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Determination of *In vitro* Anti-inflammatory Activity of Marine Microalgae: *Nannochloropsis* sp.

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Abstract

Objectives: To determine the *in vitro* anti-inflammatory activity of solvent extract of the marine microalgae, *Nannochloropsis* sp. **Materials and methods:** The preliminary assays for anti-inflammatory activity such as protein denaturation inhibition and membrane stabilization were evaluated in the various solvent extracts (petroleum ether, aqueous, isopropanol, methanol, ethyl acetate) of the microalgae, *Nannochloropsis* sp. and *in vitro* anti-inflammatory effect was determined in ethyl acetate extract against murine macrophage (RAW 264.7) cells. The compounds present in the extract were analyzed through GCMS. **Results:** The ethyl acetate extract exhibited good protection against protein denaturation at an inhibition rate of 72.66 ± 0.46% and provides significant membrane stabilization by inhibiting hemolysis by 92.02 ± 0.10 %. The *in vitro* anti-inflammatory activity was found to be dose dependent by inhibiting the inflammatory mediators such as COX (62.08 ± 0.035%), LOX (65.84 ± 0.19%) and also reduced production of the MPO (0.000797 ± 0.00003 IU/ml) and iNOS (90.44 ± 0.19%) thereby cellular nitrite level to 342.54 ± 0.49 µg/ml. The GCMS analysis for bioactive compounds showed a total of 16 compounds in which phenols and lipids are the major components. **Application:** The result suggests that the microalgae contain potential anti-inflammatory compounds which can be explored in therapeutics against inflammation induced diseases.

Keywords: *Nannochloropsis* sp., Microalgae, Anti-inflammatory, Membrane Stabilization.

1. Introduction

Inflammation is a defensive mechanism of an organism against local injury and infections, which can progress to painful or chronically harmful diseases requiring pharmacological

treatment [1]. Various immune cells such as macrophages, neutrophils, and lymphocytes are activated during inflammation [2]. It is characterized by redness, pain, heat, swelling, and loss of function in the injured area. Increased production of free radicals during inflammation causes an imbalance between the oxidizing molecules and the antioxidant system of the body. This oxidative stress leads to damage in the cellular components [3]. Eicosanoids and cytokines have significant role differentiation, development, and regulation of immune cells and are mediators of immune response [4]. Inflammation can progress to painful or chronically harmful diseases such as psoriasis, rheumatoid arthritis, and colitis, which can cause death and disability in the world [5]. Protein denaturation is one of the causes of inflammation [6]. One of the strategies to regulate inflammation is to inhibit the release of lysosomal constituents [7].

Marine organisms are one of the interested areas in research, especially in microalgae for the past decades as they produce pharmacologically active metabolites. Microalgal molecules such as carotenoids, phycobilins, polyunsaturated fatty acids, proteins, polysaccharides, vitamins, and sterols possess several health benefits and therefore are used in many sectors such as nutraceutical, pharmaceutical, and functional foods [8]. The microalgae, *Nannochloropsis* sp. are used in aquaculture as they are rich in omega-3 fatty acids [9]. They are the excellent source of eicosapentanoic acid (EPA). EPA and docosahexaenoic acid (DHA) were found to have positive effects on, anti-inflammatory activity, prevention of heart disease brain vision health and development [10]. The current work aimed at determining anti-inflammatory property of the marine microalgae, *Nannochloropsis* sp. and identify the chemical constituents by GC–MS analysis.

2. Materials and Methods

2.1. Microalgae Extract Preparation Using Different Organic Solvents

Marine microalgae, *Nannochloropsis* sp. were collected from the Centre for Marine Fisheries Research Institute (CMFRI) Tuticorin, Tamilnadu, India. The microalgae were sub-cultured in Conway medium and maintained as a pure culture. The microalgae cells were obtained by centrifugation. The pellet was collected and air dried under room temperature to get a fine powder. Dried microalgae cells of 10 g were extracted in 100 ml of different organic solvents specifically methanol, aqueous, isopropanol, petroleum ether, and ethyl acetate and was kept at 4 °C for 7 days with occasional shaking. It was then filtered using Whatman no. 1 filter paper. The filtrate was kept at room temperature to evaporate excess solvent. The concentrated filtrate was used for the further experiments.

