein sample where the lane 1 is loaded BSA (Bovine Serum Albumin) standard with molecular weight of 67 kDa, then lane 2, lane 3 and lane 4 is the crude sample with different concentration. The average molecular weight of the crude protein is about 50 to 80 kDa (Fig.7).

The Fig. 8 depict the SDS-PAGE pattern of an purified protein sample where the lane 1 is loaded with BSA (Bovine Serum Albumin) standard with molecular weight 67 kDa, then lane 2, lane 3, lane 4, lane 5 and lane 6 is the crude sample with different concentration and the average molecular weight of the crude protein is 60 to 70 k Da. This is in accordance with the protein fractions was observed by Benelhadj et al10.

Atomic Absorption Spectroscopy

The mineral content of crude and purified proteins was depicted in table 2, the purified protein sample shows higher concentration of protein and calcium contents. The present result was associated with the result of Reshma21 who stated that the aqueous extract whole seaweed samples gives higher yield and mineral contents, the difference of the results was due to species difference.

Minimum inhibitory concentration (MIC)

The different concentrations of purified protein from S. fusiformis was subjected to antimicrobial activity on the basis of minimum inhibitory concentration (MIC) towards different microorganisms, such as E. coli, Staphylococcus aureus, Klebseilla pneumoniae, Acinobacter and Pseudomonas aeruginosa. The MIC was observed as a significant and promising against all selected microorganisms where the maximum inhibitory activity of purified protein was shown against Acitobacter and Pseudomonas (Fig. 9). The antimicrobial activity of purified protein sample was maximum by increasing concentration of the concentration of proteins (Manigandan et al.).[18]

In vitro antioxidant activity

S.No	Particulars	Protein (%)	Calcium (%)	Phosphorous (%)	Salt (%)
1.	Crude Protein	12.72	1.00	0.35	15.51
2.	Purified Protein	63.48	1.58	--	6.19

Table 2: Analysis of mineral content

Antioxidant activity was determined with purified protein from S. fusiformis. There were many methods to determine the antioxidant activity and are different in terms of their assay principles and experimental conditions.

DPPH radical scavenging activity

The purified protein from S. fusiformis was studied for the ability to scavenge the DPPH radicals as shown in Fig.10. About 200 µl concentration of purified protein showed significant ($P<0.001$) elevated levels of scavenging activity when compared to the 50 µl concentration whereas the standard of 100 µl of ascorbic acid has high concentration DPPH scavenging activity. Thus significant variations in the DPPH scavenging abilities of the bioactive compounds isolated from S. fusiformis was seen as it may be due to variation in the type of extraction and differences in the zone of collection of S. fusiformis. Karthik et al.[17] also confirming the present results, the scavenging activity of marine samples differ in each other.

H_2O_2 radical scavenging activity

The H_2O_2 radical scavenging activity of purified protein extracted from S. fusiformis was illustrated in the Fig.10. The purified protein showed significant ($P<0.001$) increase in the H_2O_2 scavenging activity at the concentrations of 200µl, closely followed by concentrations of 100µl and 50µl. The 100µl of ascorbic acid exhibited higher H_2O_2 radical scavenging activity when compared with standard, resulting in the free radicals that have been generated. It is also due to the differences in extraction procedures like usage of a different solvent and filtration procedures etc. The H_2O_2 radical activity of the present study was low when compared to the result of Manigandan et al.18, the difference of the activity was due to nature of seaweed sample.

Total antioxidant capacity

The total antioxidant activity of the purified protein extracted from S. fusiformis was represented in Fig.10. The purified protein showed significant ($P<0.001$) increase in the total antioxidant activity at the concentration of 200µl, closely followed by concentrations of 100 µl and 50 µl respectively. The present results are accordance with the result of Reshma21, stated that the in vitro antioxidant was maximum by increasing the concentration of the seaweed extract.

Phosphomolybdate assay

The scavenging phosphomolybdate activity of purified protein extracted from S. fusiformis was shown in Fig.11. The purified protein from S. fusiformis showed significant ($P<0.001$) increase in the scavenging phosphomolybdate activity at the concentration of 200 µl, closely followed by concentrations of 100 µl and 50 µl. The standard antioxidant ascorbic acid exhibited scavenging phosphomolybdate activity which was compared with purified protein from S. fisiformis.

