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INVITRO ASSESSMENT OF THE ANTI-INFLAMMATORY POTENTIAL OF *SPIRULINA PLATENSIS* EXTRACT BY HUMAN RED BLOOD CELL MEMBRANE STABILIZATION AND PROTEIN DENATURATION METHOD

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Abstract

Phytochemical research is more focused on the urge for alternative natural phytochemicals that can replace synthetic chemical drugs to minimize the side effects. Controlling the side effects during the treatment is one of the challenges facing medical experts. In the last few decades, medicinal plant research based on traditional, natural, and folk medicines in written form or transferred from ancestors has been of interest to many researchers. It is engaged to explore the most essential basic medicinal properties of medicinal plants or other natural resources, such as antioxidant, anti-inflammatory, analgesic, and immunomodulatory properties. The present investigation aimed to assess the in vitro anti-inflammatory properties of the whole ethanolic extract of Spirulina platensis from Lonar Lake in Maharashtra, India. Spirulina was cultured in the laboratory using a modified Zarrouk medium. The maximum growth is achieved in modified Zarrouk's medium at pH 9.2. The algal ethanolic extract was screened for its anti-inflammation activity by using a protein denaturation inhibition assay and an HRBC membrane stabilization assay. Spirulina whole ethanolic extract proved to have anti-inflammatory potential.

Key words: Ethanolic whole extract, Anti-inflammatory activity, Sodium diclofenac, HRBC membrane stabilizing assay, Protein denaturation inhibition.

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Introduction

Inflammation is a sign of a protective innate and adaptive immune response by mammalian tissue towards chemical, biological, and mechanical injury. Biological antigens, such as different infectious agents including bacteria, fungi, viruses, and parasites. Mechanical injury due to hitting, biting, and other accidental injuries, as well as excess heat, cold, or trauma to which the body responds by inflaming the affected tissue. Other reasons for inflammation are interaction with etiological agents like chemotoxins due to insect bites, snake bites, and other animal bites, and interaction with various allergens as well as pollutants. Inflammation is a result of a cascade initiated by the immune response of the host body to eliminate the antigen (Rock, 2011). Inflammation is marked by heat, redness, pain, swelling, and fever, which are the consequences of the various cascades activated as a part of the host immune response (MacGlashan, 2003). Various mediators of the immune response are secreted by immune system cells in response to antigenic stimuli, such as activated T lymphocytes, B lymphocytes, NK cells, macrophages, monocytes, neutrophils, basophils, eosinophils, fibroblasts, endothelial cells, etc. These mediators are responsible for the stimulation of a cascade of different physicochemical changes in the host body; these include chemoattractant, vasodilators, cytokines, chemokines, C-reactive proteins, etc. (Vignali, 2012) The progression of inflammation is mediated by these mediators. The initial response of the body's inflammation is acute inflammation. Controlled inflammation is thus a part of the protective mechanism needed for tissue damage repair and regeneration. Acute inflammation progresses into chronic inflammation and leads to chronic diseases such as hay fever, arthritis, atherosclerosis, rheumatoid arthritis (RA), carcinomas, and various degenerative diseases like Alzheimer's. (Fernandes, 2015) (Heppner, 2015) Even though inflammation is the process that proceeds to cure, it causes discomfort and pain. Many steroidal, nonsteroidal, and immunosuppressive drugs are recommended to relieve and control inflammation. These drugs are predominantly synthetic chemicals. The target of these drugs is the various mediators of the inflammatory cascade. Long-term use of these drugs causes serious side effects. (Dinarello, 2010)

Indian ayurvedic literature, folk medicines, and other natural therapies explain the uses of plants and various natural products for their anti-inflammatory properties, such as honey (Yaghoobi, 2013) (Vallianou, 2014). Turmeric (*Curcuma longa*), red sandalwood (*Pterocarpus santalinus*), Allium sativum, etc. (Jayanthi, 2011). Phytochemical research during the last few decades has focused on exploiting various plants mentioned in the literature and their parts, such as roots, tubers, stems, bark, leaves, flowers, and fruits, for their medicinal values (More and Baig, 2013; Baig, 2022). Depleting forest land is a major issue for the crisis of these rare medicinal plants. Algae are a plentiful natural resource that can be extensively researched for their medicinal potential.

Algae are present in almost all natural habitats, and various phytochemicals have been tested for their pharmacological properties, such as antioxidant, anti-inflammatory, analgesic, immunomodulatory, etc. Protein denaturation is one of the reasons for an inflammatory response (Sangeetha, 2016) and the prevention of the denaturation of proteins is also one of the mechanisms of action of many NSAIDs (non-steroidal anti-inflammatory drugs) (Dalgleish, 2002).