2.2. Protein Denaturation Inhibition Assay

The assay was done by the method followed by [11]. To 0.1 ml of various solvent extract added 0.45 ml bovine serum albumin and incubated at 37 °C for 30 min. 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube after cooling. Turbidity was measured

spectrophotometrically at 660 nm. The percentage inhibition of protein denaturation was calculated by the following formula. Salicylic acid was taken as standard drug.

% of inhibition = Absorbance of control – Absorbance of test/Absorbance of control × 100

2.3. Membrane Stabilization Assay

2.3.1. Preparation of Red Blood Cells (RBCs) Suspension

The assay was performed by the method followed by [12]. The fresh whole human blood (10 ml) was collected and transferred in a centrifuge tube containing heparin. The tubes were centrifuged at 3000 rpm for 10 min and were washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline.

2.3.2. Heat Induced Hemolysis

1 ml of various solvent extracts was added to 1 ml of 10% RBC suspension. Diclofenac sodium was taken as a standard drug. The reaction mixture was centrifuged and incubated in a water bath at 56 °C for 30 min. The tubes were cooled and centrifuged at 2500 rpm for 5 min. The absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates. The % of membrane stabilization activity was calculated by the following formula. % of inhibition = Absorbance of control – Absorbance of test/ Absorbance of control × 100.

2.4. Determination of *In vitro* anti-inflammatory Activity

Murine macrophage cells (RAW 264.7) were initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecco's modified Eagles medium (DMEM). The cell line was cultured in DMEM supplemented with 10% FBS, l-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100 U/ml), Streptomycin (100 µg/ml), and Amphotericin B (2.5 µg/ml) and were incubated at 37 °C in a humidified 5% CO_2 incubator. When the cell growth reached up to 60% confluence, the cells were induced with 1 µL lipopolysaccharide (LPS: 1 µg/ml). Different concentration (25, 50, 100 µg/ml) of sample solution and diclofenac sodium, a standard anti-inflammatory drug, in varying concentration corresponding to the sample was added to the LPS stimulated cell. After 24 h of incubation, the anti-inflammatory assays were performed using the cell lysate.

2.4.1. Cyclooxygenase (COX) Activity

The COX activity was assayed by [13]. Tris–HCl buffer (pH 8), glutathione 5 mM/L, and hemoglobin 5 mM/L was added to 100 µl cell lysate and incubated for 1 min at 25 °C. Arachidonic acid of 200 mM/L was added to initiate the reaction and incubated for 20 min

at 37 °C. The reaction was terminated by the addition of 10% trichloroacetic acid (200 µL) in 1 N hydrochloric acid. After the centrifugal separation, 200 µL of 1% thiobarbiturate was added and the tubes were boiled for 20 min and centrifuged for three minutes. COX activity was determined by reading absorbance at 632 nm.

% of inhibition = Absorbance of control – Absorbance of test/Absorbance of control × 100

2.4.2. Lipoxygenase (LOX) Activity

The determination of LOX activity was done as per [14]. To 50 µL of cell lysate, added Tris–HCl buffer (pH 7.4), and sodium linoleate (200 µL) to make the reaction mixture (2 ml final volume). The LOX activity was measured as an increase of absorbance at 234 nm, which reflects the formation of 5-hydroxyeicosatetraenoic acid.

% of inhibition = Absorbance of control – Absorbance of test/Absorbance of control × 100

2.4.3. Myeloperoxidase (MPO) Activity

The determination of MPO activity was done as per the method described by [15]. Cell lysate was homogenized with 50 mM potassium phosphate buffer and 0.57% hexadecyl trimethyl ammonium bromide (HTAB). The samples were centrifuged at 2000 *g* for 30 min at 4 °C, and collected the supernatant. The sample containing MPO was activated by the addition of 50 mM phosphate buffer (pH 6) containing 1.67 mg/ml guaiacol and 0.0005% H_2O. The change in absorbance was measured at 460 nm. MPO activity was presented as units per ml of cell lysate. One unit of MPO activity was defined as that degrading 1 µM of peroxide per minute at 25 °C.