FRAP reducing power activity

The FRAP reducing power activity of purified protein extracted from the S. fusiformis was calculated and the result was expressed as percentage. The FRAP reducing power activity was brought about by the reduction of the Mo6+ ions to form a green phosphate/molybdenum complex. The purified protein exhibited significantly ($P<0.001$) increased FRAP reducing power activity at 200 µl followed

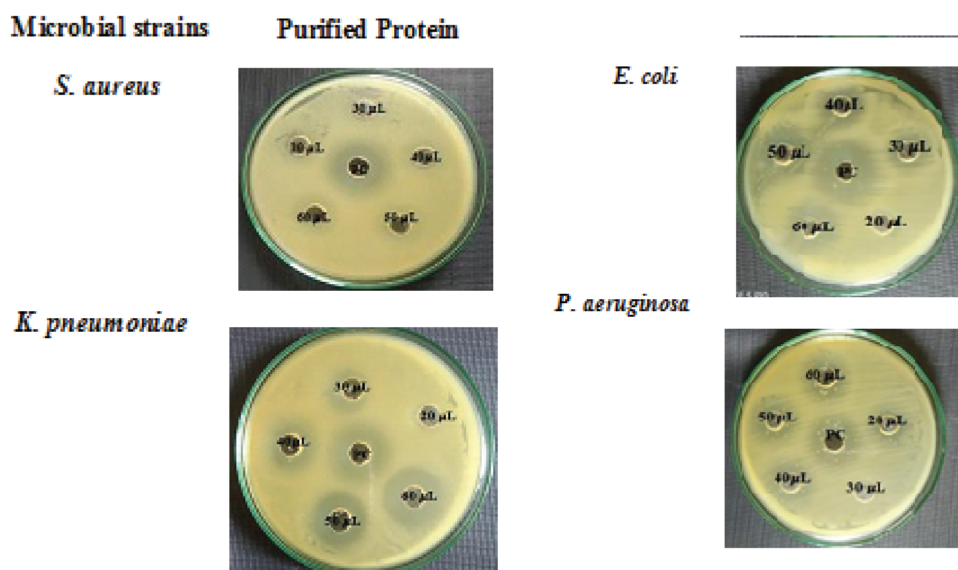

Figure 9: Minimum inhibitory concentration (MIC) of purified protein (20, 30, 40, 50, 60 µg/ml) assigned respectively in wells 1-5 and PC- Ampicillin)