Spirulina is a very primitive cyanophyte (blue-green algae) that originated 3.5 billion years ago and utilizes carbon dioxide in water as a nutrient under extreme environmental conditions such as high alkalinity (pH 9 to 11) and high intensity of sunlight (Silveira, 2007). The

phytochemical constituents of *Spirulina* made it a novel natural source of vitamins, proteins, and minerals, which added it's therapeutic and pharmacological significance in drug discovery. Long ago, *S. platensis* was harvested as a food supplement in the form of cake (making a dry mass) in Africa (Cifferi, 1983).

The most important phytoconstituents include pigments such as carotenoids, phycobiliproteins, chlorophyll, etc. Phycobiliproteins are Phycocyanin, phycoerythrin, and allophycocyanin which are made up of polypeptides. Apart from the high protein content, the presence of vitamins, minerals, carotenoids, linolenic acids, and essential fatty acids upgrades the nutritional value of *Spirulina*. (Volkmann, 2008) This research article explores the preliminary anti-inflammatory properties of ethanolic whole extract of *Spirulina platensis* from Lonar Lake in Buldhana district, Maharashtra, India, by using an protein denaturation inhibition assay and an HRBC (Human Red Blood Cell) membrane stabilizing assay.

Material and Methods:

Cultivation of Spirulina platensis:

A Pure laboratory culture of Spirulina platensis was obtained from a water sample from Lonar lake in Buldhana, Maharashtra, India. After initial identification and characterization. S. platensis was cultured in modified Zarrouk's medium under controlled light and dark conditions and maximum growth was achieved according to the media prepared by Raoof et al., (2006). Standard Zarrouk's medium was modified by adding other supplementary chemicals to enhance growth rate, such as sodium nitrite (2.50g/l), sodium chloride (0.50g/l), super phosphate (1.25g/l), magnesium sulphate (0.15g/l) sodium bicarbonate (8g/l), and calcium chloride (0.04g/l). Illumination of white light at 50 mmol photons/m^2/s with the help of the fluorescent lamp, at 30 °C. Maximum growth in terms of biomass is achieved within 10 days and was estimated spectrophotometrically. (Silveira, 2006,). The biomass of S. platensis was obtained by filtration and air-dried in the shadow. A Fine powder of dried mass was dissolved in ethanol in a closed glass container and was allowed to mix on a shaker for 48 hours and extracted later. The pure crude ethanolic extract in powder form was obtained and used for further investigations.

Preparation of drug:

Standard drug (Sodium Diclofenac 100-500µg/ml) and Spirulina Methanolic extracts (100-500 µg/ml) were prepared in isotonic saline (0.9 % NaCl) or Hartmann's solution to final concentration. (Banerjee S, 2014)

Preparation of Human Red Blood Cell Suspension (10 % v/v):

Fresh whole blood sample (5 ml) from a healthy human volunteer who had abstained from NSAID use for two weeks prior to the experiment provided was collected in a heparinized centrifuge tube. The blood sample was centrifuged at 3000 rpm for 10 minutes at room temperature, and then it was washed three times with normal saline using an equal volume each time. The packed red blood cells were then gently washed with fresh normal saline (0.9% w/v NaCl), while the supernatant containing plasma and leucocytes was carefully removed. The human erythrocyte suspension (10% v/v) was made by repeatedly washing and centrifuging five times until the supernatant was clear. (Sadique J, 1989) (Varghese, 2017)

Protein denaturation activity:

In the protein denaturation method, Spirulina extract in several concentrations (100, 200, 300, 400, and 500 µg/mL) was used to check its protein denaturation inhibition activity. A 5%w/v aqueous solution of bovine serum albumin (BSA) in phosphate-buffered saline was used as a standard protein solution. The pH of the reaction mixture was adjusted to 7. The sample mixtures (5 % BSA Solution + Spirulina ethanolic extract of various

concentrations, i.e. from 100 to 500 µg/mL) were incubated at 37 ºC for 20 minutes prior to denaturation and then heated to 51ºC for 20 minutes. Instead without Spirulina extract, BSA only in distilled water, was used as a negative control. After cooling under running tap water, the turbidity of the samples was measured spectrophotometrically at 660 nm. Diclofenac sodium was used as a standard drug. (Osman, 2016) The experiments were performed in triplicate.

