

Evaluation of Immunomodulatory Activity of Spirulina in Experimental Animals

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Abstract: Immunomodulatory Activity of Spirulina was evaluated by Hemaagglutinating antibody titer, Delay Type Hypersensitivity and Carbon clearance test. Swiss albino mice use for the study for Hemaagglutinating antibody titer, Delay Type Hypersensitivity model each model contain 8 groups of treatment of drug. While carbon clearance model contain 5 groups of animals of treatment of drug. All groups given spirulina orally. In Hemaagglutinating antibody titer model Spirulina significantly exhibited more antibody titer when compared with control. In Delay Type Hypersensitivity Spirulina significantly decreased % increase in paw edema when compare with control. In Carbon clearance test Spirulina exhibit significantly increase in phagocytic index when compare with control. Thus, Present study reveals that Spirulina holds promise as an immunomodulatory agent.

Keywords: immunomodulatory, Hemaagglutinating antibody titer, Delay Type Hypersensitivity Phagocytic index

1. Introduction

The human immune system is a truly amazing constellation of responses to attacks from outside the body. It has many facets, a number of which can change to optimize the response to these unwanted intrusions. The system is remarkably effective, most of the time. This note will give you a brief outline of some of the processes involved.

An antigen is any substance that elicits an immune response, from a virus to a sliver. The immune system has a series of dual natures, the most important of which is self/non-self recognition. The others are: general/specific, natural/adaptive = innate/acquired, cell-mediated/humoral, active/passive, primary/secondary. Parts of the immune system are antigen-specific (they recognize and act against particular antigens), systemic (not confined to the initial infection site, but work throughout the body), and have memory (recognize and mount an even stronger attack to the same antigen the next time). Self/non-self recognition is achieved by having every cell display a marker based on the major histocompatibility complex (MHC). Any cell not displaying this marker is treated as non-self and attacked. The process is so effective that undigested proteins are treated as antigens.

Sometimes the process breaks down and the immune system attacks self-cells. This is the case of autoimmune diseases like multiple sclerosis, systemic lupus erythematosus, and some forms of arthritis and diabetes. There are cases where the immune response to innocuous substances is inappropriate. This is the case of allergies and the simple substance that elicits the response is called an allergen.^[1,2]

Spirulina

Spirulina are multicellular and filamentous blue-green algae that has gained considerable popularity in the health food industry and increasingly as a protein and vitamin supplement to aquaculture diets. It grows in water, can be harvested and processed easily and has very high macro- and micro-nutrient contents. It has long been used as a dietary supplement by people living close to the alkaline lakes where it is naturally found – for instance those living

adjacent to Lake Chad in the Kanem region have very low levels of malnutrition, despite living on a spartan millet-based diet. This traditional food, known as *dihé*, was rediscovered in Chad by a European scientific mission, and is now widely cultured throughout the world. In many countries of Africa, it is still used as human food as a major source of protein and is collected from natural water, dried and eaten. It has gained considerable popularity in the human health food industry and in many countries of Asia it is used as protein supplement and as health food.^[3,4,5]

Introduction and Historical Perspective of Spirulina

Spirulina is a primitive organism originating some 3.5 billion years ago that has established the ability to utilize carbon dioxide dissolved in seawater as a nutrient source for their reproduction. Spirulina is a photosynthesizing cyanophyte (blue-green algae) that grows vigorously in strong sunshine under high temperatures and highly alkaline conditions.

Historical use

In the sixteenth century, when the Spanish invaders conquered Mexico, they discovered that the Aztecs living in the Valley of Mexico in the capital Tenochtitlan were collecting a “new food” from the lake. Spanish chroniclers described fishermen with fine nets collecting this blue coloured “techuitlatl” from the lagoons and making a blue-green cake from it. Other legends say Aztec messenger runners took spirulina on their marathons. Techuitlatl was mentioned by naturalists until the end of the sixteenth century, but not after that, probably reflecting the loss of the lakes as they were drained for urban and agricultural development. The only remnant today, Lake Texcoco, still has a living algae spirulina population.^[6,7]

The Kanembu population living along the shores of Lake Chad collects the wet algae in clay pots, drain out the water through bags of cloth and spread out the algae in the sandy shore of the lake for sun drying.

The semi-dried algae is then cut into small squares and taken to the villages, where the drying is completed on mats in the sun. When dry, women take these algae cakes for sale in the

local market. Dihé is crumbled and mixed with a sauce of tomatoes and peppers, and poured over millet, beans, fish or meat and is eaten by the Kanembu in 70 percent of their meals. Pregnant women eat dihé cakes directly because they believe its dark colour will screen their unborn baby from the eyes of sorcerers. Spirulina is also applied externally as a poultice for treating certain diseases. Abdulqader, Barsanti and Tredici (2000) further noted that the local trading value of the dihé annually harvested from Lake Kossorom in Chad (about 40 tonnes) amounts to more than US \$100,000, which represents an important contribution to the economy of the area.^[8,9]

Re discovery of Spirulina

In 1940, a French phycologist Dangeard published a report on the consumption of dihé by the Kanembu people near Lake Chad. Dangeard also noted these same algae populated a number of lakes in the Rift Valley of East Africa, and was the main food for the flamingos living around those lakes.^[10]

Twenty-five years later during 1964-65, a botanist on a Belgian Trans-Saharan expedition, Jean Léonard, reported finding a curious greenish, edible cakes being sold in native markets of Fort-Lamy (now N'Djamena) in Chad. When locals said these cakes came from areas near Lake Chad, Léonard recognized the connection between the algal blooms and dried cakes sold in the market.^[11]

