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# Marine macroalgae: As nutraceutical supplements with potential anti-inflammatory properties

**Mythri, E[a], Lavanya Rayapu[a], Sambasiva Reddy Chenchula[a], Sriramulu Arava[c], Kajal Chakraborty[b,*]and Lokanatha Valluru[a,*]**

[a]Department of Biotechnology, Dravidian University, Kuppam-517426, AP, India.
[b]Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin-682001, Kerala, India.
[c]Department of Biotechnology, Rayalaseema University, Kurnool-518007, A.P., India
***Corresponding Author's Email:** lokanath.valluru@gmail.com and kajal_cmfri@yahoo.com

**ABSTRACT**

*Marine macroalgae we reutilized widely for its high biological activities in primordial systems of medicine. The current study evaluates the anti-inflammatory activity of marine macroalgae extracts (FS4, AE3, PH, SF1 and FM4) in acute and chronic inflammation studies of rats. The acute (carrageenan (Cg) induced paw edema method) and chronic inflammation (Cotton pellet (Cp) induced granuloma) in rats were treated with marine macroalgae extracts. The biochemical analysis such as alanine aminotransferase(ALT), aspartate aminotransferase (AST), creatinine, total protein, and urea were estimated in both inflammation studies. Superoxide dismutase (SOD), Lipid peroxidase (LPx), Glutathione reduced (GSH) and myeloperoxidase (MPO) were studied in paw tissue of acute inflammation rats. Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), SOD, catalase (CAT), GSH, LPx were estimated in the tissues of chronic inflammation rats. The oral administration of marine macroalgae extracts has showed significant anti-inflammatory activity in acute and chronic inflammations. The altered biochemical estimations were recovered after treating with marine macroalgae extracts. Among five potential marine macroalgae extracts the oligosaccharide extract of Gracilaria opuntia (FM4) has shown significant ($p<0.05$) reduction in acute and chronic inflammation studies. Hence, FM4 was purified and characterized as sulphated galactopyran.Thus, marine macroalgae were demonstrated to be the valuable nutraceutical supplements with potential activities against inflammation.*

***Key words:*** *Marine macroalgae, Inflammation, Cotton pellet, Carrageenan, Sulphated galactopyran.*

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## INTRODUCTION

Inflammation is a multifaceted pathophysiological process that conciliated by various signaling molecules produced by macrophages, leucocytes and mast cells. The edema is formed due to active complement factors that results in extravasations of proteins and fluids, and intensification of leukocytes at the inflammatory regions.[1] Chronic inflammation leads to cardiovascular diseases, cancer, lung and neurodegenerative diseases.[2] Although steroidal and non-steroidal anti-inflammatory drugs (NSAIDs), particularly aspirin, indomethacin, diclofenac etc., are some of the highly effective drugs treating the inflammatory diseases, but they have numerous pernicious drawbacks such as stomach bleeding and gastrointestinal ulcers.[3] This leads to increase in the research, on the development of alternative medicines from the natural products without any side effects.

Now-a-days research has been spotlighted on advocacy of anti-inflammatory drugs from the natural sources. However, there are numerous natural products that show the anti-inflammatory properties with fewer side effects, especially from marine organisms which are rich source of bioactive potential compounds that differs structurally with more pharmaceutical usage.[4] Among them, macroalgae are the affluent sources of bioactive compounds, and these products are majorly used in medical and biomedical research.[5]Marine macroalgae are rich in polysaccharides which are utilized in food, cosmetic and pharmaceutical industries and also in microbiology and biotechnology research. It was proved that macro molecules have showed abundant biological activities important to human health, such as antiviral, anti-tumoral, anti-inflammatory, apoptic activity and anticoagulant activity.[6]

This study consequently envisages to evaluate the anti-inflammatory properties of the 5 extracts of aqueous (FS4), ethanolic (AE3), methanolic (PH1 and SF1) and aqueous extract with oligosaccharide conjugate properties (FM4) obtained from the different species of marine macroalgae.

## MATERIAL AND METHODS

### Chemicals

O-dianisidinedihydrochloride, Carrageenan, hydrogen peroxide,thiobarbituric acid (TBA), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), glutathione (GSH),ethylene diamine tetra acetic acid (EDTA),trichloro acetic acid (TCA), were procured from Himedia (Bangalore, India) and Sigma Aldrich (St. Louis, Missouri, USA). C-reactive protein test kit was procured from Biovendor, LaboratorniMedicina. Australia.

### Maintenance of animals

Male albino wistar rats (*Rattusnorvigicus*) weighing 190±10g, were procured from Sri Venkateswara Enterprises Pvt. Ltd. (Bangalore, Karnataka, India). The animals were fed with standard rodent diet and free access to water throughout the experiment and overnight (12h) fasted before the day of the experiment. The study was approved by IAEC, PES Medical College, Kuppam, India. (Vide No. PESIMSR/Pharma/IAEC/20/2014-2015/date 08.12.14).

### Preparation of Marine macroalgae extracts:

The marine macroalgae *Gracilaria opuntia*, *Turbinaria conoides* (AE3 and SF1) and *Sargassum wightii* (PH1) were collected from Gulf of Mannar of mandapam region located between 8°48' N, 78°9' E and 9°14' N, 79°14' Eon southeast coast of India. The samples (2 Kg) were washed in double distilled $H_2O$to remove the epiphytes and were shade dried and powdered. The powdered samples of AE3 and SF1and PH1(1000 g) were extracted with n-hexane (600 mL x 2), at room temperature for 24 h, and pigments were separated. The residues were filtered (Whatmann No. 1) and were extracted 3 times with methanol (MeOH) and ethanol (EtOH)(50-60°C, 3h) respectively. With the help of rotary vacuum evaporator (Heidolf, Germany) the pooled filtrate was concentrated (50°C) to afford dark brown viscous mass of MeOH (112 g) and EtOH fractions (96 g) respectively. Aqueous extract of *G. opuntia* (FS4) was prepared by extracting the dried seaweed powder (500 g) with hot water (2 L) at 80°C for 3 h. To remove the solid residues the contents were cooled and centrifuged (8500 rpm for 15 min, 4°C, SorvellBiofugeStratos, Thermo Scientific, USA) and that were freeze-dried to get the crude aqueous extract (25 g). The aqueous extract of *G. opuntia* was concentrated before being precipitated with alcohol (500 mL). The precipitate was lyophilized to get a dried oligosaccharide fraction of *G. opuntia* (FM4). This was powdered and packed in vacuum packed bags and stored in refrigerator until further use.