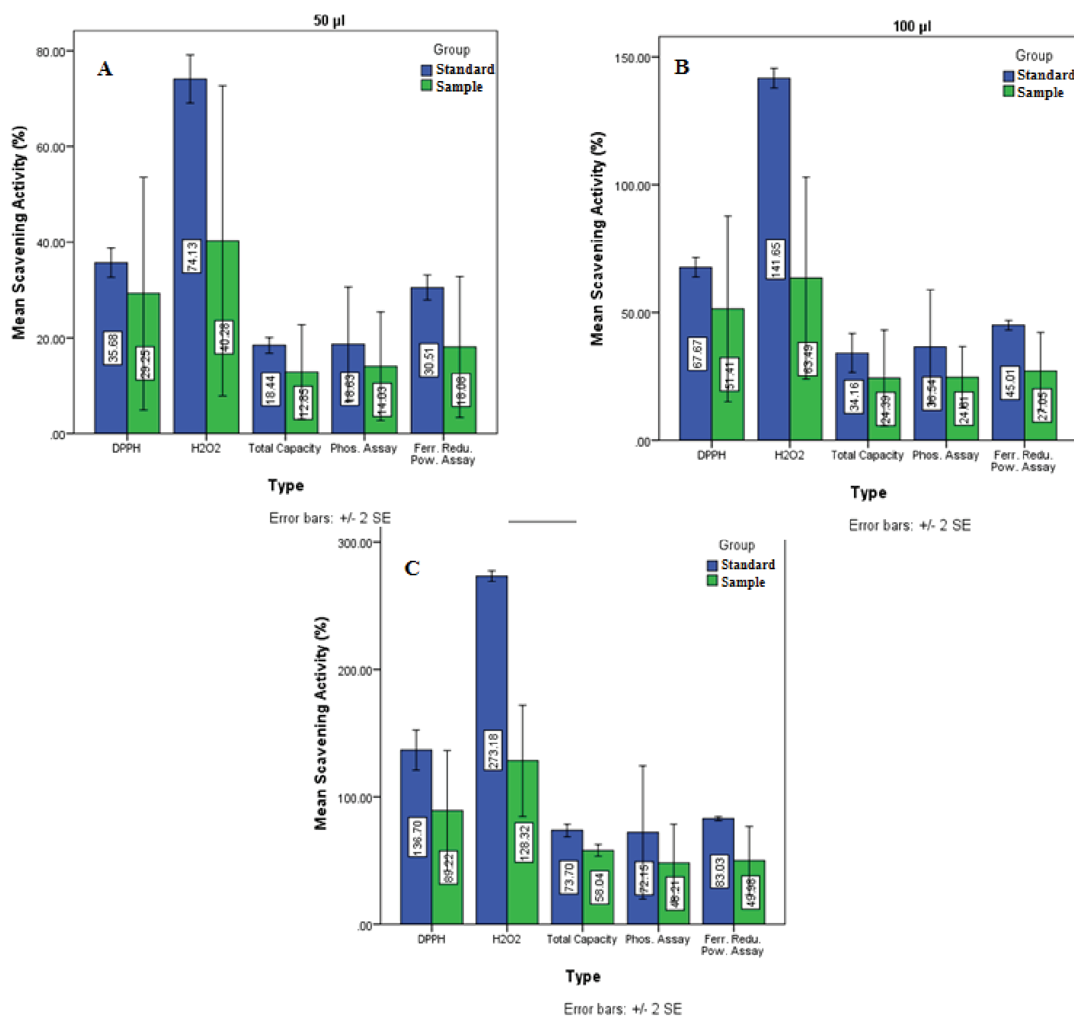

Figure 10: (A-C) In vitro Anti-oxidant activity of purified protein from S. fusiformis

by100 µl. The antioxidant activity was brought about by the ability to scavenge the free radicals and thus in the current study, the purified protein of S. fusiformis showed significant antioxidant activity by scavenging the reactive oxygen species. The in vitro antioxidant activity of purified protein from S. fusiformis was less when compared with previous report of agar from seaweed21 where seaweed was macroalga and S. fusiformis was microalga.

In vivo antioxidant activity

The in vivo anti-oxidant defense system like SOD, CAT, GPx and GSH in zebrafish larva induced with LPS (group II) exhibited a significant (P<0.05) decrease in the tissues of the zebrafish larva when compared to normal (group I) zebrafish larva. Group III zebrafish larva showed a significant (P<0.05) increase in the activities of all these parameters. Cellular life in an oxygenated background has necessitated the progression of effective cellular tactics to identify and detoxify metabolites of ROS. These effects can disturb significantly a host of physiological practices and metabolic pathway locally bounded up with the zebrafish larva of inflammation disorders. For example, excess ROS can produce lipid peroxidation in vivo. The significant (P<0.05) rise in the activities of the primary enzymatic anti-oxidant defenses – SOD, CAT, GPx and GSH in the sample co-administrated groups (group III) and betamethasone (group IV) treated zebrafish larva. It has been suggested that small amount of purified protein enhanced the antioxidant activity of SOD. The GSH activity [Fig.11 (A-D)] of LPS induced (group II) fish was significantly (P<0.05) lower than control, sample with different concentration and betame-thasone administrated groups of larva. The level of GSH has the significant factor for quantifying the level of antioxidative activity in vivo. GSH can be curtained in almost every cell of the body and have the tremendous antioxidant and detoxifying activity. It cannot only be eradicate free radicals in vivo but also boost up the immunity level also.[27] On the bases of the previous report21 the in vivo antioxidant properties of agar seem to be similar with the purified protein from S. fusiformis.