Percent inhibition of protein denaturation was calculated as follows- (Osman, 2016)

% Inhibition of denaturation = $(1 - \frac{D}{C}) \times 100$

Where D = the absorbance of test sample

C = the absorbance of negative control

(Without the test sample or reference drug).

Membrane stabilization assay:

Cell membrane lysis is one of the effects of inflammation to release lysosomal content as a part of the inflammatory cascade, resulting in the destabilization of the biological membrane. Stabilization of these membranes is one of the mechanisms of anti-inflammatory drugs. The RBC membrane is a useful study system as a biological membrane. Any injury such as heat, hypotonicity, or interaction with phenyl hydrazine, methyl salicylate, etc. results in destruction of the RBC membrane and the release of hemoglobin (Labu, 2015) (Sarveswaran, 2017). The HRBC membrane stabilizing activity assay was carried out in which Human Red Blood Cells (HRBC) membrane gets lysed in a hypotonic medium or by heat and interaction with methyl salicylate, or phenyl hydrazine, results in hemolysis and hemoglobin oxidation. Inhibition of RBC hemolysis due to hypotonicity and heat were taken as a measure of the mechanism of anti-inflammatory activity studies. (Sarveswaran, 2017) (Yoganandam, 2010)

Heat-induced hemolysis:

The heat-induced hemolysis experiment includes the reaction mixture (2 ml) consists of a 1 ml test sample, control, and a standard drug of different concentrations (100 to 500 µg/ml *Spirulina* whole extract, saline, and aspirin 100 to 500 µg/ml respectively) and 1 ml of 10% RBCs suspension were mixed well and allowed to set for 20 minutes prior to lysis treatment. Then, the reaction mixtures were incubated in a water bath at 56 ºC for 30 minutes. At the end of the incubation, the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 minutes and the absorbance of the supernatants was recorded at 560 nm. The experiment was performed in triplicate for the entire sample.

The Percent inhibition of Hemolysis was calculated as follows-

Percent inhibition= $\frac{\text{Absorbance control - Absorbance sample}}{\text{Absorbance Control}} \times 100$

Hypotonicity-induced hemolysis:

A hypotonic solution was prepared in distilled water by dissolve the powdered *Spirulina* ethanolic extract was employed in this test. The centrifuge tubes were filled with the hypotonic solution (5 ml) containing graduated doses of the extracts (100, 200, 300, 400, and 500 g/ml). Additionally, duplicate pairs (per dose) of centrifuge tubes were filled with isotonic solution (5 ml) that contained graded doses of the extracts (100 to 500 g/ml). Five millilitres each of the distilled water and 5 ml of sodium diclofenac solution in concentrations ranging from 100 to 500 g/ml served as control. Each tube was added with 0.1 ml of erythrocyte suspension and was gently mixed. The solutions were centrifuged for 3 minutes at 1300 rpm after being incubated for 1 hour

at room temperature (37°C). Using a spectrophotometer (Chemito UV 2100), the absorbance (OD - optical density) of the hemoglobin concentration of the supernatant was calculated to be 560 nm. Assuming that the amount of hemolysis produced in the presence of distilled water was 100%, the percent hemolysis was calculated.

Percentage protection= $100 - \frac{OD\ of\ Sample}{OD\ of\ Control} \times 100$

Results :

In protein Denaturation inhibition assay ,heat induced denaturation of BSA (Bovine Serum Albumin) was the parameter characterized spectrophotometrically. Denaturation of protein results in increased absorption in the protein solution without spirulina content. And the fall in absorption was the indictor to denaturation inhibition with different concentration of spirulina ethanolic extract.

Both the methods used to study the disintegration of HRBCs membrane were heat induced destabilization and hypotonicity induced membrane destabilization, the parameter observed was percent hemolysis that studied spectrophotometrically. Inhibition of the destabilization of HRBC membrane at different concentration of Spirulina ethanolic extract was observed.

Table 1: Anti-inflammatory activity of ethanolic extract of Spirulina platensis (% inhibition of protein denaturation by using BSA denaturation method)

Concentration µg/ml	**% inhibition of protein denaturation**	
	Standard (SDF)	SEE
100	70	66
200	80	76
300	85	81
400	93	90
500	99	95