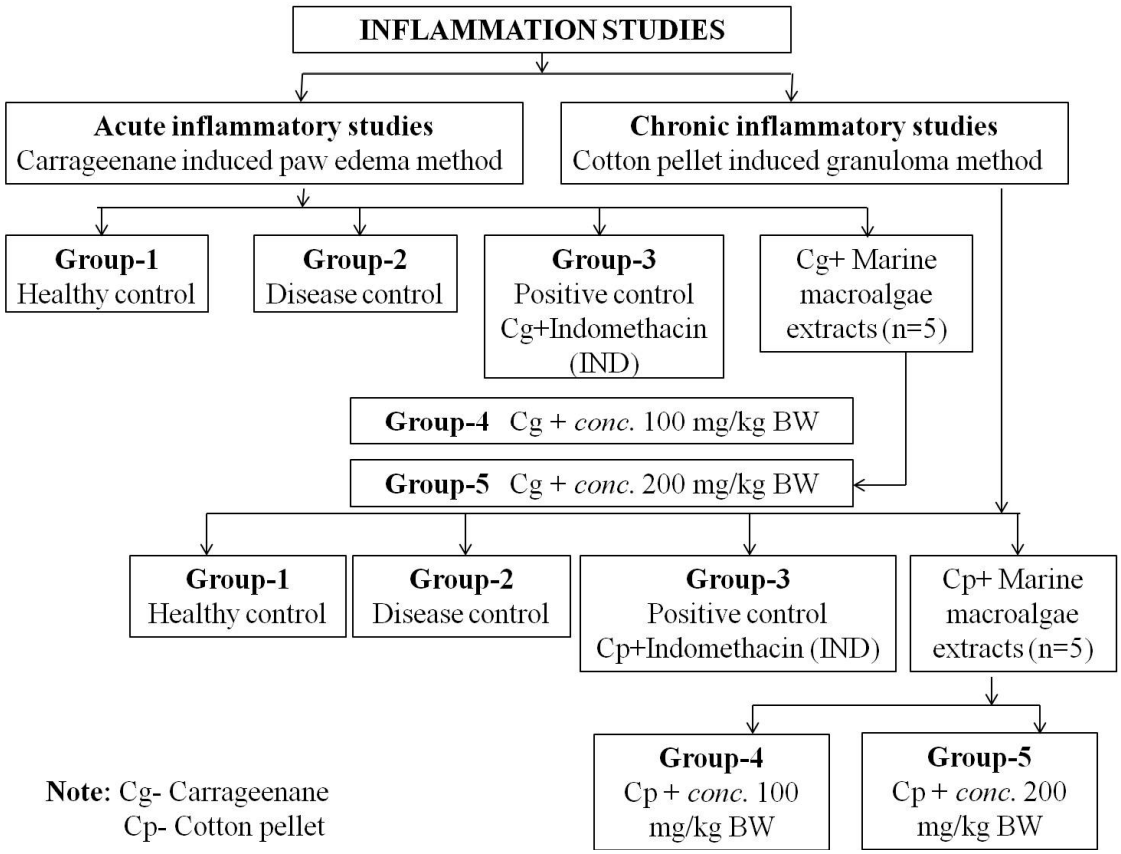
### Anti-inflammatory activities

### Dose determination and survival analysis

Rats were divided into groups, with 5 animals in each group. The FM4 was administered orally at possible maximum dose following a standard method.[7] The survival rate was observed continuously for 14 days to detect the mortality rate and behavioral changes. After induction of cotton pellet (Cp), a group was treated with saline and served as control. Based on preliminary toxicity tests, the doses of 100, 150, 200, 250, 300 mg/Kg of FM4 extract were chosen for further experiments. The Kaplan-Maier survival analysis curve was done in graphpad prism version 6.07.

### Experimental Design

The treatment was initiated after inducing inflammation with carrageenan for acute studies and cotton pellet for granuloma method for chronic inflammatory studies by selecting different marine macroalgae extracts (n=5). The animals were separated into 5 groups with 5 rats in each group for acute and chronic studies. The experimental design is as follows:




**Inflammation studies**

The acute inflammatory effect was evaluated by carrageenan induced rat paw edema method by using plethysmometer (Model No. L57410, Global Biotech Inc. USA) according to the method of Winter et al.[8] The chronic inflammatory effect was evaluated by cotton pellet induced granuloma according to the method of Goldstein et al. [9] The anti-inflammatory effect of marine macroalgae extracts (AE3, FS4, FM4, PH1 and SF1) were analyzed in both acute and chronic studies.

**Hematological and biochemical studies**

Before cervical dislocation of rats, blood was collected from retro-orbital vein for the hematological estimations in serum creatinine, total protein, urea, ALT and AST by using INVITRO 250 automated clinical chemistry analyzer (Ortho Clinical Diagnostics, Bangalore, INDIA) in both acute and chronic studies. MPO[10], SOD[11], LPx[12]activities were estimated in hind paw tissue of rats. SOD, LPx, GSH and CAT[13] were determined in chronic inflammation by Cp induced granuloma of rat tissue such as liver, kidney and heart.ESR was evaluated using Westergren method [14] in Cp induced rats blood. Statistical analysis was done by one-way ANOVA.

**Histopathalogy**

For histological examination, tissues like liver, kidney and heart were excised by euthanizing rats after 14 days of treatment. The tissues were fixed in 10% formaldehyde and dehydrated in graduated ethanol (50-100%), cleared in xylene and paraffin embedding. The sections (5 µm) were stained with eosin and haematoxylindye and examined under microscope.

**Purification and characterization of oligosaccharide fraction of *G. opuntia*:**

The precipitated oligosaccharide conjugate of *G. opuntia* (FM4) was purified by anion exchange column chromatography using DEAE (diethylamineoethyl)-cellulose anion exchange resin. A glass column (25 x 4 cm) with a mesh sieve was mounted vertically on a stand and rinsed with water. DEAE-cellulose (3 g) was made into slurry with minimum amount of water and loaded into the column. The polysaccharide fraction (1 g) was dissolved in 2-3 ml of water to make a suspension and loaded into the previously packed column. The column was initially eluted with water to get the first fraction. This was followed by step-wise elusion with 0.1, 0.3, 0.5 and 1 M sodium chloride (NaCl) gradient until the absence of positive reaction of the phenol-sulphuric acid assay[15] in the test tubes containing eluted fractions. The eluted fractions were lyophilized to get the purified oligosaccharide fractions. The oligosaccharide enriched fraction was referred as sulphated galactopyran and the motif was characterized from *G. opuntia* by using fourier-transform infrared (FTIR) (Perkin-Elmer 16 PC spectrometer, Boston, USA) and Nuclear magnetic resonance (NMR) spectroscopy.

**Statistical analysis**

The obtained data was analyzed by using the statistical package of social sciences (SPSS, 16.0 version). Comparison between the control and experimental animals results were done by one-way ANOVA followed by Tukey's multiple comparison test. When $p<0.01$, considered as significant difference. All the values were expressed as mean ± S.D (n=5).

**RESULTS**

In the present investigation the aqueous and oligosaccharide extracts of *G. opuntia* (FS4 and FM4), ethanolic extracts of *T.conoides* (AE3) and methanolic extracts of *S.wightii*(PH1), *T.conoides* (SF1) were screened for acute and chronic inflammatory studies in rats (Table. 1).

The dose determination test of marine macroalgae compounds were studied in Cpinduced granuloma of rats. Rats were treated with five different concentrations ofFM4 such as 100, 150, 200, 250, 300 mg/Kg BW. In the present study all different concentrations have shown recovery compared to control and Indomethacin (IND) groups. Amongfive concentrations200 mg/Kg has shown significant ($p<0.01$) recovery compared to the other concentrations based on the survival analysis (Fig. 1).

The acute studies of Cg induced paw edema test results demonstrated that there was a significant ($p<0.05$) increase of paw size from 0.26±0.02 to 2.24±0.19 at 3 h in control group. It was reduced in marine macroalgae extracts (AE3, FS4, FM4, PH1 and SF1) treated groups. Among five marine macroalgae extracts FM4 200mg/Kg BW concentration has shown reduction in paw size significantly ($P<0.05$) at 3 hr (0.98 ± 0.08) compared to indomethacin (IND) treated group (Table.2 and Fig.2). The percentage of inhibition was found to be increased in marine macroalgae treated groups at 3rd and 4th h compared to control and IND treated groups. FM4 treated groups has significantly ($p<0.05$) increased in percentage of inhibition (60.2% and 66.3%) respectively compared to other marine macroalgae treatedgroups, control and IND group (Table.3).

The modulations of antioxidant enzyme activity levels were estimated in acute inflammatory studies of Cg induced paw edema of rats and marine macroalgae extracts treated groups in the subplantar region of rats left hind paw (Fig. 3). The SOD and GSH levels were decreased significantly ($p<0.05$), whilst the malondialdehyde (MDA) levels in lipid peroxidation (LPx) activity and Myeloperoxidase (MPO) levels were increased respectively. Marine macroalgae extracts of AE3, FS4, FM4, PH1 and SF1 (200 mg/Kg BW) recovered the enzyme activities significantly in experimental groups compared to control group. Among five marine macroalgae extracts treatment, FM4 showed greater ability in reducing the altered levels of antioxidant enzymes activity such as SOD (3.93±0.92), GSH (3.89±0.29), LPx (5.36±0.96) and MPO (8.92±0.84) in left hind paw tissue respectively (Fig. 3).