In-vivo anti-inflammatory Activity

Zebrafish embryos toxicity was assessed with increasing concentrations of purified protein in

96-well U-bottom microplates. All the embryos were exposed to purified protein of different concentrations (100, 200, 300 mg/ml) based on the previous report17. The exposure on higher concentration of 300 µg/ml for 48, 72, 96 hpf were showed for malformations. The LC50 of Zebrafish embryo was observed to be 200 µg/ml. D. rerio embryos were in line with the results of a previous study reported.[28]

The zebrafish embryo toxicity were validated based on the developmental malformations such as tail curvature, delayed hatching and yolk sac deformities shown in (Fig.13). From the above figure, the present study was concluded that the purified protein from S. fusiformis has not shown toxicity up to 200 µg/ml and this concentration of purified protein may be taken for further study.

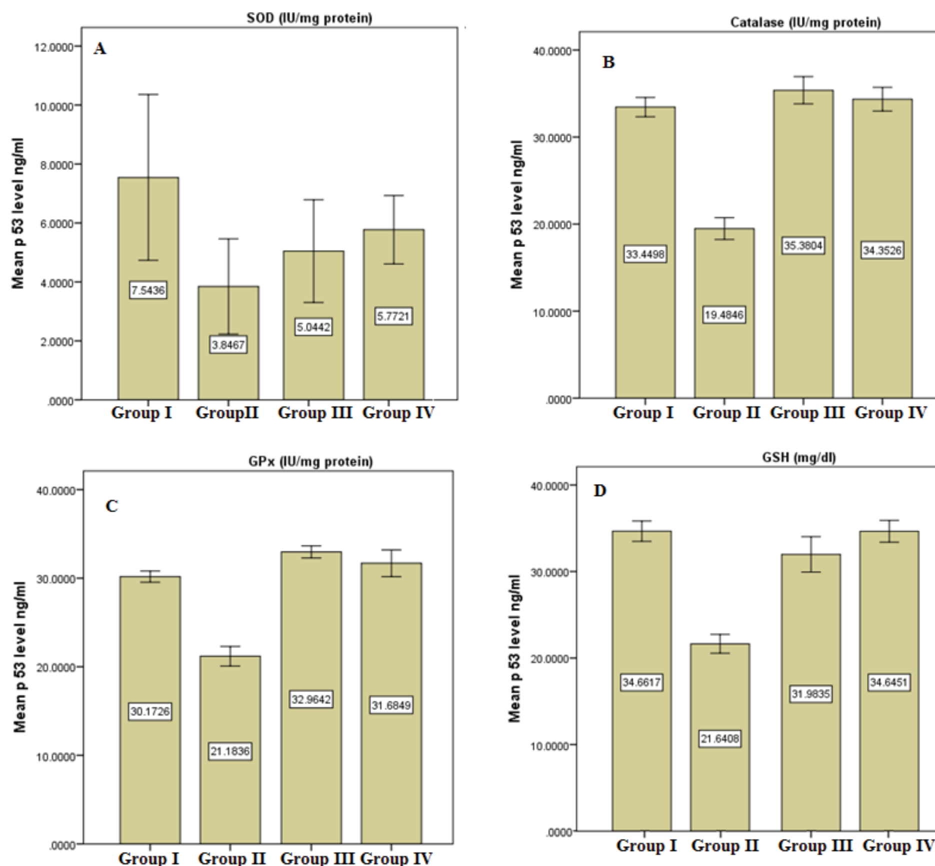

Figure 11: (A-D) In vivo antioxidant activity of experimental zebrafish larva Group I - Control; Group II - LPS induced; Group III - 200 mg of purified protein; Group IV - Positive (Betamethasone)