*SEE= Spirulina Ethanolic Extract

*SDF= Sodium Diclofenac

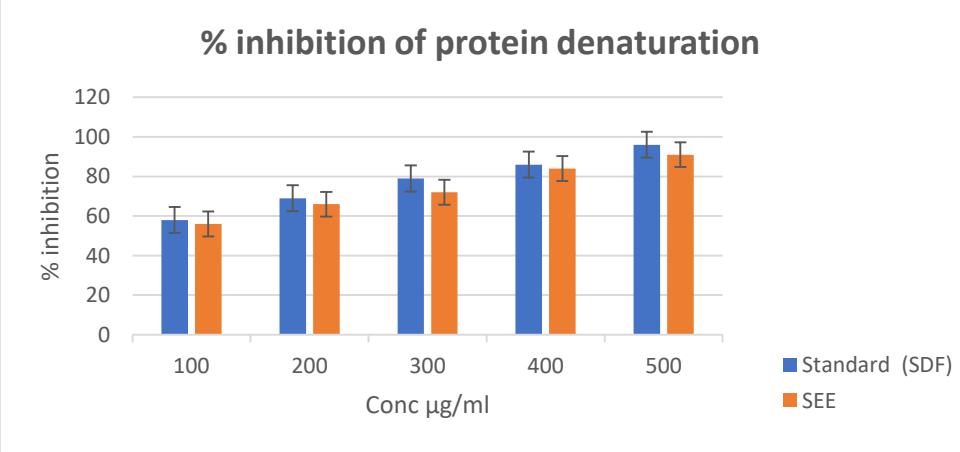


Table.2: Effect of different concentrations of Spirulina ethanolic extract and standard (sodium diclofenac) on HRBC membrane by using heat induced hemolysis of erythrocyte

Concentration µg/ml	% inhibition of HRBC haemolysis	
	Standard (SDF)	SEE
100	60	53
200	70	66
300	78	73
400	84	80
500	90	87

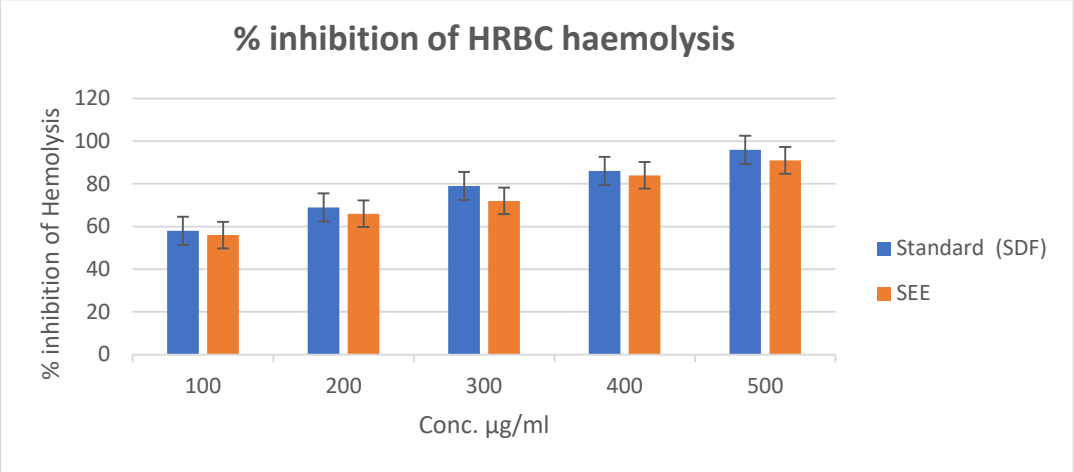
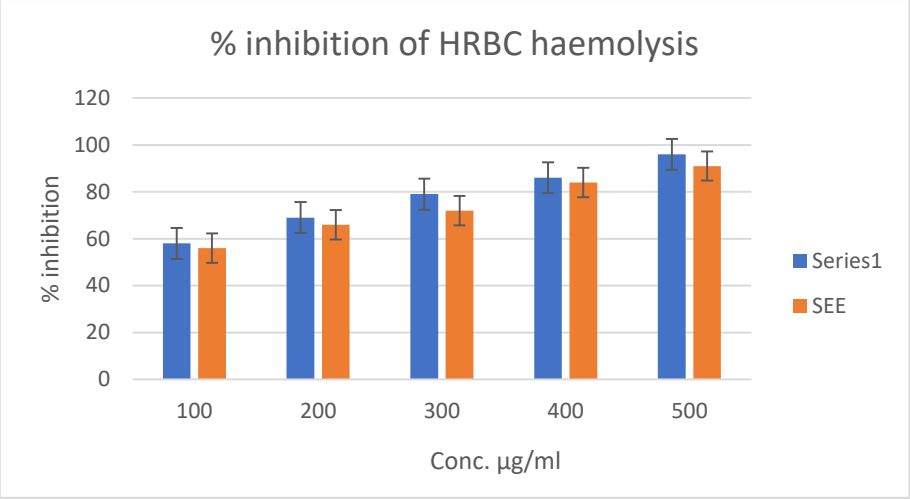


Table 3 : Effect of different concentrations of Spirulina ethanolic extract and standard (sodium diclofenac) on HRBC membrane by using Hypotonicity induced hemolysis of erythrocyte.