Table. 4show the effect of marine macroalgae extracts and indomethacin (IND) on granulomatous tissue growth in Cp induced granuloma of rats. The oral administration of marine macroalgae (AE3, FS4, FM4, PH1 and SF1) at dose 200mg/Kg BW showed significant ($p<0.01$) reduction in the formation of granuloma tissue on the implanted Cp compared to control group and IND group (54.26%). FM4 has shown significant ($p<0.01$) decrease in granuloma tissue growth compared to that of other treated groups and percentage of inhibition of granuloma tissue is 57.26%.

The Erythrocyte sedimentation test (ESR) and C-reactive protein (CRP) are the biomarkers of systemic chronic inflammation. Chronic inflammation studies of Cp induced granuloma rats blood and serum has shown significant ($p<0.01$) increased in control group (46.82±3.63; 8.46±0.43). These increased ESR and CRP levels were decreased significantly ($p<0.01$) upon treatment of marine macroalgae (AE3, FS4, FM4, PH1 and SF1) extracts for 14 days. Among five extracts FM4 has shown significant decrease in ESR (20.49±1.08) and CRP (4.96±0.53) levels (Fig. 4).

The hematological perturbations of acute inflammatory studies of Cg induced paw edema and Cp induced granuloma of rats blood serum such ALT (90.83±3.58; 100.83±3.09), AST (187.26±10.42; 197.26±12.42), Creatinine (2.56±0.18; 3.56±0.18), total protein (Cg &Cp- 6.2±0.28) and urea (96.28±7.28; 102.28±7.28) has shown significant ($p<0.05$) increased in control group. These levels were reduced significantly ($p<0.01$) after treating with marine macroalgae extracts (AE3, FS4, FM4, PH1 and SF1). Among five extracts FM4 has shown significant ($p<0.01$) decrease in acute and chronic inflammatory studies of ALT (50.28±2.7; 43.53±4.16), AST (50.27±9.82; 48.53±7.84), Creatinine (0.79±0.16; 0.68±0.19), total protein (6.03±0.16; 6.32±0.36) and urea (39.68±8.23; 32.92±6.25) in blood serum of rats respectively (Fig. 5 and Fig.6).

The inflections of anti-oxidant enzymes activity levels were estimated in Cp induced granuloma of rats and marine macroalgae extracts (AE3, FS4, FM4, PH1 and SF1) treated groups in the liver, kidney and heart tissues (Fig.7). The antioxidant enzyme levels such as SOD (3.01 ± 0.19, 4.66 ± 0.98, 0.98 ± 0.28), CAT (68.08 ± 6.13, 58.55 ± 5.72, 7.58 ± 1.24), GSH (42.42 ± 1.69, 21.26 ± 1.72, 24.2 ± 2.14) were significantly ($p<0.01$) decreased, whilst the levels of malondialdehyde (MDA) in LPx (19.58 ± 2.77, 38.63

± 2.86, 36.62 ± 3.62) activity in Cp induced inflammation rat liver, kidney and heart tissues respectively were increased. The AE3, FS4, FM4, PH1 and SF1 (200 mg/Kg BW) recovered the enzyme activities significantly in the selected tissues of experimental groups when compared to control rats. Among these five treatments, FM4 showed greater ability in reducing the altered levels of antioxidant enzymes activities such as SOD (5.86 ± 0.11, 7.60 ± 0.46, 2.32 ± 0.21), CAT (91.68 ± 4.23, 82.68 ± 2.09, 12.06 ± 2.32), GSH (63.91 ± 3.28, 31.96 ± 0.98, 43.07 ± 3.53) and LPx (7.86 ± 1.43, 12.12 ± 2.26, 17.10 ± 3.2) in liver, kidney and heart tissues respectively (Fig.7).

The Kaplan-Maier method was used for the estimation of the survival analysis.[16] In the survival analysis, the survival percentage of control-100%, diseased-20%, IND-40%, AE3-40%, FS4-60%, FM4-80%, PH1-40% and SF1-20% (Fig. 8). FM4 has shown more survival rate compared to other marine macroalgae extracts. FM4when orally administrated in possible doses of 100, 150, 200, 250, 300 mg/Kg body weight in Cp induced granuloma of rats the mortality rate was obtained. Among five concentrations 200mg/kg BW has shown significant survival rate (80%) compared to disease control (20%) and IND (40%) groups (Fig. 8).

Histopathalogical changes in liver, kidney and heart tissues of Cp induced granuloma and treated rats are shown in Fig. 9. Marked hepatocellular damage, extensive vacuolization and disordered liver structure were observed in liver of chronic inflammatory induced rats (Fig. 9. A, B, C). In Cp induced inflammatory rat kidney, mesangial cells were damaged and uneven thickening of the basement membrane were observed (Fig. 9. D, E, F). In Heart, the myocardial fibers were disarrayed and focal destruction in myocytes were observed in chronic inflammatory rats. However these changes were re-established with the treatment of FM4 marine macroalgae compound(Fig. 9. G, H, I).

Among five compounds FM4 extract obtained from *G. opuntia* has shown significant recovery of chronic inflammation. Hence FM4 was purified and characterized.

The Oligosaccharide fraction of FM4 from the red marine macroalgae *G. opuntia* (FM4) was isolated by column chromatographic method and followed by step-wise elusion with sodium chloride gradient until the dearth of a positive reaction of the phenol-sulphuric acid assay.[15] The eluted fractions were lyophilized to get the purified oligosaccharide fractions. The carbohydrate enriched fraction referred as sulphated polygalactan. The structure of the sulphated polygalactan was elucidated by the extensive analysis of $^{1}$H-NMR spectra. The polysaccharide fraction has resolved signals of anomeric protons (δ 4.4 - 5.5), methylene and methane hydrogens (δ 3.6 – 4.9) of the sulphated polygalactan moiety in $^{1}$H-NMR spectra. Characteristic signal at δ 3.4 has revealed the region of additional number of alkoxy (- OCH3) replacements in the sulfated polygalactans. In addition to the characteristic peaks for sulphated polygalactan units, xylose and anhydrogalactose units were in minor constituents in the fraction, which were often found in red seaweed polysaccharides. The presence of positively and negatively charged ions of sulphate groups in the sulphated galactan moiety has sustained the structure. The FT-IR spectra demonstrated a strong absorption band at 1210-1260 $cm^{-1}$ corresponds to S=O groups (sulphate ester groups). Characteristic bands for –OH groups at 3200-3400 $cm^{-1}$ has substantiated the structure of the sulphated polygalactan. The sulphated galactopyran motif of *G. opuntia* was designed based on the detailed NMR experiments[17](Fig. 10).

## DISCUSSION

Now-a-days the utility of traditional medicines was massively increased and marine macroalgae are the abundant source of bioactive compounds that might leads to synthesis of novel drugs.[18]The present study was carried out to evaluate the traditional assert of marine macroalgae extracts as an anti-inflammatory drug. The dose determination test of the marine macroalgae compounds were studied in Cp induced granuloma of rats. The rats were treated with five different concentrations of theFM4 such as 100, 150, 200, 250, 300 mg/Kg BW. The present studies showed that all the different concentrations have shown recovery compared to control and IND groups. Upon dose determination and survival analysis the extracts were safe upto 200mg/kg BW concentration (Fig. 1).