In 1967 spirulina was established as a “wonderful future food source” in the International Association of Applied Microbiology. Analysis of the nutritional properties of spirulina showed first and foremost an exceptionally high protein content, of the order of 60–70 percent of its dry weight; it also showed the excellent quality of its proteins (balanced essential amino acid content). This first data was enough to launch many research projects for industrial purposes in the 1970s, because micro-organisms (yeast, chlorella, spirulina, some bacteria and moulds) seemed at that time to be the most direct route to inexpensive proteins – the iconic “single cell proteins”.^[12]

At the same time when Léonard rediscovered spirulina in Africa, a request was received from a company named Sosa-Textcoco Ltd by the “Institutfrançais du pétrole” to study a bloom of algae occurring in the evaporation ponds of their sodium bicarbonate production facility in a lake near Mexico City. As a result, the first systematic and detailed study of the growth requirements and physiology of spirulina was performed. This study, which was a part of Ph.D. thesis by was the basis for establishing the first large-scale production plant of spirulina.^[12]

While finally no micro-organism fulfilled its promise of cheap protein, spirulina continued to give rise to research and increasing production, reflecting its perceived nutritional assets.

Nutritional supplementation

One health problem that is of great concern, especially in developing countries, is malnutrition. Severe forms of malnutrition are expressed as protein energy malnutrition defects such as kwashiorkor, marasmus and marasmic kwashiorkor.

Apart from protein deficiencies, affected children usually do not have a complete balanced diet which includes the micronutrients such as vitamins and minerals that are essential for normal growth and development. The consequences of malnutrition represent a global problem, which affects morbidity as well as mortality. Increased tissue production of prostaglandin E2 as a result of high intake of linoleic acid in a polyunsaturated fatty acid deficient diet, causes inhibition of the proliferation and cytokine production of Th1 cells, the mediators of cellular immunity.¹⁰ Diet associated inhibition of the Th1 subset is a major contributor to the high prevalence of these diseases in sub-Saharan areas.¹¹ Spirulina is rich in proteins, carbohydrates, polyunsaturated fatty acids, sterols and some more vital elements such as calcium, iron, zinc, magnesium, manganese and selenium.

It is a natural source of vitamin B12, vitamin E, ascorbic acid, tocopherols and a whole spectrum of natural mixed carotene and xanthophylls phytopigments. Spirulina as a supplement serves to provide these nutrition requirements and seems to be a ‘wonder food’.^[13]

2. Materials

Apparatus and Equipments

- 1) Tarsor's immunology plates “U” bottomed 96 wells (code 934396).
- 2) Labline Variable volume (10–50 µl) Micropipette.
- 3) Analytical weighing balance (Shimatzu.Model AY-220)
- 4) UV-Visible spectrophotometer (Jasco.Model V-630)
- 5) Cooling centrifuge (Remi C-24).
- 6) B.O.D. Incubator (Labline. Ind.)
- 7) Digital Plethysmometer (Orchid.Ltd.)
- 8) The common laboratory glassware of Borosil glass, cotton and syringes (sterile, whenever necessary).
- 9) Tuberculin syringe, Microcapillary tubes.
- 10) To prevent the personal contamination standard working and operating procedures were strictly followed. Personal hygiene was maintained.

Drugs and Chemicals

- 1) Spirulina was obtained as a gift sample from **MAXCIMA** Ltd. Rajan Pharmaceuticals D II block M.I.D.C. Chinchwad.
- 2) Salbutamol was obtained as a gift sample from PDVVPF's college of pharmacy, Ahmednagar.
- 3) Cyclophosphamide was procured as a marketed product **CYCLOXAN** of biochem pharmaceuticals industries Ltd. Daman.
- 4) Pelikan fountain Indian Ink (Hannovar ,Germany)

Procurement of Drugs:

- 1) Spirulina was gifted by **MAXCIMA** Ltd. Rajan Pharmaceuticals D II block M.I.D.C. Chinchwad.
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- 3) Salbutamol was obtained as a gift sample from PDVVPF's college of pharmacy, Ahmednagar.

Dose Selection of Drugs

Spirulina usually prescribed as an antioxidant drug in the dose range 3gm per day dose depending upon the health of a patient. Salbutamol is usually prescribed as bronchodilator in the dose range of 0.1-2 mg for the treatment of asthma. In the present study, dose selection of Spirulina and has been fixed on the basis of, doses given to mice as an antioxidant treatment and doses that affect the spreading ability of macrophages in mice in the dose of 100 mg/kg (p.o.) and 200 mg/kg (p.o.) Dose of Salbutamol has been fixed on the basis of the dose that lowering cAMP level significantly in mice i.e. (2 mg/kg, p.o.).^[14]

Spirulina 100 mg/kg, Spirulina 200 mg/kg and Salbutamol were dissolved in distilled water and was given orally.

Experimental Animals

Swiss albino mice of inbred colony obtained from (National Institute of Biosciences, Pune) of either sex weighing 20-25 gm were housed in groups of 5 to 6. Mice were maintained at standard laboratory conditions. All mice were fed with synthetic pelleted diet, and clean tap water. Mice were maintained at 22° ± 1°C with 60% relative humidity, and at day and night cycle of 12 hour each. The animals were allowed to acclimatize to laboratory conditions prior to experimentation. All experiments were conducted during the light period of 12/12 hours of the day/night cycle.^[15]

2.2 Methods (Screening Section)

Humoral immune response

A) Hemagglutinating antibody (H.A) titer:^[15]

Table 1: Experimental Protocol (Grouping and treatment)