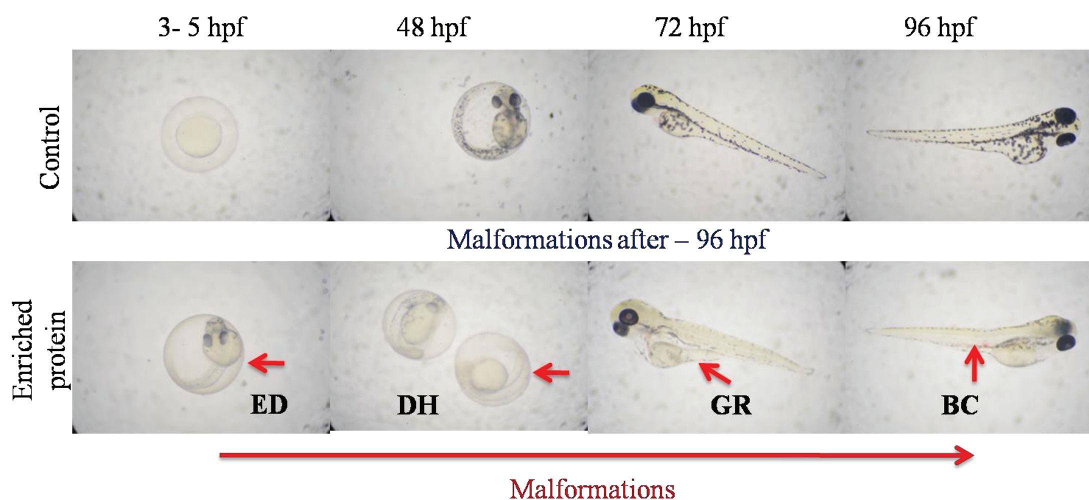

Figure 12: Toxicity of the zebrafish embryos

Histology

The histology of the sections revealed the inflammatory processes in the control, LPS treated and purified protein from S. fusiformis pretreated zebra fish larva. The larval sections stained with hematoxylin and eosin established the inflammation in LPS induced inflammation in zebra fish larva, which remained restored in a dose-dependent method of purified protein-pretreated LPS induced inflammation. The inflammation pathogenesis was illustrated with decreased intestinal vasculature, increased leukocyte recruitment, increased cellular proliferation and skin damage (Fig.13). H & E staining of the sections revealed pronounced inflammation by increase in intestine size, while increased goblet cell population (stained dark blue) was observed by AB staining in LPS induced zebra fish larva. However, the histological sections of larva treated with purified protein showed significant reduction in AB staining, demonstrating potential preventive effects of inflammation.