Cg induced paw edema is a classical acute inflammation model that has been extensively used for the steroid and non-steroid anti-inflammatory drug studies.[19]Cg-induced inflammation has significant prophetic value to inhibit the mediators causing acute inflammation. By injecting the Cg at subplantar region of rat left hind paw, the edema was produced and it is a biphasic over 4 or more hours. In the earlier phase it release serotonin and histamine and the late phase is continued by prostaglandins and leucotrienes. Kinines provide continuation between the two phases.[20]The anti-inflammatory drugs acts on the late phase of the acute inflammation. The FM4 extract has shown significant inhibition in the late phase ofCg induced paw edema (Table 2 & 3; Fig. 2). This might be due to the regulation of prostaglandins and leucotrienes.[21] It was deep-rootedby the MPO activity in the subplantar tissue from rat paw (Fig. 2). The MPO tissue levels are well harmonized marker of tissues neutrophil infiltration, and they correlate with the disease severity.[22]

The transudative and proliferative components of the chronic inflammation were evaluated by Cp granuloma method. The wet weight of the pellets associates with transude, the dry weight correlates with granuloma tissues. The chronic inflammation occurs due to increase in proliferative cells. These cells can be either spread or in granuloma form. Non-steroidal anti-inflammatory drugs reduce the size of granuloma resulting in cellular reaction by inhibiting granulocytes infiltration, avoiding the generation of collagen fibers and repressing muco-polysaccharides.[23] The FM4 extract showed significant anti-inflammatory activity compared to other marine macroalgae extracts in Cp-induced granuloma chronic inflammatory conditions, which reveal its effect in reducing the increased number of fibroblasts and synthesis of collagen and muco-polysaccharides during the formation of granuloma tissue (Table. 4).

CRP and ESR levels were varied in chronic studies (Fig. 4). The increased levels of serum CRP were found during the active inflammation or cell damage is an acute phase protein that is identified as significant biomarker for different inflammatory and degenerative diseases.[24] The decreased levels of CRP during marine macroalgae treatment propose that it can control the inflammation mediated chronic complications(Fig. 4). The ESR levels have been raised in the control group, increase in WBC count and other characteristic hematological alterations such as the decrease in Hb count etc. Upon treatment with marine macroalgae extracts the ESR levels were reduced (Fig. 4). This was previously reported in *Sargassum wightii* and *Barringtonia racemose.*[25,26]

As well as, this study evaluated the integrity of rats subjected to marine macroalgae treatment in acute and chronic toxicity assay using biochemical, and histopathalogical parameters. A lysosomal enzyme plays a major role in the progress of acute and chronic inflammations.[27] In Cg and Cp induced granuloma animal model, the marine macroalgae (AE3, FS4, FM4, PH1 and SF1) treatment significantly decreased the increased levels of enzymes activities such as ALT, AST, creatinine, urea and there was no change in total protein (Fig. 5 and Fig.6).

In the chronic inflammation conditions, large amount of superoxide and H2O2 radicals are produced due to the accumulation of granulocytes and macrophages, resulting in cell damage as of decline in antioxidant enzyme levelsand increase in lipid peroxidation.[28, 29]SOD and CAT plays a significant role in the superoxide anion and H2O2detoxification respectively, protecting the cells against damage caused due to oxidative free radicals. Marine macroalgae extracts increased the depletion of SOD levels in rat tissues such as liver, kidney and heart, possibly by competing in scavenging for free radicals, whichresults in preserving the integrity of cellular membranes (Fig. 7). A declined GSH levels in tissues of rat were recovered by treating with marine macroalgae extracts (Fig. 7). Lipid peroxidation is considered as vital mechanism of the damage caused during chronic inflammation. [29] Marine macroalgae extracts treatment has shown significant decrease in LPx levels, thus it can defend the development of free radicals and reduce the inflammations (Fig. 7). The antioxidant activities of different tissues were previously reported in *Sargassum hemiphyllum*. [30]

Histopathalogical changes in liver, kidney and heart tissues of Cp induced granuloma and treated rats are shown in Fig. 9. Marked hepatocellular damage, extensive vacuolization and disordered liver structure were observed in liver of chronic inflammatory induced rats (Fig. 9. A, B, C). In Cp induced inflammatory rat kidney, damage in mesangial cells and uneven thickening of the basement membrane were observed (Fig. 9. D, E, F). In Heart, myocardial fibers were disarrayed and focal destruction in myocytes were observed in chronic inflammatory rats. However these changes were reestablished with the treatment of FM4 marine macroalgae compound (Fig. 9. G, H, I). It was previously reported that sulfated polysaccharides from other marine macroalgae were considered as safe on toxicological evaluation.[31]

Among five extracts AE3, FS4, FM4, PH1 and SF1, oligosaccharide conjugate of *Gracilaria opuntia* (FM4) has showed the best anti-inflammatory properties. Hence FM4 compound was purified and characterized. The proposed structure of *Gracilaria opuntia* (FM4) was sulphated galactopyran was elucidated for further analysis and future work.

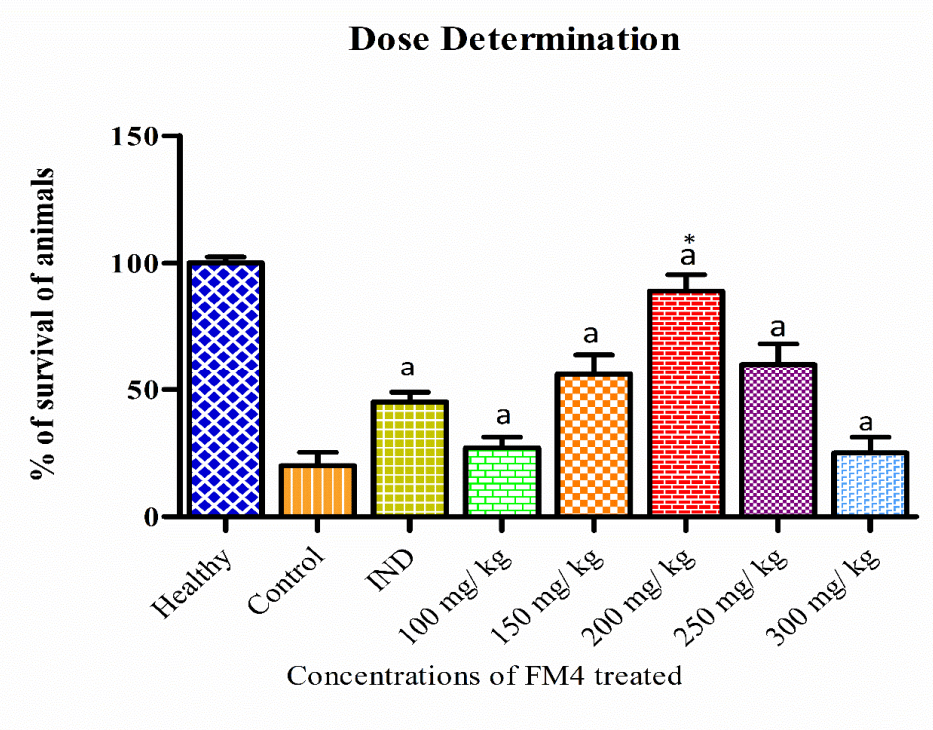



**Fig. 1.Dose determination of *Gracilaria opuntia* (FM4) extract in chronic inflammation studies in cotton pellet induced rats.** Among five different concentrations 200mg/Kg BW concentrations has shown effective recovery compared to IND and control groups.The Values are expressed as mean ± SD of 5 individual observations. a- Comparison between control and treated groups ($p<0.05$). * Comparison between control and 200mg/kg groups ($p<0.05$). Compared to other groups the 200mg/Kg concentration have more animal survival rate.