2.4.4. Inducible Nitric Oxide Synthase (iNOS) Activity

Nitric oxide synthase was determined by the method described by [16]. Cell lysate was homogenized in 2 ml of HEPES buffer. To 0.1 ml of Arginine substrate, 0.1 ml manganese chloride, 0.1 ml 30 µg dithiothreitol (DTT), 0.1 ml NADPH, 0.1 ml tetrahydropterin, 0.1 ml oxygenated haemoglobin and 0.1 ml enzyme (sample) was added. Increase in absorbance was recorded at 401 nm.

2.4.5. Estimation of Cellular Nitrite Levels

The level of nitrite level was estimated by [17]. The reaction mixture containing 0.5 ml of cell lysate and 0.1 ml of sulphosalicylic acid was vortexed well for 30 min. Centrifuged the samples at 5000 rpm for 15 min and the protein-free supernatant was used for the estimation of nitrite levels. To 200 µl of the supernatant, 30 µl of 10% NaOH and 300 µl of Tris–HCl buffer and mixed well. To this, 530 µl of Griess reagent was added and incubated in the dark for 10–15 min. The absorbance was measured at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained.

2.5. GC–MS Analysis of Microalgae Extract

The GC–MS analysis of ethyl acetate extract of *Nannochloropsis* sp. (EAN) was carried out using GC–MS QP2010 Ultra (Shimadzu) with Rxi-5Sil MS fused-silica capillary column of 30 m length, 0.25 mm diameter, and 0.25 mm film thickness. An electron ionization system with ionization energy of 70 eV was used for analysis. Helium gas (99.99%) was used as the carrier gas at the constant flow-rate of 1 ml/min. The temperatures of the injector and mass transfer line were set at 250 °C and 240 °C, respectively. The oven temperature was programmed from 80 °C to 200 °C at 3 °C/min, and finally increased to 260 °C at 10 °C/min. Aliquots of 1 ml of the diluted samples were injected in split mode with a split ratio of 1:10 and with a mass scan range of 45–900 AMU. The total running time of the GC–MS analysis was 51 min.

2.6. Statistical Analysis

The data were analyzed and expressed as means ± SD. The EC50 values were calculated from linear regression analysis.

3. Result

3.1. Protein Denaturation Inhibition Assay

The various solvent extracts of *Nannochloropsis* sp. at 500 µg/ml concentration were evaluated for protein denaturation inhibition activity and is shown in Figure 1. EAN exhibited highest protection against protein denaturation of about 72.66 ± 0.46% inhibition among the various solvent extracts which was followed by methanol and isopropanol, petroleum ether and aqueous extract. The extracts were compared with salicylic which showed an inhibition of about 96.13 ± 0.23%. All experiments were performed in triplicates and the results were expressed as mean ± SD.

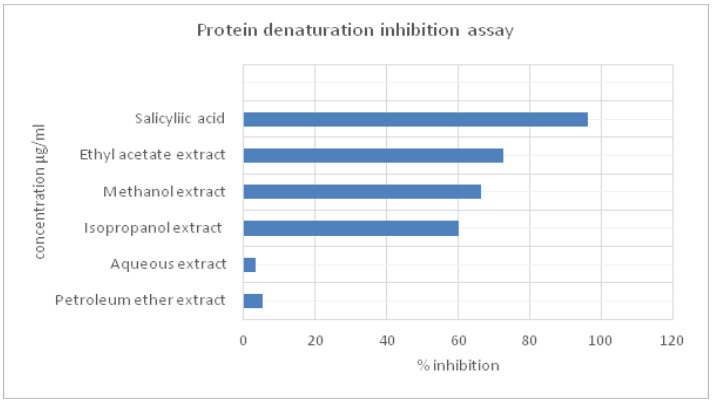

FIGURE 1. Protein denaturation inhibition assay.