GR.NO	Group (N=6)	Treatment and Dose/Day.	Treatment Schedule
I	Control	Distilled water 1ml/Kg (p.o.)	1 st to 21 st Day.
II	Cyp. Control	Cyclophosphamide (Cyp.) 100mg/Kg (p.o.)	Single dose, each on day 9 th and day 16 th .
III	SPI Treated	Spirulina 100 mg/Kg (p.o.)	1 st to 21 st Day.
IV	SPI Treated	Spirulina 200 mg/Kg (p.o.)	1 st to 21 st Day.
V	SAL Treated	Salbutamol 2 mg/Kg (p.o.)	1 st to 21 st Day.
VI	SPI + Cyp. Treated	SPI 100 mg/Kg (p.o.) + Cyp.100mg/Kg (p.o.)	SPI from 1 st to 21 st day + Single dose of Cyclophosphamide each on 9 th and 16 th day.
VII	SPI + Cyp. Treated	SPI 200 mg/Kg (p.o.) + Cyp.100mg/Kg (p.o.)	SPI from 1 st to 21 st day + Single dose of Cyclophosphamide each on 9 th and 16 th day.
VIII	SAL + Cyp. Treated	SAL 2 mg/Kg (p.o.) + Cyp.100mg/Kg (p.o.)	SAL from 1 st to 21 st day + Single dose of Cyclophosphamide each on 9 th and 16 th day.

Cyp. Control : Cyclophosphamide Control.

SPI (100mg/kg) Treated : SPI (100mg/kg) Treated.

SPI (200mg/kg) Treated : SPI (200mg/kg) Treated.

SAL Treated : Salbutamol Treated.

SPI (100mg/kg) + Cyp.Treated : SPI (100mg/kg) + Cyclophosphamide Treated.

SPI (200mg/kg) Treated + Cyp.Treated : SPI (200mg/kg) Treated + Cyclophosphamide Treated.

SAL + Cyp.Treated : Salbutamol + Cyclophosphamide Treated.

Treatment Schedule:

Group I: **Control** :- Animals in the group I received distilled water orally in the dose of 1ml/Kg /day from 1st to 21st Day.

Group II: **Cyclophosphamide (Cyp.) control** :- Animals in the group II received Cyclophosphamide (Cyp.)100 mg/Kg/day as a single oral dose each on day 9th and day 16th.

Group III: **Spirulina 100mg/kg (SPI) Treated** :- Animals in the group III received Spirulina 100 mg/Kg/day as a single p.o. dose daily from 1st to 21st Day.

Group IV: **Spirulina 200mg/kg(SPI) Treated** :- Animals in the group IV received Spirulina 200 mg/Kg /day as a single oral dose daily from 1st to 21st Day.

Group V: **Salbutamol(SAL) Treated** :- Animals in the group V received Salbutamol 2 mg/Kg /day as a single p.o. dose daily from 1st to 21st Day.

Group VI: **Spirulina 100mg/kg (SPI) + Cyclophosphamide (Cyp.) Treated** :- Animals in the group VI received Cyclophosphamide (Cyp.)100 mg/Kg/day as a single oral dose each on day 9th and day 16th and Spirulina(SPI) 100 mg/Kg /day as a single p.o. dose daily from 1st to 21st Day.

Group VII: **Spirulina 200mg/kg + Cyclophosphamide (Cyp.) Treated** :- Animals in the group VII received Cyclophosphamide (Cyp.)100 mg/Kg/day as a single oral dose each on day 9th and day 16th and Spirulina(SPI)200mg/Kg /day as a single oral dose daily from 1st to 21st Day.

Group VIII: **Salbutamol(SAL) + Cyclophosphamide (Cyp.) Treated** :- Animals in the group VIII received Cyclophosphamide (Cyp.)100 mg/Kg/day as a single oral dose each on day 7th and day 16th and Salbutamol(SAL) 2 mg/Kg/day as a single oral dose daily from 1st to 21st Day.

Preparation of Sheep RBC (SRBC):

Sheep blood is collected from Veterinary hospital at Pimpri in sterile Alsever's solution in 1:1 proportion of Alsever's solution (freshly prepared) and processed for preparation of Sheep RBC (SRBC) batch.

Formula of Alsever's solution:

Sodium chloride 4.2 g/l
Sodium citrate 8.0 g/l
Citric acid anhydrous 0.55 g/l
Glucose 20.5 g/l

Antigen challenge:

On 7th and 14th day of the study, all the groups I to VIII were immunized with sheep RBC's (SRBC) in normal saline (0.5X10⁹/100 g) of body weight intraperitoneally.^[17]

Immunological Studies:

The animals were divided into eight groups consisting of six animals each. All the groups were treated with their respective treatment as described above. Cyclophosphamide was administered orally in a dose of Cyp.100 mg/Kg, two days after the primary and secondary immunization with sheep's RBC (SRBC).

Humoral Immune Response:

In the hemagglutination test serum, contained anti SRBC antibodies mainly IgG and IgM was employed for the estimation of humoral immune response. Microtiter plates having 96 wells having 12 wells in each row and 'U' bottom, were used for estimation of antibody titer. Two rows i.e.24 wells were used for the dilution of each serum sample.

0.2 to 0.4 ml blood was withdrawn on day 14th and 21st from retro-orbital plexus under mild ether anesthesia from all antigenically challenged mice. Blood was centrifuged to obtain serum, normal saline was used as a diluent and SRBC count was adjusted to (0.025X10⁹ cells/ml). Each well of a microtiter plate was filled initially with 20 µl of saline. 20 µl of serum was mixed with 20 µl of saline in the first well of microtiter plate. Subsequently the 20µl diluted serum was

removed from first well and added to the next well to get twofold dilutions of the antibodies present in the serum. Further twofold dilutions of this diluted serum were similarly carried out till the last well of the second row (24th well), so that the antibody concentration of any of the dilutions is half of the previous dilution. 20 µl SRBC (0.25X10⁹ cells) were added to each of these dilutions and the plates were incubated at 37°C for one hour and then observed for hemagglutination. The highest dilution giving hemagglutination was taken as the antibody titer. The antibody titers were expressed in the graded manner, the minimum dilution (1/2) being ranked as 1, and mean ranks of different groups were compared for statistical significance.