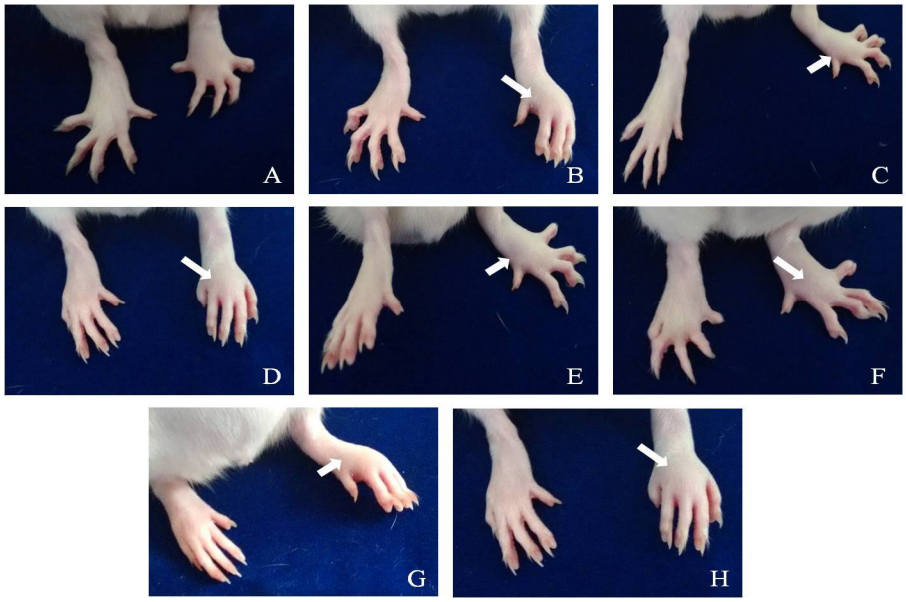



**Fig.2.Photographs of acute inflammation of carrageenan induced and treated rats hind paw at 3rd hour.** Arrows in the photos indicated edema of rat paw. (A) Healthy rat, (B) Carrageenan (Cg) induced, (C) Indomethacin treated (5mg/Kg), (D) Cg+AE3, (E) Cg+FS4, (F) Cg+FM4, (G) Cg+PH1 and (H) Cg+SF1.

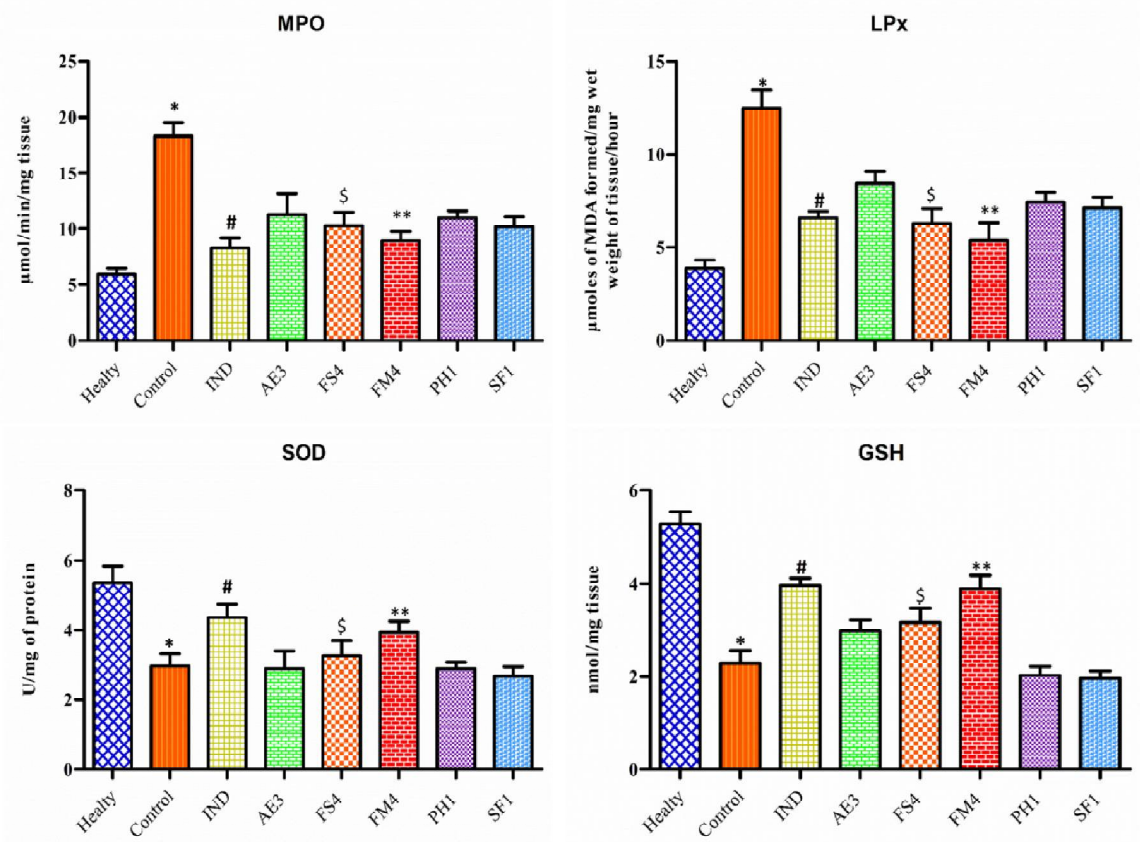



**Fig. 3. Effect of marine macroalgae extracts on antioxidant enzyme activities of carrageenane induced paw tissue of rats in acute inflammation:**The increased levels of MPO, LPx and decreased levels of SOD and GSH were recovered after treating with marine macroalage extracts. Data was expressed in mean ± SD of 5 individual observations. *Comparision between Healthy and control (Cp) p<0.01. #comparision between control and IND p<0.01. $comparision between control and FS4 p<0.01. **Comparision between control and FM4 p<0.01.

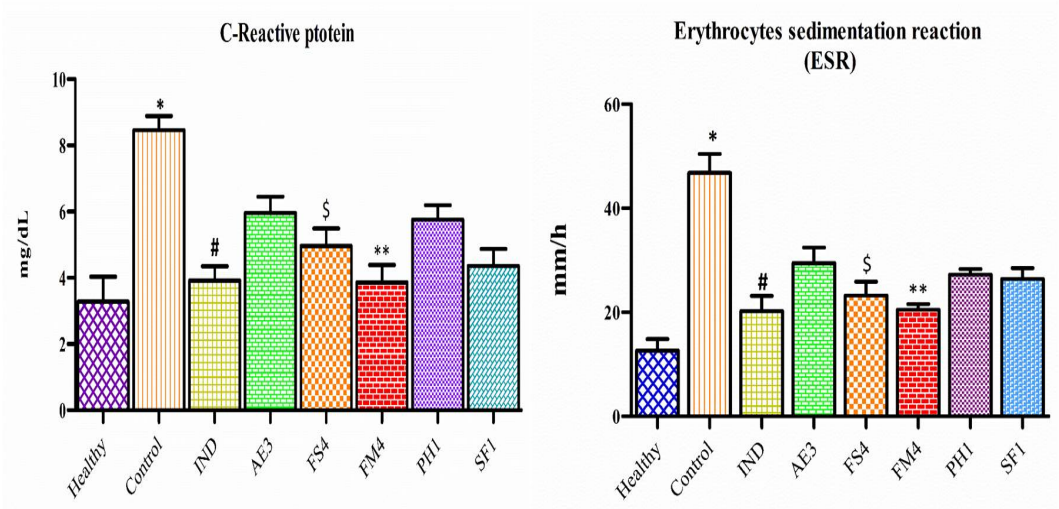



**Fig. 4. Estimation of C-reactive protein and Erythrocytes sedimentation reaction (ESR) in fasting blood of different experimental groups treated with marine macroalgae in chronic inflammation of cotton pellet-induced granuloma of rats.** The increased levels of CRP and ESR were decreased after treating with marine macroalgae extracts. FM4 has shown more significant reduction in CRP and ESR levels compared to other groups. The Values are expressed as mean ± SD of 5 individual observations. * Comparison between and control and healthy groups (p<0.05). # Comparison between control and IND groups (p<0.05). $ Comparison between control and FS4 groups (p<0.05). ** Comparison between control and FM4 groups (p<0.05).