3.2. Membrane Stabilization Assay

One of the well-known causes of inflammation is membrane disruption. Figure 2 shows the membrane stabilization activity of various extracts of *Nannochloropsis* sp. at a concentration of 500 µg/ml. The highest activity was noticed in EAN with an inhibition of hemolysis at 92.02 ± 0.10%, which was followed by methanol, isopropanol, aqueous extract, and least activity was seen in petroleum ether extract. The activity of various extract was compared with the control drug, diclofenac sodium which showed an inhibition of membrane disruption of 96.37 ± 0.26%.

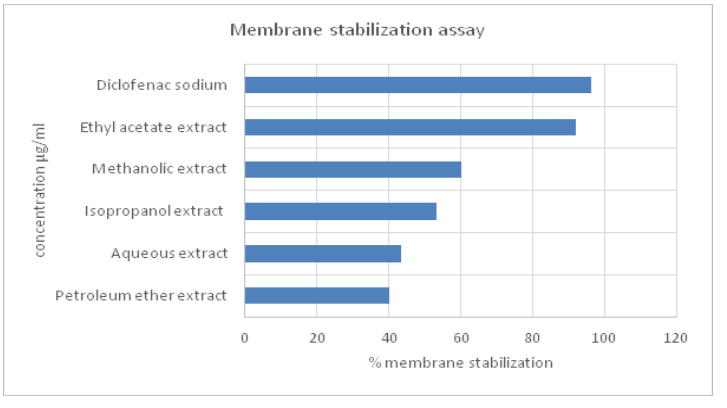

FIGURE 2. Membrane stabilization assay.

3.3. Determination of *In vitro* Anti-inflammatory Activity

3.3.1. COX Inhibitory Activity

Cox is the enzyme which produces the inflammatory agents such as prostaglandins from arachidonic acid. In the present study, EAN showed an inhibition of 62.08 ± 0.03% at 100 mg/ml concentration against the enzyme COX in a dose-dependent manner which is compared with the standard drug diclofenac sodium which showed an inhibition of about 87.49 ± 0.19%. Figure 3 represents the anti-inflammatory effect of EAN. The EC50 value of extract and diclofenac was found to be 23.28 µg/ml and 12.67 µg/ml, respectively.

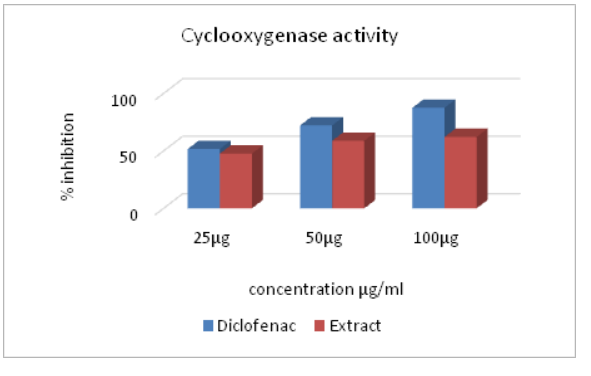

FIGURE 3. Cyclooxygenase activity.

3.3.2. LOX Inhibitory Activity

The EAN exhibited 65.84 ± 0.19% inhibition against lipoxygenase activity at 100 mg/ml concentration thereby blocked the formation of leukotrienes. The dose-dependent inhibition of extract was compared with the standard drug diclofenac sodium which showed an inhibition of 98.20 ± 0.08% and EC50 value at 18.16 µg/ml. The EC50 value of extract was found to be 20.81 µg/ml. The anti-lipoxygenase activity of EAN at varying concentration is shown in Figure 4. The present study indicates the good ability of the extract to reduce the activity of lipoxygenase.