Antibody titer obtained on day 14th after 1st challenge (on 14th day) and on day 21st after 2nd challenge (on 20th day) was considered as primary and secondary humoral immune response respectively.

Statistical Analysis:

The results are expressed as mean ± SEM. Data on primary antibody titer were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparison test. Value of *P* less than 5% (*P*<0.05) was considered statistically significant.

Cell mediated immune response.**B) Delayed Type Hypersensitivity (DTH):^[16]****Table 2:** Experimental Protocol (Grouping and treatment schedule)

GR.NO.	Group (N=6)	Treatment And Dose Per Day.	Treatment Schedule
I	Control	Distilled water 1ml/Kg (p.o.)	1 st to 21 st Day.
II	Cyp. Control	Cyclophosphamide(Cyp.) 100 mg/kg (p.o.)	Single dose, on day 14 th two hours after immunization with Sheep RBC(SRBC).
III	SPI Treated	Spirulina 100 mg/Kg (p.o.)	1 st to 21 st Day.
IV	SPI Treated	Spirulina 200 mg/Kg (p.o.)	1 st to 21 st Day.
V	SAL Treated	Salbutamol 2 mg/Kg (p.o.)	1 st to 21 st Day.
VI	SPI + Cyp. Treated	Spirulina 100 mg/Kg (p.o.)+ Cyp.100mg/kg (p.o.)	SPI given p.o. from 1 st to 21 st Day + Cyclophosphamide as a Single dose, on day 14 th two hours after immunization with (SRBC).
VII	SPI + Cyp. Treated	Spirulina 200 mg/Kg (p.o.) + Cyp.100mg/kg (p.o.)	SPI given orally from 1 st to 21 st Day + Cyclophosphamide as a Single dose, on day 14 th two hours after immunization with (SRBC).
VIII	SAL + Cyp. Treated	SAL 2 mg/Kg (p.o.) + Cyp.100mg/kg (p.o.)	SAL given p.o. from 1 st to 21 st Day + Cyclophosphamide as a Single dose, on day 14 th two hours after immunization with (SRBC).

Cyp. Control : Cyclophosphamide Control.

SPI 100mg/kg Treated : Spirulina 100mg/kg Treated.

SPI 200mg/kg Treated : Spirulina 200mg/kg Treated.

SAL Treated : Salbutamol Treated.

SPI 100mg/kg + Cyp.Treated : SPI 100mg/kg + Cyclophosphamide Treated.

SPI 200mg/kg + Cyp.Treated : Famotidine + Cyclophosphamide Treated.

SAL + Cyp.Treated : Salbutamol + Cyclophosphamide Treated.

Treatment Schedule :

Group I: **Control** :- Animals in the group I received distilled water orally in the dose of 1ml/Kg /day from 1st to 21st Day.

Group II: **Cyclophosphamide (Cyp.) control** :- Animals in the group II received Cyclophosphamide (Cyp.)100mg/kg as a single oral dose single dose given orally on day 14th two hours after immunization with Sheep RBC (SRBC).

Group III: **Spirulina 100mg/kg (SPI) Treated** :- Animals in the group III received Spirulina (SPI) 100 mg/Kg /day as a single dose p.o. from 1st to 21st Day.

Group IV: **Spirulina 200mg/kg (SPI) Treated** :- Animals in the group IV received Spirulina (SPI) 200 mg/Kg /day as a single dose orally from 1st to 21st Day.

Group V: **Salbutamol(SAL) Treated** :- Animals in the group V received Salbutamol(SAL) 2 mg/Kg /day as a single dose p.o. from 1st to 21st Day.

Group VI: **Spirulina 100mg/kg (SPI)+ Cyclophosphamide (Cyp.) Treated** :- Animals in the group VI received Cyclophosphamide (Cyp.) as a single dose given orally on day 14th two hours after immunization with (SRBC) and Spirulina (SPI) 100 mg/Kg /day as a single p.o. dose daily from 1st to 21st Day.

Group VII: **Spirulina 200mg/kg (SPI) + Cyclophosphamide (Cyp.) Treated** :- Animals in the group VII received Cyclophosphamide (Cyp.) as a single dose given orally on day 14th two hours after immunization with (SRBC) and Spirulina (SPI) 200mg/Kg/day as a single oral dose daily from 1st to 21st Day.

Group VIII: **Salbutamol(SAL) + Cyclophosphamide (Cyp.) Treated** :- Animals in the group VIII received Cyclophosphamide (Cyp.) as a Single dose given orally on day 14th two hours after immunization with (SRBC) and Salbutamol(SAL) 2 mg/Kg/day as a single p.o. dose daily from 1st to 21st Day.

Preparation Sheep RBC (SRBC)

Sheep RBCs (SRBC) were prepared for the estimation of Hemagglutination Test, same were used for the current screening model.

Antigen challenge

On 14th day of the study, all the groups I to VIII were immunized with SRBC (0.5X10⁹/100gm.body weight) subcutaneously, in normal saline.^[29]

Immunological Studies

The animals were divided into eight groups consisting of six animals each. All the groups were treated with their

respective treatment as described above. Cyclophosphamide was administered orally in a dose of 100 mg/kg, two hours after the primary and secondary immunization with sheep RBC (SRBC).