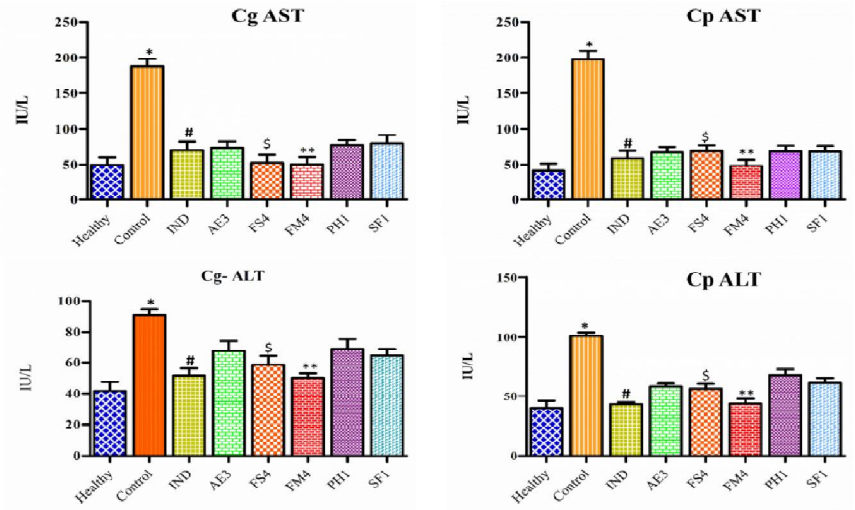



**Fig. 5.Estimation of Serum ALT and AST in blood of acute inflammation of carrageenan (Cg)-induced paw edema and chronic inflammation of cotton pellet (Cp) induced granuloma of rats after treating with marine macroalgae extracts.** The increased levels of ALT and AST in control group after Cg and Cp induction were recovered with the treatment of marine macroalgae. The values are expressed as mean ± SD of 5 individual observations. * Comparison between healthy and control ($p<0.05$). # Comparison between control and IND groups ($p<0.05$). $ Comparison between control and FS4 groups ($p<0.05$). ** Comparison between control and FM4 groups ($p<0.05$).

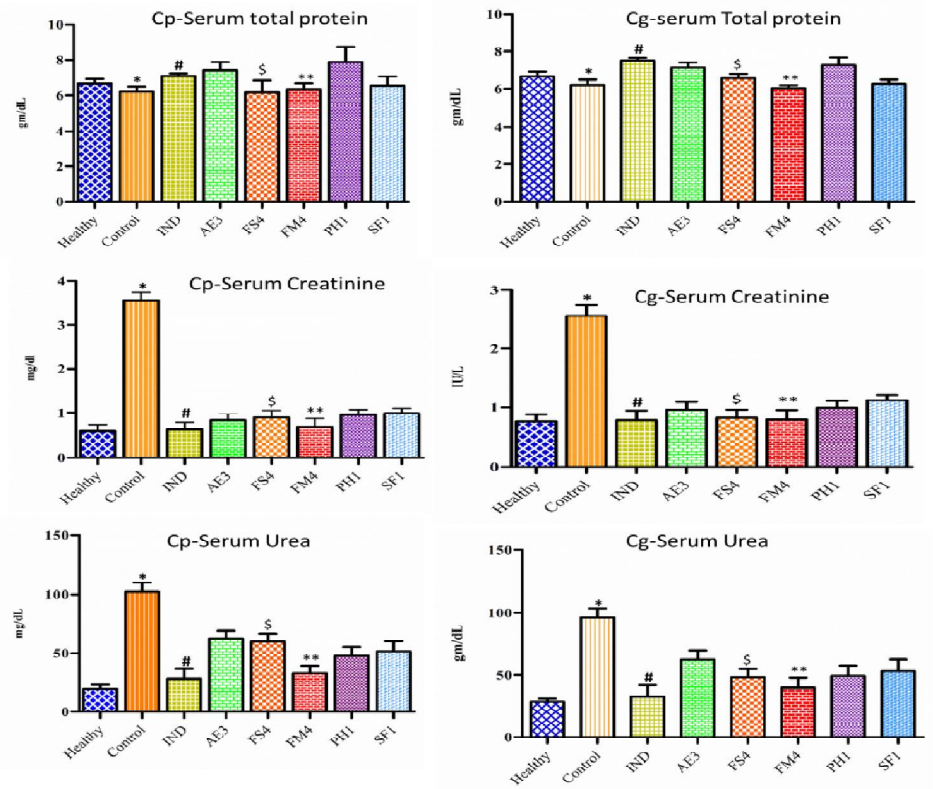



**Fig. 6. Evaluation of biochemical changes in blood ofacute inflammation in carrageenan (Cg)-induced paw edema and chronic inflammation in cotton pellet (Cp) induced granuloma of rats after treating with marine macroalgae extracts.** The increased levels of Serum Creatinine and urea were significantly decreased after treating with marine macroalgae compounds, but there was no significant change in total protein levels compared to control and treated groups. The Values are expressed as mean ± SD of 5 individual observations. * Comparison between healthy and control ($p<0.05$). # Comparison between control and IND groups ($p<0.05$). $ Comparison between control and FS4 groups ($p<0.05$). ** Comparison between control and FM4 groups ($p<0.05$).

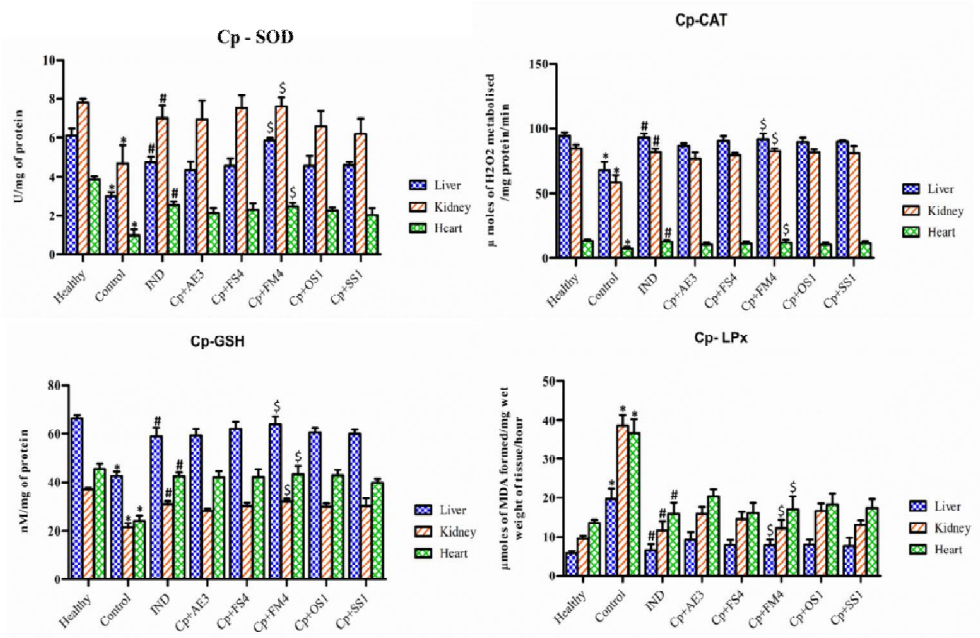



**Fig.7.Analysis of marine macroalgae compound on antioxidant enzyme activities in Liver, Kidney and Heart tissues of chronic inflammatory studies of cotton pellet induced granuloma of rats:** After induction of inflammation, the SOD, CAT, GSH levels were decreased and MDA levels were increased compared to normal rats. On the treatment with the five potential macroalgae extracts the enzymes activities were recovered in chronic inflammatory rats compared to their controls. Data was expressed in mean ±SD (n=5). *Comparison between Healthy and control (Cp) p<0.01. # Comparison between Cp+IND and FM4 p<0.01. $ Comparison between Cp and FM4 p<0.01. There is no significance between Cp+IND and FM4 treated groups.