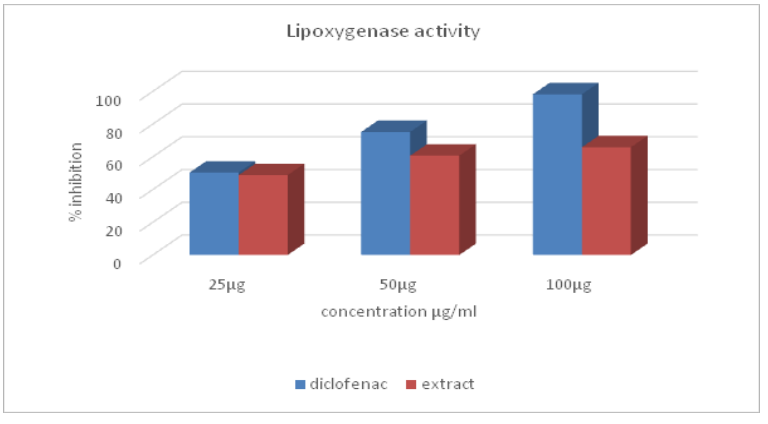

FIGURE 4. Lipoxygenase activity.

3.3.3. Estimation of MPO

The EAN was found to reduce the enzyme activity to 0.000792 ± 0.000033 U/ml and the standard drug diclofenac showed enzyme activity to 0.000481 ± 0.00006 U/ml at a concentration of 100 µg/ml. The extract reduced the production of MPO in a dose-dependent manner, thereby prevented the generation of oxidants. Figure 5 shows the myeloperoxidase inhibitory activity of the extract and the standard drug diclofenac.

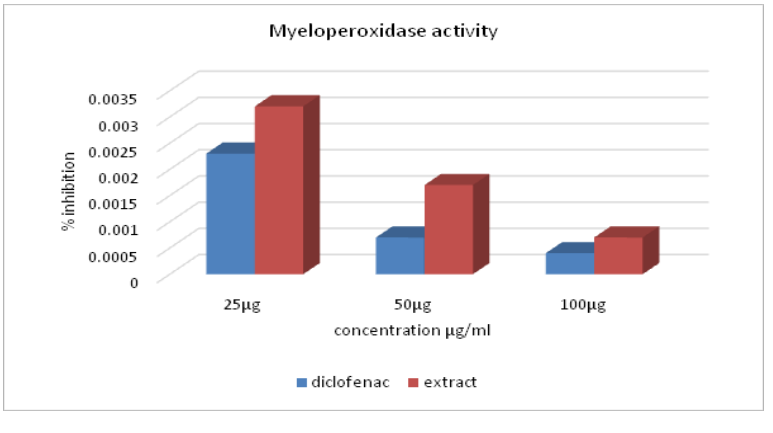

FIGURE 5. Myeloperoxidase activity.

3.3.4. iNOS Inhibitory Activity

iNOS are stimulated in response to inflammatory mediators. The EAN was found to have significant inhibitory effect against iNOS of 90.44 ± 0.19% at 100 µg concentration which showed an EC50 value of 23.06 µg/ml and the drug diclofenac exhibited an inhibition of 97.4 ± 0.33% is shown in Figure 6. The EC50 value of diclofenac was found to be 16.02 µg/ml. The present study indicates that the extract is a potent inhibitor of iNOS in a dose-dependent manner.

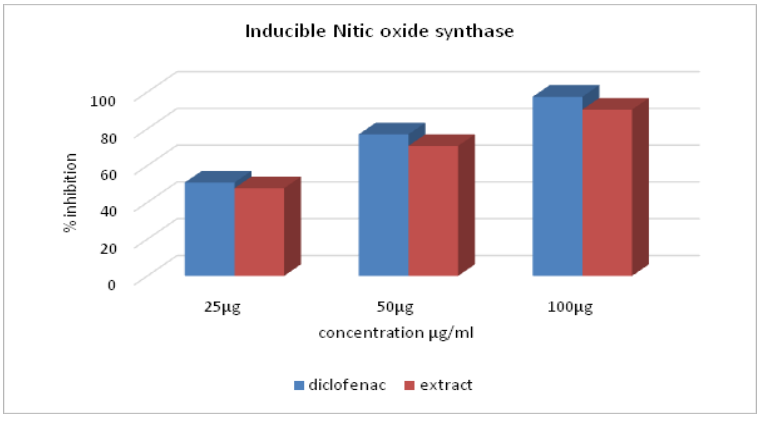

FIGURE 6. Inducible nitric oxide synthase activity.