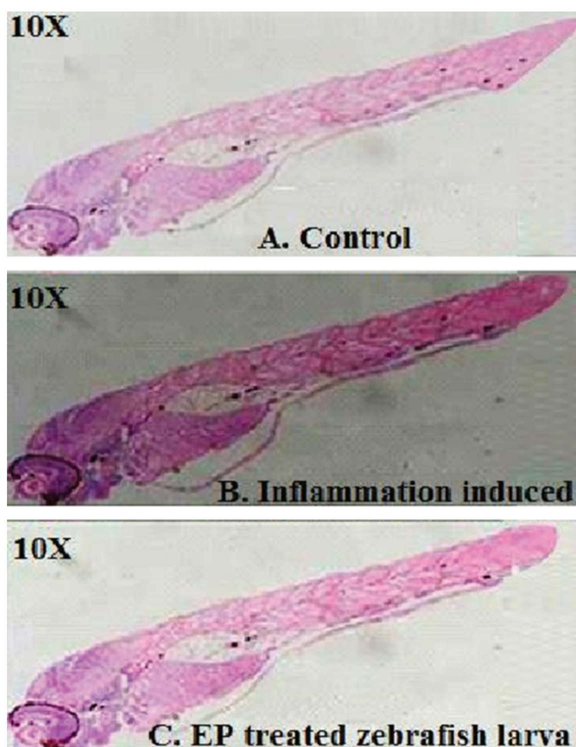

Figure 13: Hematoxylin and Eosin staining

Conclusions

The natural products play an important role in prevention and /or potential therapeutic application in various diseases like inflammation and allergy. In present study the purified protein was extracted from S. fusiformisis by the ultrasound extraction method where the protein content was observed as 0.1174%. It has been fractionated for the soluble protein in the buffer. Then the total concentration and molecular weight has been determined. The in vitro antioxidant activity of purified protein from S. fusiformis was significantly high at 200µl among the selected concentration. The purified protein also exhibited commendable inhibition in the growth of microbiota associated with inflammation marked by definite zone of inhibitions, comparable to the levels of positive control. The in vivo antioxidant activity of purified protein showed significantly high where the LPS treated showed decreased anti-oxidant enzymes in the experimental zebrafish larva. Further the efficacy of anti-inflammatory and anti-oxidant activity of purified protein was evidenced in vivo by staining assays. In conclusion, the purified protein from S. fusiformis has the immense potential as an anti-oxidant and anti-inflammatory agent with less adverse effects. The traditional natural marine based source may play a vital role in the future as novel drug for the inflammatory and various diseases with further studies in clinical trial management.

Acknowledgement

The authors gratefully acknowledge Dr. R. Saravanan, Faculty of Allied Health Sciences, CARE for providing the direction and permitted to use the Zebra fish facility.

Conflict of interest

No conflict of interest to declare among the authors.

References

1. Ahmed AU. An overview of inflammation: mechanism and consequences Front. Biol. 2011; 6: 274. https://doi.org/10.1007/s11515-011-1123-9

2. Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008: 454: 428-435. DOI: 10.1038/nature07201

3. Jayawardena TU, Fernando IPS, et al. Isolation and purification of fucoidan fraction in Turbinaria ornata from the Maldives; Inflammation inhibitory potential under LPS stimulated conditions in in-vitro and in-vivo models. Int. J. Biol. Macromol. 2019; 131: 614-623. DOI: 10.1016/j.ijbiomac. 2019.03.105

4. Yang C, Yu L, et al. Pyrroloquinoline quinone (PQQ) inhibits lipopolysaccharide induced inflammation in part via downregulated NF-κB and p38 / JNK activation in microglial and attenuates microglia activation in lipopolysaccharide treatment mice. PLoS One. 2014; 9: e109502. DOI: 10.1371/journal.pone. 0109502

5. Lin CY, Wang WH, Chen SH, et al. Lipopolysaccharide-Induced Nitric Oxide, Prostaglandin E2, and Cytokine Production of Mouse and Human Macrophages Are Suppressed by Pheophytin-b. Int. J. Mol. Sci. 2017; 18: 2637. DOI: 10.3390/ijms18122637

6. Vane JR and Botting R.M. Anti-inflammatory drugs and their mechanism of action. Inflamm. Res. 1998: 47; S78–S87. https://doi.org/10.1007/s000110050284

7. Maingandan V, Karthik R, Saravanan R. Marine carbohydrate based therapeutics for Alzheimer disease – Mini Review. J. Neuro. Sci. 2015: S10; 1-6 DOI: 10.21767/2171-6625.S10010

8. Brennan L, Owende P. Biofuels from micro-algae - A review of technologies for production, processing, and extractions of biofuels and co- products. Renew. Sustain. Energy Rev. 2010: 14; 557–577. https://doi.org/10.1016/j.rser.2009.10.009

9. Wu Q, Liu L Miron A, et al. The antioxidant, immunomodulatory, and anti-inflammatory activities of Spirulina: an overview. Arch. Toxicol. 2016: 90; 1817–1840. https://doi.org/10.1007/s00204-016-1744-5

10. Benelhadj S, Gharsallaoui A, Degraeve P, Attia H, Ghorbel D. Effect of pH on the functional properties of Arthrospira (Spirulina) platensis protein isolate. Food Chem. 2016: 194; 1056-1063. https://doi.org/10.1016/j.foodchem.2015.08.133

11. Siva Kiran RR, Madhu GM, Satyanarayana SV. Spirulina in combating Protein Energy Malnutrition (PEM) and Protein Energy Wasting (PEW) - A review. J. Nut. Res. 2015: 3; 62-79. DOI: 10.13140/RG.2.1.3149.0325