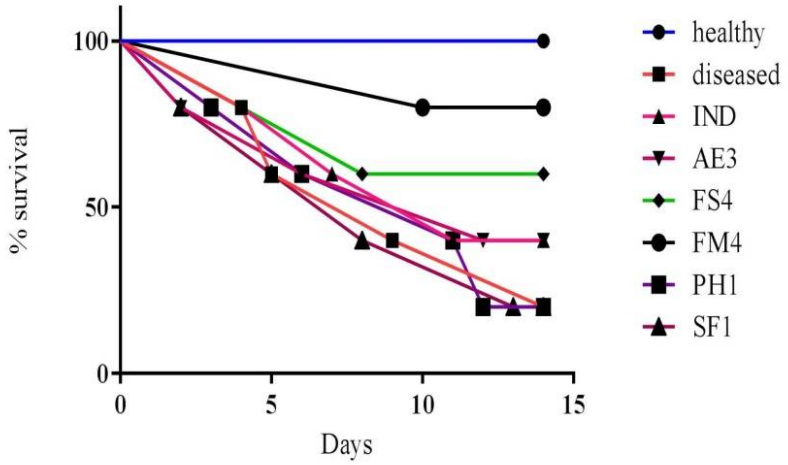



**Fig. 8. Survival analyses during chronic inflammation in cotton pellet induced granuloma rats treated with marine macroalgae extracts (AE3, FS4, FM4, PH1 & SF1).**Kaplan-Maier method was used for the estimation of survival analysis between Healthy, Diseased, IND and marine macroalgae extracts treated groups. Groups calculated whereas control-100%, Diseased-20%, IND-40%, AE3-40%, FS4-60%, FM4-80%, PH1-40% and SF1-20%.

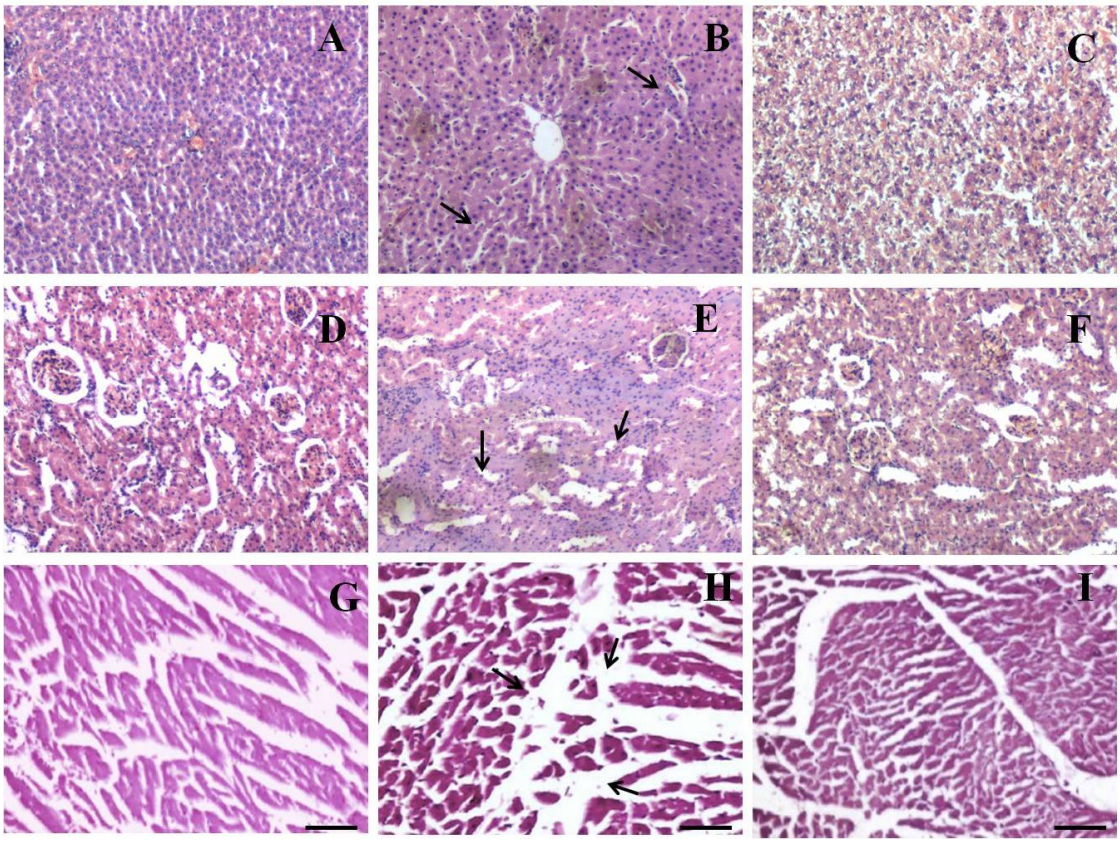



**Fig. 9.Photomicrographs of Chronic inflammation of cotton pellet induced granuloma of rat tissues such as Liver, Kidney and Heart.** (A) control, (B) Diseased control and (C) FM4 treated in liver tissue, showing inflammation of hepatocytes and recovered by treating with FM4 marine macroalgae compound. (D) Control, (E) Diseased control and (F) FM4 treated in kidney, showing inflammation in mesangial cells and this was regenerated after treating with FM4. (G) Control, (H) Disease control and (I) FM4 treated in heart, showing disarrated myocardial fibers, focul destruction in myocytes and this was recovered with the FM4 compound treatment. Scale bar = 5 µm; Lens= 10x.

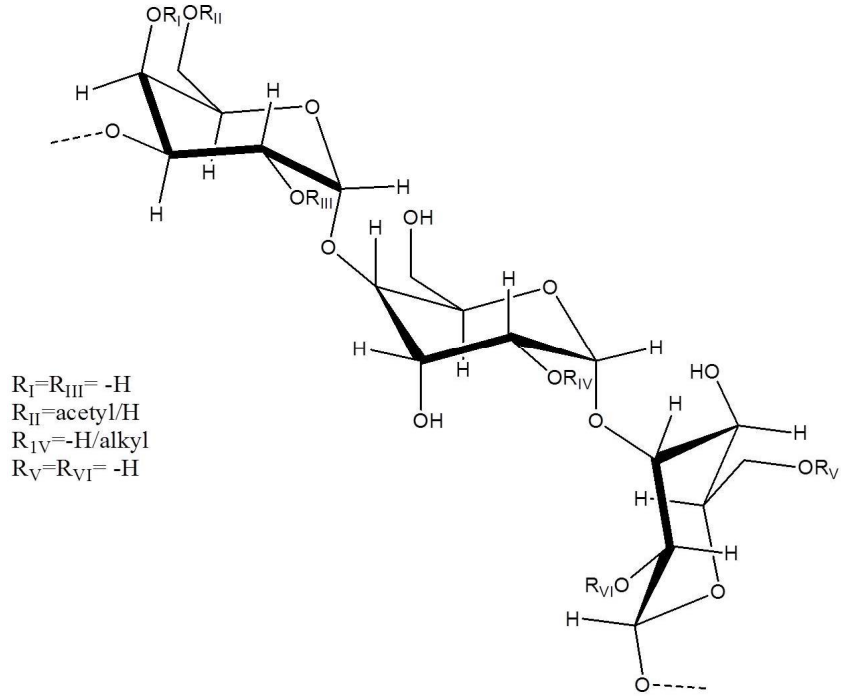



**Fig. 10.** Proposed structure of sulphated galactopyran of *Gracilaria opuntia*(FM4).

**Table 1:** Solvent (methanol, ethanol) and aqueous extracts of red and brown seaweeds

| Sample code | Genus name |
|---|---|
| **Aqueous extracts** | |
| FS4 | *Gracilaria opuntia* (Red seaweed) |
| FM4 | *Gracilaria opuntia* (Red seaweed) (oligosaccharide conjugate*) |
| **Methanolic extracts (MeOH)** | |
| PH1 | *Sargassum wightii* (Brown seaweed) |
| SF1 | *Turbinaria conoides* (Brown seaweed) |
| **Ethanolic extracts (MeOH)** | |
| AE3 | *Turbinaria conoides* (Brown seaweed) |

*Oligosaccharide conjugates of seaweeds were derived from the ethanol precipitated residue of the supernatant of the aqueous extracts. For detailed description the methodology section to be referred.