3.3.5. Estimation of Cellular Nitrite Level

The EAN effectively reduced the cellular nitrite at a concentration of 100 µg/ml to 342.54 ± 0.49 µg/ml which is lesser than the standard drug (339.2 ± 0.98 µg/ml). Figure 7 represents the cellular nitrite level after the treatment with EAN and the drug diclofenac. The EAN exhibited a remarkable ability to reduce the cellular nitrite level.

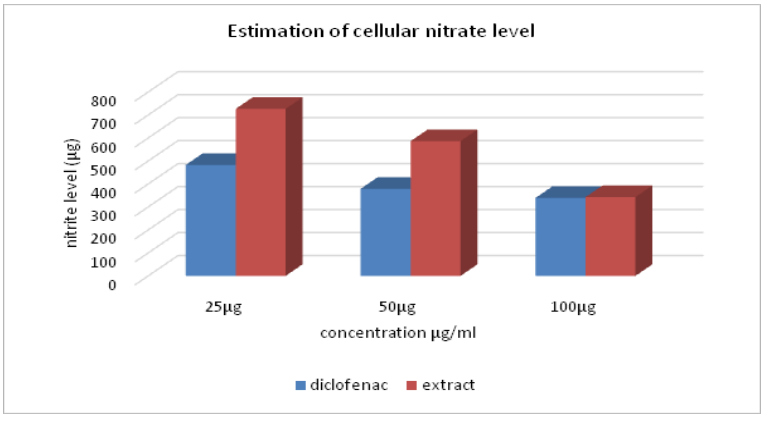

FIGURE 7. Estimation of cellular nitrite level.

3.4. GC–MS Analysis

GC–MS is a powerful tool for identification and determination of phytochemicals. Among the 16 compounds identified, phenol, 2,4-bis(1,1-dimethylethyl) obtained at a retention time of 21.431 min, area of 498345 and area %- 26.07 is the most prevailing major compound. These phenolic compounds were reported in the literature to have bioactive potential. Other major components include heneicosane, heptadecane, hexatriacontane, tetracosane and O O'-biphenol, 4,4',6,6'-tetra-t-butyl. The remaining compounds such as undecanol, 1-pentadecane, dodecane ,1-nonadecane, dibutyl phthalate, heptacasanol, eicosane, n-tetracasanol showed the area percentage on and under 5.19. The list of compounds obtained through GCMS were shown in Table 1. The peak obtained through GCMS analysis is shown in Figure 8.

TABLE 1. GCMS analysis of EAN

Peak	R time	Area	Area %	Name
1	4.486	59271	3.10	Decane
2	9.533	62091	3.25	1-Undecane
3	9.821	57622	3.01	Dodecane
4	17.009	99116	5.19	Pentadecene
5	21.431	498345	26.09	Phenol,2,4,bis(1,1-dimethylethyl)
6	24.776	133383	6.98	Heptadecene
7	32.057	83760	4.38	Nonadecene
8	37.246	82644	4.32	Dibutyl phthalate
9	38.721	92534	4.84	1-Nonadecene
10	43.435	97912	5.12	1-Heptacosanol
11	43.534	79194	4.14	Eicosane
12	44.941	114546	5.99	Tetracosane
13	45.515	106310	5.56	0 0'-Bisphenol,4,4',6,6'-tetra-t-butyl
14	46.053	67353	3.52	n-Tetracosanol-1
15	46.115	152158	7.96	Heneicosane
16	48.632	124993	6.54	Hexatriacontane
		1911232	100.00	