12. Lupatini LA, Colla LM, Canana C, Collaa E. Potential application of Microalgae Spirulina Platensis as a protein source. J. Sci. Food Agricult. 2016: 99; 3672-3680. DOI: 10.1002/jsfa.7987

13. Thamilmaraiselvi B, Steffi PF. Investigation of phytochemical constituents in Spirulina fusiformis for antibacterial activity. Nat. J. Physiol. Pharm. Pharmacol. 2018: 8; 1491-1495. DOI: 10.5455/njppp.2018.8.0417030072018

14. Yucetepe A, Saroglu O, Bildik F, Ozcelik B, Daskaya-Dikmen C. Optimisation of ultrasound-assisted extraction of protein from Spirulina platensis using RSM. Czech J Food Sci. 28; 2018: 36: 98-108. https://doi.org/10.17221/64/2017-CJFS

15. Saravanan R. Isolation of low-molecular weight heparin/heparin sulphate from marine sources. Adv. Food. Nutr. Res. 2014: 72; 45-60. doi: 10.1016/B978-0-12-800269-8.00003-8

16. Bradford MM. A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-Dye Binding. Anal. Biochem. 1976: 72; 248-254. DOI:10.1006/abio.1976.9999

17. Karthik R, Manigandan V, Sheeba R, Saravanan R, Rajesh PR. Structural characterization and comparative biomedical properties of phloroglucinol from Indian brown seaweeds. J. Appl. Phycol. 2016; 28: 3561-3573. https://doi.org/10.1007/s10811-016-0851-2

18. ManigandanV, Velusamy A, Rubanya P, Karthik R, Karthi S, Sri Ramkumar V, Umamaheswari S, Saravanan R, Arivalagan P. Antioxidant, anticoagulant and mosquitocidal properties of water soluble polysaccharides (WSPs) from Indian seaweeds. Proces. Biochem. 2019; 84: 196-204.

19. Sánchez E, Rivas Morales C, Castillo S, Leos-Rivas C, García-Becerra L, Ortiz Martínez DM. Antibacterial and Antibiofilm Activity of Methanolic Plant Extracts against Nosocomial Microorganisms. Evid. Based Compl. Alternat. Med. 2016: 1572697. doi: 10.1155/2016/1572697

20. S. Li, Y. Zhao, L. et al. Antioxidant activity of Lactobacillus plantarum strains isolated from traditional Chinese fermented foods. Food Chem. 2012: 135; 1914-1919. DOI: 10.1016/j.foodchem.2012.06.048

21. Reshma BS. Evalution of antioxidant and anti-skin aging properties of agar from brown seaweed L. digitata (Hudson) in aging mice induced by D-galactose. M.Sc. Dissertation submitted to Chettinad Academy of Research and Education. 2018; 16-22 (Unpublished data)

22. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantification of antioxidant capacity through the formation of a phosphomolybdenum complex. Specific application to the determination of vitamin E. Anal. Biochem. 1999: 269; 337-341. DOI: 10.1006/abio.1999.4019

23. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 1996: 239; 70-76. DOI: 10.1006/abio.1996.0292

24. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev. Dyn. 1995: 203: 253-310. DOI: 10.1002/aja.1002030302

25. Oehlers SH, Flores MV, et al. chemically induced intestinal damage models in zebrafish larvae. Zebrafish 2013: 10:184-193. doi: 10.1089/zeb.2012.0824.

26. Scholz S. Zebrafish embryos as an alternative model for screening of drug-induced organ toxicity. Arch Toxicol. 2013: 87; 767–769. https://doi.org/10.1007/s00204-013-1044-2

27. Lavanya G, Voravuthikunchai SP, Towatana NH. Acetone extract from Rhodomyrtus tomentosa: a potent natural antioxidant. Evidence-Based Complement. Alt. Med. 2012: 1; 1-9. https://doi.org/10.1155/2012/535479

28. Novoa B, Bowman TV, Zon L, Figueras A. LPS response and tolerance in the zebrafish (Danio rerio). Fish Shellfish Immunol. 2009 Feb; 26(2): 326–331. DOI: 10.1016/j.fsi.2008.12.004