**Table 2:** Evaluation of the anti-inflammatory efficiency of marine macroalgae in acute inflammation of carrageenane-induced paw edema in rats.

| Experimental groups | Conc. (mg/kg BW) | Inflammatory paw volume (mL) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0 h | 1st h | 2nd h | 3rd h | 5th h | 12th h | 24th h |
| Healthy | Saline | 0.10±0.18 | 0.10±0.18 | 0.10±0.18 | 0.10±0.18 | 0.10±0.18 | 0.10±0.18 | 0.10±0.18 |
| Control | Saline | 0.26 ±0.02# | 0.42±0.06# | 0.81±0.29# | 2.24±0.19# | 2.26±0.08# | 0.92±0.36# | 0.76±0.16# |
| IND | 5 | 0.16 ±0.05 | 0.46±0.16 | 0.54±0.26 | 1.28±0.06* | 0.79±0.12* | 0.36±0.32 | 0.24±0.28* |
| AE3 | 100 | 0.17 ±0.03 | 0.63±0.18 | 0.87±0.09 | 1.98±0.23* | 1.16±0.16* | 1.43±0.12 | 0.36±0.06* |
| AE3 | 200 | 0.13± 0.06 | 0.56±0.09 | 0.86±0.28 | 1.02±0.13*,a | 0.99±0.12*,a | 0.48±0.20 | 0.32±0.04*,a |
| FS4 | 100 | 0.18±0.12 | 0.58±0.06 | 0.76±0.24 | 1.26±0.19* | 1.21±0.21* | 0.98±0.09 | 0.21±0.26* |
| FS4 | 200 | 0.16±0.15 | 0.49±0.24 | 0.85±0.26 | 1.01±0.02*,a | 0.82±0.26*,a | 0.39±0.23 | 0.28±0.21*,a |
| FM4 | 100 | 0.21±0.13 | 0.51±0.26 | 0.58±0.28 | 1.06±0.05* | 0.98±0.08* | 0.76±0.15 | 0.28±0.13* |
| FM4 | 200 | 0.26±0.14 | 0.48±0.28 | 0.72±0.09 | 0.98±0.08*,a | 0.76±0.12*,a | 0.46±0.07 | 0.19±0.16*,a |
| PH1 | 100 | 0.19±0.09 | 0.68±0.12 | 0.89±0.18 | 2.16±0.07* | 1.68±0.06* | 0.86±0.16 | 0.28±0.11* |
| PH1 | 200 | 0.18±0.06 | 0.51±0.16 | 0.96±0.06 | 1.12±0.06*,a | 0.98±0.12*,a | 0.73±0.18 | 0.36±0.18*,a |
| SF1 | 100 | 0.20±0.08 | 0.48±0.29 | 0.99±0.08 | 2.02±0.07* | 1.42±0.16* | 0.98±0.17 | 0.31±0.15* |
| SF1 | 200 | 0.21±0.07 | 0.56±0.18 | 0.98±0.16 | 1.09±0.12*,a | 0.96±0.09*,a | 0.69±0.19 | 0.25±0.13*,a |

Before receiving the injection of carrageenane (500 µg/paw) the positive control received indomethacin and remaining groups received the marine macroalgae extracts (100 and 200 mg/kg BW). The Values are expressed as mean ± SD of 5 individual observations. # Comparison between healthy and control ($p<0.05$). *comparison between control and treated groups ($p<0.05$). a-Comparison between 100 and 200 mg/kg BW treated groups. 200 mg/kg BW treated groups showed significant reduction in paw volume compared to 100 mg/kg BW treatment

**Table 3:** Percentage of inhibition during acute inflammation in carrageenane induced paw edema of rats.

| Groups | Concentrations (mg/Kg BW) | % inhibition | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1st | 2nd | 3rd | 5th | 12th | 24th |
| Healthy | - | - | - | - | - | - | - |
| Control | - | - | - | - | - | - | - |
| IND | 5 | 36.1 | 47 | 42.8 | 65 | 74.8 | 75 |
| AE3 | 100 | 9.7 | 14.7 | 11.6 | 48.6 | 51.7 | 62.5 |
| AE3 | 200 | 26.3 | 15.6 | 44.6 | 56.1 | 66.4 | 66.6 |
| FS4 | 100 | 31.39 | 38.2 | 55 | 56.6 | 58.7 | 70.8 |
| FS4 | 200 | 19.4 | 25.4 | 43.7 | 46.4 | 50.3 | 78.1 |
| FM4 | 100 | 29.1 | 43.1 | 57.1 | 63.7 | 63.6 | 70.8 |
| **FM4** | **200** | **33.3** | **50** | **60.2** | **66.3** | **67.8** | **80.2** |
| PH1 | 100 | 5.5 | 12.7 | 8 | 25.6 | 39.8 | 70.8 |
| PH1 | 200 | 29.1 | 5.8 | 50 | 56.6 | 57.3 | 62.5 |
| SF1 | 100 | 33.3 | 2.9 | 9.8 | 37.1 | 37.7 | 67.7 |
| SF1 | 200 | 26.3 | 2.9 | 51.3 | 57.5 | 58.0 | 73.9 |

**Table 4:** Evaluation of anti-inflammatory activity of marine macroalgae in chronic inflammatory studies of cotton pellet granuloma in rats.

| Groups | Concentrations (Mg/Kg BW) | Weight of dry cotton pellet granuloma (mg) | % inhibition |
|---|---|---|---|
| Healthy | - | - | - |
| Control | - | 132.14 ± 12.48* | - |
| IND | 5 | 64.25 ± 6.82# | 54.26 |
| AE3 | 100 | 91.48 ± 8.23 | 19.26 |
| AE3 | 200 | 84.28 ± 5.2 | 32.61 |
| FS4 | 100 | 86.91 ± 4.2 | 34.85 |
| FS4 | 200 | 82.24 ± 6.8$ | 32.61 |
| FM4 | 100 | 104 ± 4.6 | 21.21 |
| **FM4** | **200** | **66.56 ± 7.82**** | **57.26** |
| PH1 | 100 | 118.28 ± 5.28 | 10.61 |
| PH1 | 200 | 96.81 ± 6.52 | 20.46 |
| SF1 | 100 | 104.86 ± 4.28 | 21.41 |
| SF1 | 200 | 98.64 ± 5.10 | 20.81 |

A sterile cotton pellet weighing 20 mg was implanted subcutaneously into the groin region of rats to initiate the chronic inflammation. The control group receive saline, the IND group receives indomethacin (5 mg/kg BW) and remaining groups received the marine macroalgae extracts (100 and 200 mg/kg BW) orally for 14 days. Values are expressed as mean ± SD of 5 individual observations. * Comparison between Healthy and control (STZ) $p<0.01$. # Comparison between control and IND $p<0.01$. $ Comparison between control and FS4 $p<0.01$. ** Comparison between control and FM4 $p<0.01$. FM4 (200 mg/kg BW) showed significant inhibition of granuloma compared to the control group.

## CONCLUSION

In conclusion marine macroalgae were demonstrated to be valuable natural source of bioactive compounds with potential activities against inflammation, and therefore might be utilized to prepare the novel functional ingredients for nutraceutical supplements against inflammation. Recently,consumers are attracted towards natural bioactive compounds. Thus, marine macroalgae might be the alternative source for synthetic components alternatively used as new functional foods. This study showed that the ethanolic extract of *T. conoides* (AE3), methanolic extracts of *S.wightii*and *T. conoides*(PH1 and SF1), aqueous extract of *G. opuntia* (FS4) and oligosaccharide extract of *G. opuntia* (FM4) have significantly reduced the acute and chronic inflammations. The crude extracts of marine macroalgae have capability of reducing inflammation and related oxidative stress. Among different treatments, oligosaccharide extract derived from *G.opuntia*(FM4) showed significantly greater anti-inflammatory properties.Hence, it could be used as nutraceutical supplement or natural green remedy against inflammation.

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## CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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