Cellular Immune Response:

Foot pad reaction method in mice was used for detection of cellular immune response. On day 20, injection of sheep RBC (0.025x10⁹ cells) in the sub-planter region of right hind paw in the volume of 0.03 ml and normal saline in left hind paw in same volume. Foot pad reaction was assessed after 24 hr. i.e. on day 21, in terms of increase in the thickness of footpad due to oedema caused as a result of hypersensitivity reaction, with the help of a Digital Plethysmometer according to method. The footpad reaction was expressed as the difference in the thickness (m.m) between the right foot pad injected with SRBC and the left footpad injected with normal saline.

Statistical Analysis:

The results are expressed as mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparison test. Value of *P* less than 5% (*P*<0.05) was considered statistically significant.

Non-Specific Immune response:

C) Carbon Clearance Test:^[17]

Table 3: Experimental Protocol (Grouping and treatment schedule)

GR.NO.	Group (N=5)	Treatment and Dose Per Day	Treatment Schedule
I	Vehicle Control	Distilled water 1ml/Kg (p.o.)	1 st to 7 th Day.
II	SPI Treated	Spirulina 100 mg/Kg (p.o.)	1 st to 7 th Day.
III	SPI Treated	Spirulina 200 mg/Kg (p.o.)	1 st to 7 th Day.
IV	SAL Treated	Salbutamol 2 mg/Kg (p.o.)	1 st to 7 th Day.

SPI 100mg/kg Treated : Spirulina 100mg/kg Treated.

SPI 200mg/kg Treated : Spirulina 200mg/kg Treated.

SAL Treated : Salbutamol Treated.

Treatment Schedule

Group I: **Control** :- Animals in the group I received distilled water orally in the dose of 1ml/Kg /day from 1st to 7th Day.

Group II: **Spirulina 100mg/kg (SPI) Treated** :- Animals in the group II received Spirulina (SPI) 100 mg/Kg /day as a single p.o. dose daily from 1st to 7th Day.

Group III: **Spirulina 200mg/kg (SPI) Treated** :- Animals in the group III received Spirulina (SPI) 200 mg/Kg /day as a single oral dose daily from 1st to 7th Day.

Group IV: **Salbutamol (SAL) Treated** :- Animals in the group IV received Salbutamol(SAL) 2 mg/Kg /day as a single p.o. dose daily from 1st to 7th Day.

The animals were divided into 4 groups consisting 5 animals each. All the groups were treated with their respective treatment as described above. On day 7, 3 hours after the last dose all the animals of each group was given colloidal carbon intravenously in a volume of 1ml/100g. Blood samples were then collected (25 µl) from retro orbital plexus at 0 and 30 minutes after injection of colloidal carbon ink and lysed in distilled water (3ml). The optical density was

measured spectrophotometrically at 650 nm. The phagocytic index was calculated from the following equation

$$K = \frac{(\ln OD1 - \ln OD2)}{(t2 - t1)}$$

Where OD1 and OD2 are the optical density at time t1 and t2 respectively.

Statistical Analysis

The results are expressed as mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparison test. Value of *P* less than 5% (*P*<0.05) was considered statistically significant.

3. Results

Assessment of Humoral immune response:

A) Primary Humoral Immune Response:

At the end of 14 days prophylactic treatment of Spirulina 100mg/kg, Spirulina 200mg/kg and Salbutamol, the animals of each group were subjected to primary and secondary antigen challenge by Sheep RBCs. Primary antibody titer was determined and shown in the Table No.4.

Mice in the cyclophosphamide control group had shown significantly less antibody titer (^{##}*p*< 0.01) when compared to

control. In the group of mice with normal immune status, Spirulina 100mg/kg and Spirulina 200mg/kg showed significant ($*p < 0.05$) rise in primary antibody titer, Salbutamol did not produce significant rise in primary antibody titer. Where as in the groups of mice treated with cyclophosphamide *i.e.* immunosuppressed, groups treated with Spirulina 100mg/kg ($***p < 0.001$) and Spirulina 200mg/kg ($***p < 0.001$), significantly raised primary antibody titer but Salbutamol has shown nonsignificant reduction in primary antibody titer when compared with cyclophosphamide control, (immunosuppressed control group).

Spirulina (100 mg/kg. p.o.) in combination with cyclophosphamide *i.e.* an immunosuppressed Spirulina treated

group enhanced the primary antibody titer significantly ($***p < 0.001$) when compared with cyclophosphamide control.

Spirulina (200 mg/kg. p.o.) in combination with cyclophosphamide *i.e.* an immunosuppressed Spirulina treated group enhanced the primary antibody titer significantly ($***p < 0.001$) when compared with cyclophosphamide control.

Salbutamol (2mg/kg. p.o.) in combination with cyclophosphamide *i.e.* an immunosuppressed Salbutamol treated group decreased the primary antibody titer when compared with cyclophosphamide control but statistically non-significant.