Concentration µg/ml	% inhibition of HRBC haemolysis	
	Standard (SDF)	SEE
100	58	56
200	69	66
300	79	72
400	86	84
500	96	91



* SEE=Spirulina Ethanolic Extract

** SDF=Sodium Diclofenac

Table 2 and table 3 shows comparative HRBC membrane stabilization with Spirulina ethanolic extract with standard sodium diclofenac.

It was observed that percent inhibition of protein denaturation for different concentration of spirulina extract compared with standard drug sodium diclofenac. The spirulina extract exhibited good anti-inflammatory properties that are comparable with standard. Hypotonicity as well as heat induced membrane destabilization was found to be inhibited by spirulina extract and nearly competent with the standard drug.

Discussion

Protein denaturation inhibitors are known to work by reducing the activity of many inflammatory mediators that are involved in the inflammation process. Inflammation has been linked to the denaturation of proteins, which is widely known. The ability of medications like phenylbutazone, salicylic acid, and sodium diclofenac to prevent protein denaturation is dose dependent. Numerous phytochemicals have been found to have potent anti-inflammatory properties on par with those of synthetic anti-inflammatory medications.

Protein denaturation is one of the factors that contributes to rheumatoid arthritis, and one of the in vitro assays used to evaluate anti-inflammatory medications is inhibition of denaturation. The existing medications, referred to as nonsteroidal anti-inflammatory medicines (NSAID), work by preventing prostaglandin from doing its job. An autocoid that is secreted extracellularly and causes discomfort is prostaglandin. Anti-inflammatory drugs prevent the production of these atracoids by either blocking the COX enzyme or preventing the destruction of the lysosomal membrane. The majority of anti-inflammatory medications stabilize the plasma membrane of mammalian erythrocytes, which prevents hemolysis brought on by hypotonicity and heat.

Damage to cell membrane due to lysosomal release is a well-marked consequence of inflammatory response. Inhibition of membrane destabilization is one of the mechanisms of action of anti-inflammatory substances. Red Blood cell membrane is well studied model for membrane stabilization experiments (Umapathy, et al., 2010) Damage to cell membrane makes cell more fragile and susceptible to other secondary damages such as free radical interaction, results in losing the control on regulation of osmosis, diffusion, active and passive transport via membrane. This may lead to destabilization of cell membrane. (Gambhire, Juvekar, & Wankhede, 2009)

Many researchers evaluated different plants and their individual parts as well as components and crude extracts preventing protein denaturation to prove their anti-inflammatory property.

Oyedepo et.al. (1995) studied the anti-inflammatory properties of *Fagra zanthoxiloides*, *Olax subscorpioidea*, and *Tetrapleura tetraptera*.These extracts had been proved for membrane stabilization action against heat and hypotonicity induced RBC lysis. (Oyedepo, 1995) studies by (Umapathy, et al. (2010) *Albuca setosa* aqueous extract shown to protect heat induced erythrocyte membrane lysis (Umapathy, et al., 2010) Ethanolic extract of *Wedelia trilobata* (Govindappa, Naga, Poojashri, Sadananda, & Chandrappa, 2011) *Semecarpus anacardium* bark (Kumar, Bevara, Laxmikoteswramma, & Malla, 2013) studied for their protein denaturation inhibition property by using BSA, and *Albucas etosaon* by egg albumin. (Umapathy et al., 2010)

Inflammation-induced membrane lysis caused by heat and a hypotonic solution was prevented by the Spirulina extract at doses of 100 to 500 g/ml. When inflammatory mediators are causing tissues to become more permeable, membrane stabilization helps to stop the leaking of fluids and serum proteins into the tissues. The phytoconstituents in the whole ethanolic extract of Spirulina platensis exhibit anti-inflammatory property as the Spirulina extract stabilizes the red blood cells membrane and prevents hypotonicity-induced hemolysis.

CONCLUSION

Ethanolic whole extract of Spirulina platensis have anti-inflammatory potential which has been proved by its protein denaturation inhibition and erythrocyte membrane stabilization ability. This preliminary result needs to study more with other invitro as well in-vivo research methods. Characterization of individual components of algal extract need to be explore for similar activity to find out individual as well as synergetic potentials.

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