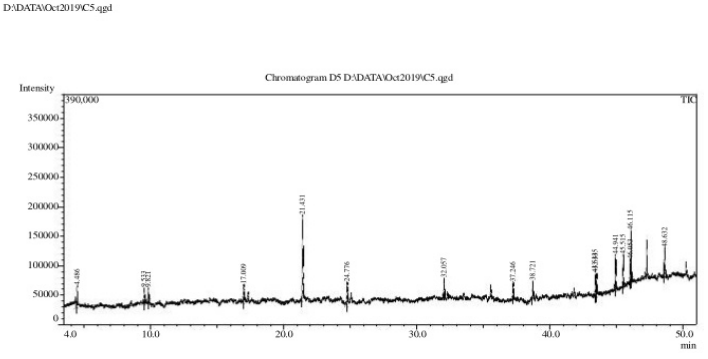

FIGURE 8. GCMS analysis of EAN.

4. Discussion

Inflammation is the defensive response of the body and several mechanisms are involved in it. It is characterized by redness, pain, heat and swelling and leads to loss of function in the injured area. Denaturation of protein and membrane disruption are found to have a role in inflammation. In the present study, initially various extracts of the microalgae, *Nannochloropsis* sp. were screened for anti-inflammatory activity. Denaturation of proteins was reported to be one of the causes of inflammation [18]. Diseases related to protein denaturation include Alzheimer's, Parkinson's, Huntington's as well as dementia [19]. In the protein denaturation inhibition assay, the EAN exhibited good protection against protein denaturation. There is a resemblance between RBC membrane and lysosomal membrane and so the stabilization activity has the possibility to inhibit content of neutrophils at the site of inflammation [20]. Membrane stabilization assay, the EAN showed significant activity among the various extract. From the membrane stabilization assay, it is confirmed that the EAN can provide cell membrane integrity and viability and thus prevent inflammation. As the EAN exhibited significant activity among the various extracts in the above two mentioned assays, the same was used to evaluate *in vitro* anti-inflammatory effect in murine macrophage cells. During inflammation, as part of their defensive roles, leukocytes release their lysosomal enzymes, including proteases, causing further tissue damage and subsequent inflammation [21]. The inflamed cells produce pro-inflammatory cytokines as well as stimulate the expression of nitric oxide synthase isoform, iNOS and COX [22]. Leukotrienes are derived from arachidonic acid via lipoxygenase pathway and are involved in the pathogenesis of several inflammatory diseases like asthma, psoriasis, rheumatoid arthritis, and inflammatory bowel diseases [23]. The regulation of this enzyme is important in the inhibition of inflammation and the agents that inhibits these enzymes acts as anti-inflammatory agents. The EAN showed significant level of inhibition of pro-inflammatory enzymes such as COX and LOX in a dose-dependent manner. Oxidative stress is a well-known cause of inflammation. After oxidative stress and different inflammatory responses, MPO enzyme is released into the extracellular fluid which causes several types of tissue injuries and pathogenesis of chronic diseases such as rheumatoid arthritis, cardiovascular diseases, liver diseases, diabetes, and cancer have been reported to be linked with MPO-derived oxidants [24]. The antioxidants present in the EAN effectively reduced the oxidants and thereby reduced the MPO production. iNOS produces NO in response to a wide array of stimuli, most prominently endotoxin and endogenous proinflammatory mediator which causes tissue damage during inflammation [25]. The EAN showed remarkable level of inhibition of iNOS as well as cellular nitrite level which were dose dependent. The GCMS study showed the presence of phenols and fatty acid as the major content of the extract which may have role in the anti-inflammatory activity.

5. Conclusion

The present study indicates that various extract of *Nannochloropsis* sp. showed significant anti-inflammatory activity in a dose-dependent manner. Among the various solvent extracts, the EAN exhibited highest protection against protein denaturation and provides

good stabilization of membranes. The EAN exhibited significant inhibitory activity on enzymes COX and LOX which is indicated by the reduction of cellular nitrite level, inducible nitric oxide synthase and myeloperoxidase, significantly. The phenolic and fatty acid components of the microalgae were identified by GCMS which may be attributing the anti-inflammatory property.

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