Table 4: Effect of Spirulina treatment on primary antibody titer

S.NO	Group	Treatment	Dose and Route	Antibody Titer
1	Control	Distilled water	1ml/Kg (p.o.)	5.40 ± 0.244
2	Cyp. Control	Cyclophosphamide (Cyp.)	100 mg/Kg (p.o.)	3.20 ± 0.375 ^{##}
3	SPI Treated	Spirulina (SPI)	100 mg/Kg (p.o.)	6.200 ± 0.2 [*]
4	SPI Treated	Spirulina (SPI)	200 mg/Kg (p.o.)	7.80 ± 0.374
5	SAL Treated	Salbutamol (SAL)	2 mg/Kg (p.o.)	5.00 ± 0.316
6	SPI + Cyp. Treated	Spirulina+ Cyclophosphamide	SPI 100 mg/Kg (p.o.)+ Cyp.100 mg/Kg (p.o.)	5.40 ± 0.24 ^{***}
7	SPI + Cyp. Treated	Spirulina+ Cyclophosphamide	SPI 200 mg/Kg (p.o.)+ Cyp.100 mg/kg (p.o.)	5.40 ± 0.24 ^{***}
8	SAL + Cyp Treated	Salbutamol+ Cyclophosphamide	SAL2mg/Kg (p.o.)+ Cyp100mg/kg (p.o.)	3.63 ± 0.25

Values are expressed as Mean ± S.E.M.

* = $p < 0.05$, and *** = $p < 0.001$

When immunosuppressed drug treated groups were compared with cyclophosphamide control.

^{##} = $p < 0.01$

When Cyclophosphamide control compared with control.

(Statistically analysed by one-way ANOVA followed by Tukey-Kramer multiple comparisons test.

B) Secondary Humoral Immune Response:

At the end of 14 days prophylactic treatment of Spirulina 100mg/kg, Spirulina 200mg/kg and Salbutamol, the animals of each group were subjected to antigen primary and secondary antigen challenge by sheep RBCs. Secondary antibody titer is determined and is shown in the Table no 5.

Mice in the cyclophosphamide control group had shown significantly less antibody titer ($^{##}p < 0.01$) when compared to control. In the group of mice with normal immune status, Spirulina 100mg/kg ($^{*}p < 0.01$) and Spirulina 200mg/kg ($^{*}p < 0.05$) produced significant rise in secondary antibody titer. Where Salbutamol ($^{*}p < 0.01$) shown significant reduction in secondary antibody titer same as in the groups of

mice treated with cyclophosphamide *i.e.* immunosuppressed, groups treated with Spirulina, significantly raised secondary antibody titer when compared with cyclophosphamide control, an immunosuppressed control group while Salbutamol showed nonsignificant decrease in secondary antibody titer when compared with cyclophosphamide control .

Spirulina (100 mg/kg. p.o.) in combination with cyclophosphamide *i.e.* an immunosuppressed Spirulina treated group enhanced the secondary antibody titer significantly ($^{**}p < 0.01$) when compared with cyclophosphamide control.

Spirulina (200 mg/kg. p.o.) in combination with cyclophosphamide *i.e.* an immunosuppressed Spirulina treated group enhanced the secondary antibody titer significantly ($^{**}p < 0.01$) when compared with cyclophosphamide control.

Salbutamol (2 mg/kg. p.o.) in combination with cyclophosphamide *i.e.* an immunosuppressed drug treated group decreased the secondary antibody titer when compared with cyclophosphamide control but statistically not found to be significant.

Table 5: Effect of Spirulina treatment on secondary antibody titer

S.No	Group	Treatment	Dose And Route	Antibody Titer
1	Control	Distilled water	1ml/Kg (p.o.)	7.40 ± 0.245
2	Cyp.Control	Cyclophosphamide (Cyp.)	100mg/Kg (p.o.)	5.60±0.245 ^{##}
3	SPI Treated	Spirulina (SPI)	100 mg/Kg (p.o.)	10.2 ± 0.374 ^{**}
4	SPI Treated	Spirulina (SPI)	200 mg/Kg (p.o.)	12.40±0.245 [*]
5	SAL Treated	Salbutamol (SAL)	2 mg/Kg (p.o.)	6.40 ± 0.245 ^{**}
6	SPI + Cyp Treated	Spirulina (SPI)+ Cyclophosphamide (Cyp.)	SPI 100 mg/Kg (p.o.) + Cyp.100 mg/Kg (p.o.)	6.60 ± 0.245 ^{**}
7	SPI + Cyp Treated	Spirulina (SPI) + Cyclophosphamide (Cyp.)	SPI 200mg/Kg (p.o.) + Cyp.100 mg/Kg (p.o.)	6.90 ± 0.374 ^{**}
8	SAL + Cyp Treated	Salbutamol (SAL) + Cyclophosphamide (Cyp.)	SAL 2 mg/Kg (p.o.)+ Cyp.100 mg/Kg (p.o.)	4.200 ± 0.2

Values are expressed as Mean ± S.E.M.

* = $p < 0.05$ and ** = $p < 0.01$

When immunosuppressed drug Treated groups were compared with cyclophosphamide control ^{##} = $p < 0.01$

When Cyclophosphamide control was compared with Control. (Statistically analysed by one-way ANOVA followed by Tukey-Kramer multiple comparisons test.)

Assessment of Cell mediated immune response.

C) Delayed Type Hypersensitivity induced footpad oedema

Footpad oedema in Delayed Type Hypersensitivity is performed by injecting SRBCs into sub plantar region of hind limb of mice; the degree of Footpad oedema was measured after 24 hours. The DTH skin response requires antigen specific memory T cells and produces inflammation. The inflammation results from the production of local cytokines and chemotaxis at the site of injection, which results in the recruitment of large number of neutrophils and mononuclear cells.

The result shown in Table No.6 indicates that Cyclophosphamide control group has shown significant increase, ($^{##}p < 0.01$) in the mean difference, in the paw thickness as compared to control group.

In the groups of mice treated with Cyclophosphamide *i.e.* an immunosuppressed groups with two different drugs, significantly increase DTH response in terms of increase in

the mean difference in the paw thickness where Salbutamol treated group significantly decrease DTH response in terms of decrease in the mean difference in the paw thickness. Where as in the group of mice with normal immune status, Spirulina did produce significant decrease in DTH response. Spirulina 100mg/kg ($^{***}p < 0.001$) and Spirulina 200mg/kg ($^{*}p < 0.05$) while Salbutamol produced significant increase DTH response.

Spirulina (100 mg/kg. p.o.) in combination with Cyclophosphamide *i.e.* an immunosuppressed Spirulina treated group increase DTH response in terms of mean difference in the paw thickness, significantly ($^{***}p < 0.001$) when compared with Cyclophosphamide control.

Spirulina (200 mg/kg. p.o.) in combination with Cyclophosphamide *i.e.* an immunosuppressed Spirulina treated group increase DTH response in terms of mean difference in the paw thickness, significantly ($^{***}p < 0.001$) when compared with Cyclophosphamide control.

Salbutamol (2 mg/kg. p.o.) in combination with Cyclophosphamide *i.e.* an immunosuppressed Salbutamol treated group decrease DTH response in terms of mean difference in the paw thickness, non-significantly when compared with Cyclophosphamide control.

Table 6: Effect of Spirulina treatment on cell-mediated immune response by Delayed Type Hypersensitivity induced footpad oedema

S. No.	Group	Treatment	Dose and Route	% Increase In Paw Oedema
1	Control	Distilled water	1ml/Kg (p.o.)	10.74 ± 0.35
2	Cyp.Control	Cyclophosphamide (Cyp.)	100 mg/Kg (p.o.)	12.80 ± 0.25 ^{##}
3	SPI Treated	Spirulina (SPI)	100 mg/Kg (p.o.)	8.20 ± 1.73 ^{***}
4	SPI Treated	Spirulina (SPI)	200 mg/Kg (p.o.)	7.10 ± 0.35 [*]
5	SAL Treated	Salbutamol (SAL)	2 mg/Kg (p.o.)	10.00 ± 0.08 [*]
6	SPI + Cyp Treated	Spirulina (SPI)+ Cyclophosphamide	Cyp. 100 mg/Kg (p.o.) + SPI 100mg/Kg (p.o.)	9.50 ± 0.68 ^{***}
7	SPI + Cyp Treated	Spirulina (SPI)+ Cyclophosphamide	Cyp. 100 mg/Kg (p.o.) + SPI 200mg/Kg (p.o.)	9.40 ± 0.69 ^{***}
8	SAL + Cyp Treated	Salbutamol (SAL) + Cyclophosphamide	Cyp. 100 mg/Kg (p.o.) + SAL 2mg/Kg (p.o.)	9.90 ± 0.56

Values are expressed as Mean ± S.E.M.

* = $p < 0.05$ and *** = $p < 0.001$

When immunosuppressed drugs Treated groups were compared with Cyclophosphamide control

^{##} = $p < 0.01$

When Cyclophosphamide control was compared with Control.

Statistically analysed by one-way ANOVA followed by Tukey-Kramer multiple comparisons test.)

D) Carbon Clearance Test

To assess the functional changes in, macrophages of reticuloendothelial system, their phagocytic ability was determined. Table No.7 shows the effect of Spirulina on Reticulo-Endothelial System by Carbon Clearance Test. Spirulina treated group, at a dose of 100 mg/kg exhibited significantly high phagocytic index of 0.0336. Similarly 200 mg/kg dose of Spirulina treated group exhibited significantly high phagocytic index of 0.0448. Whereas Salbutamol dose of 2 mg/kg, also exhibited low phagocytic index of 0.0120 as compare to control 0.0180 but was found statistically non-significant.

Table 7: Effect of Spirulina treatment on Reticulo-Endothelial System by Carbon Clearance Test

Sr. No.	Group	Treatment and Dose Per Day.	Phagocytic Index
1	Control	Distilled water 1ml/Kg (p.o.)	0.0180 ± 0.00041
2	SPI Treated	Spirulina 100 mg/Kg (p.o.)	0.0336 ± 0.00081 ^{***}
3	SPI Treated	Spirulina 200 mg/Kg (p.o.)	0.0448 ± 0.00014 ^{***}
4	SAL Treated	Salbutamol 2 mg/Kg (p.o.)	0.0120 ± 0.00041

Values are expressed as Mean ± S.E.M.

^{***} = $p < 0.001$

Drug treated groups were compared with Control. (Statistically analysed by one way ANOVA followed by Tukey-Kramer multiple comparisons test.)

4. Discussion

Spirulina is increase the antigen-specific, as well as the total, IgA antibody level in the Peyer's patches, mesenteric lymph nodes and intestinal mucosa as well as in the spleen cells in

mice. Lower antigen-specific IgG1 and IgE antibody levels in the serum suppressing allergic reactions. Signaling responses through Toll in blood cells; increasing activity of macrophages, Natural Killer cells (NK), and neutrophils. The presence of co-operative IL-12 and IL-18 for NK-mediated IFN production.

Caspase dependent apoptosis induction in HeLa cells *in vitro*; activation of pro-apoptotic gene and down regulation of anti-apoptotic gene expression, to facilitate the transduction of tumoural apoptosis signals; activation of caspases 2, 3, 4, 6, 8, 9, and 10.

The induction of a cell-mediated immune response to a specific antigen is regulated, for the most part, by the release of cytokines. IL-12 is produced by macrophages, monocytes, dendritic cells and B cells in response to bacterial products and intracellular parasites. The biological effects of the production of IL-12 are directed at T cells and NK cells. IL-12 is responsible primarily for the subsequent production of IFN γ and tumor necrosis factor- α (TNF- α) from both NK cells and helper T cells. IL-12 also stimulates the rate at which NK cells and helper T cells proliferate following antigen activation. In addition, the lytic capacities of both NK and helper T cells are increased by the presence of IL-12. IL-12 has the specialized function of leading naive CD4 $^{+}$ T cells to differentiate toward the TH1 cell type in order to prepare for the release of IFN- γ and for the development of the cell-mediated immune response. However, IL-12 is not effective in the down regulation by means of reversing TH2 cells differentiation. IL-12 and IL-2 are both important cytokines in the regulation of a cell-mediated immune response, IL-2 being responsible for stimulating the growth and proliferation of T cells, while IL-12 stimulates the differentiation of the CD4 $^{+}$ T cells into TH1 cells. [18, 19, 20]

IL-10 was first recognized for its inhibition of T cell activation and effector functions. IL-10 was initially characterized as a cytokine produced by certain Th2 cell clones. Many other cell types, however, such as macrophages, B cells, mast cells, and keratinocytes have been shown to secrete considerable amounts of this cytokine. IL-10 down-regulates the expression of MHC class II and costimulatory molecules and inhibits the production of proinflammatory cytokines, including IL-12 by DCs and other professional antigen-presenting cells. [21, 22, 23, 24]

Spirulina increase the level of IL-12 and IL-2, on the other side reducing the level of IL-10 by decreasing the level of cAMP in dendritic cell and ultimately activate immune system.

When mice were sensitized with SRBC, an antigen gets diffused in the extra vascular space and enters the lymph node via the lymphatics. Particulate antigens are taken up by macrophages lining the sinuses or disperse in the lymphoid tissues and processed. Small highly antigenic peptides are combined with MHC class II molecule. B cells with receptors for antigen binds and internalizes it into an endosomal compartment and process and presents it on MHC class II molecules to T $_{H2}$ cells. These B cells are

triggered to proliferate, giving rise to clones of large numbers of daughter cells. Some of the cells of these expanding clones serve as memory cells, other differentiates and become plasma cells that make and secrete large quantities of specific antibody. During a primary response, IgM is secreted initially, often followed by a switch to an increasing proportion of IgG. The magnitude of secondary antibody response to the same antigen is amplified in terms of antibody production.

In the present study, the assessment of humoral immunity was carried out using hemagglutination test. The anti-SRBC antibody titer in Spirulina treated groups in the dose of 100 mg/kg, 200 mg/kg respectively, with normal immune status, was increased but statistically non-significant, while Salbutamol 2 mg/kg the anti-SRBC antibody titer was decrease but statistically non-significant as compared to control group with normal immune status.

In the immunosuppressed groups Cyclophosphamide was used as immunosuppressant as, it selectively suppresses humoral immunity by exerting depressive effect on antibody production, if given after antigenic stimulation. This may be due to interference with helper T cell activity.

In the immunosuppressed groups, Spirulina significantly protected Cyclophosphamide induced suppression of humoral immunity indicating that Spirulina counteracts suppression of both, primary and secondary humoral immune response induced by cyclophosphamide. But Salbutamol significantly mimicked Cyclophosphamide induced suppression of humoral immunity. This suggests that Spirulina exerts immunoprotective and strong immunomodulatory property and Salbutamol exerts immunosuppressive property. Spirulina stimulates humoral immune response in terms of elevation in anti SRBC antibody titer.

When activated T $_{H1}$ cells encounter certain antigens, *viz* SRBC. They secrete cytokines that induce a localised inflammatory reaction called Delayed Type Hypersensitivity. DTH comprises of two phases, an initial sensitisation phase after the primary contact with SRBC antigen. During this period T $_{H1}$ cells are activated and clonally expanded by APC with class II MHC molecule (eg. langerhans cells and macrophages are APC involved in DTH response). A subsequent exposure to the SRBC antigen induces the effector phase of the DTH response, where T $_{H1}$ cells secrete a variety of cytokines that recruits and activates macrophages and other non specific inflammatory mediators. The delay in the onset of the response reflects the time required for the cytokines to induce the recruitment and activation of macrophages.

In the model of Delayed type hypersensitivity paw edema in the groups of mice (normal immune status) treated with Spirulina, in the dose of 100 mg/kg (p.o.), 200 mg/kg (p.o.) was potentiated, when challenged with SRBC, as compared to control group but it was not found statistically significant.

Evaluation of immunomodulatory activity of Spirulina on the parameters of non specific immunity was carried out by *in vivo* phagocytosis.

In carbon clearance test, Spirulina (100 mg/kg) (p.o.) and Spirulina (200mg/kg, p.o.) treated groups, exhibited significantly high phagocytic index and Salbutamol (2 mg/kg, p.o.) showed reduction in phagocytic index as compare to control group. This indicates stimulation of the reticuloendothelial system by Spirulina treatment and depression of the reticulo endothelial system by Salbutamol. Phagocytosis by macrophages is important against the smaller parasites and its effectiveness is markedly enhanced by opsonisation of the parasite with the antibodies and complement activation leading to more rapid clearance of parasites from blood.

5. Conclusion

Spirulina (100 mg/kg, p.o.) and Spirulina (200 mg/kg, p.o.) and Salbutamol (2mg/kg, p.o.) treatment exerted a strong immunomodulatory activity in laboratory animals. Spirulina have shown a significant immunostimulant effect on specific arms of immune system. The humoral immunity and cell mediated immunity particularly in the immune compromised status were protected by Spirulina treatment.

Spirulina has also; non-specifically activated the immune system by the activation of Reticulo endothelial system. While β_2 agonist has exhibited negative effect on a non-specific immune system. So agents that increase the level of cAMP (i.e. β_2 agonist) have suppressive effect on immune system and cAMP lowering agents have stimulant effect on immune system.

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